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Characterization of Bacteriophages and Their Application as a Phage Cocktail for Biocontrol of Multidrug-resistant *Escherichia coli*

다제내성 대장균의 제어를 위한 박테리오파지의 특성 분석과 파지 칵테일의 적용에 관한 연구

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Abstract

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Multidrug-resistant (MDR) bacteria are increasing globally and necessitate the development of new treatment agents. *Escherichia coli* is a gram-negative bacterium responsible for various diseases, and the emergence of MDR *E. coli* has been reported in various countries. Bacteriophages (phages) are considered a promising alternative to antibiotics, and phage cocktails are generally used to broaden host ranges in phage treatment. In this study, phages infecting multiple MDR *E. coli* clinical isolates were isolated. The biochemical and genomic characterizations of phages were conducted, and the efficiency of infection of the phage cocktail with a broad host range was evaluated in diverse conditions. A total of twenty-one *E. coli* clinical isolates resistant to various groups of antibiotics were used in the study. Six *E. coli* phages were isolated, and based on the host range determination, the phage cocktail composed of phages ELSA1, ELSA2, and ELT4 with the broad

host range against MDR *E. coli* strains were constructed in equal ratio. The phage cocktail controlled 85.7% (18/21) of MDR *E. coli* strains. Three phages were classified as the *Myoviridae* family based on transmission electron microscopy (TEM) analysis. Phage viabilities were stable between 4-55°C and pH 3-11, respectively. In the one-step growth curve analysis, phages ELSA1, ELSA2 and ELT4 showed burst sizes of 33, 160, and 45 plaque-forming units per cell, respectively. Whole-genome sequencing of phages revealed that genome sizes of ELSA1, ELSA2 and ELT4 were 169,532, 110,683, and 165,114 bp, respectively. Results of phage receptor analysis indicate that ELSA1 uses LPS and OmpA, but ELT4 uses Tsx as receptors to infect the hosts. Phage inhibition assays using single phages and the phage cocktail against three MDR *E. coli* strains or mixed culture of them revealed that the antibacterial activities of the phage cocktail were comparable to that of single phage against MDR *E. coli* strains at three different MOIs. Also, the phage cocktail controlled mixed culture of MDR *E. coli* more effectively compared to single phage treatments. In food application, the phage cocktail treatment at a MOI of 10^4 effectively reduced MDR *E. coli* on surface of romaine lettuce at 4°C and 25°C. In the checkerboard titration assays using the phage cocktail with 8 antibiotics

(ciprofloxacin, levofloxacin, meropenem, imipenem, ceftazidime, ceftriaxone, ampicillin and piperacillin-tazobactam), phage cocktail showed synergy effects in controlling the mixed culture of three MDR *E. coli* strains when inoculated with meropenem or imipenem from carbapenems class. In conclusion, the results indicate that the phage cocktail designed in this study showed improved antibacterial activity against various MDR *E. coli* compared to single phage. Furthermore, results of food application and the checkerboard assays show that the phage cocktail used in the study is highly utilized as a biocontrol agent.

Keywords: Multi-drug resistance, *Escherichia coli*, bacteriophage, bacteriophage cocktail

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I. INTRODUCTION

Multidrug resistance (MDR) stands for resistance of bacteria to at least one antibiotic from three or more antimicrobial classes, which are increasing worldwide. It is known that inappropriate and excessive use of antibiotics caused and accelerated the emergence of MDR pathogens (Bettiol et al., 2015). According to a recent report from Centers for Disease Control and Prevention (CDC), more than 2.8 million outbreaks of antibiotic-resistant pathogens were investigated, resulting in more than 35,000 deaths in the United States (CDC, 2019). Resistance to antibiotics is usually mediated by various mechanisms: production of inactivating enzymes; changes and alterations in the antibiotic target; reduced cellular permeability; and efflux system (Alekshun et al., 2007; Gerard D Wright, 2010). Multidrug-resistant bacteria usually have multiple mechanisms associated with antibiotic resistance, and the most of MDR strains is thought to be developed from gaining of resistance genes by horizontal transfer (Aslani MM et al., 2011). Acquisition and increase of resistance to various antibiotics of pathogens has become a serious threat to public health and necessitate development of an alternative to antibiotics (Magiorakos et al., 2012).

Escherichia coli is a gram-negative, facultative anaerobic bacterium, and some of *E. coli* strains are responsible for various human diseases including urinary tract

infection (UTI) and bloodstream infections by gaining specific virulence factors (Ejikeugwu et al., 2012). Intestinal pathogenic *E. coli* (IPEC) includes shiga toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) causing diarrhea and severe complication. Also, there are extraintestinal pathogenic *E. coli* (ExPEC) such as uropathogenic *E. coli* (UPEC) and sepsis and meningitis-associated *E. coli* (MNEC) (Kaper JB et al., 2004; Karch H et al., 2005; Nataro JP et al., 1998). In addition to pathogenicity, multidrug resistance of *E. coli* poses threat to public health. According to global priority list of pathogens with antibiotic-resistance released by World Health Organization (WHO) in 2017, *Enterobacteriaceae* including *E. coli* is listed on the "Critical" group, which necessitate development of new antimicrobial agents (WHO, 2017).

Bacteriophages (phages), the viruses that specifically infect and lyse host bacteria, are considered as promising alternative to antibiotics (Brussow H, 2017). The life cycles of phage in bacterial host are divided into lytic and lysogenic cycle. Generally, lytic phages are thought to more suitable option for therapy because immediate lysis after infection. Whereas lysogenic phages can acquire toxic genes from bacterial host during phage replication. Phage therapy has multiple advantages including efficiency against MDR pathogens because of different mechanisms compared to antibiotics, high specificity for target bacteria, and costly efficiency (Matsuzaki et al., 2005). Also, phage-antibiotic synergy (PAS) is being studied to

evaluate the therapeutic potential of phage treatment by enhancing antibacterial activity (Lin, Y et al., 2018). The limitations of single phage treatment include the narrow host range and the occurrence of phage resistance. To overcome these disadvantages, phage cocktail is generally used by combining phages infecting several different strains (Chan and Abedon, 2012). The most important criterion for an ideal phage cocktail is to reduce growth of a range of host strains effectively (K. Shahin et al., 2021).

In this study, we isolated phages infecting MDR *E. coli* strains and constructed a phage cocktail with broad host range against MDR *E. coli* strains by combining three different phages ELSA1, ELSA2, and ELT4. We conducted the biochemical, genomic characterizations of phages. Antibacterial activity of the phage cocktail was assessed using MDR *E. coli* strains and compared to single phage treatments. Furthermore, efficiency of infection was evaluated using the phage cocktail on the romaine lettuce. PAS effect with the phage cocktail and antibiotics from four classes were evaluated to identify an ideal combination for improved antibacterial activity and increase utilization of the phage cocktail as a biocontrol agent.

II. MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Twenty-one MDR *E. coli* clinical isolates (EC1-EC21) used in this study were obtained from department of laboratory medicine from Yonsei university college of medicine (Seoul, Republic of Korea) and are listed in Table 1. The MDR *E. coli* strains and *E. coli* MG1655 were cultured at 37°C in Luria-Bertani (LB) media (Difco, United States) with aeration and used for isolation of *E. coli* infecting phages from collected samples.

2.2 Phage isolation and propagation

E. coli phages ELT1 and ELT3 were isolated from retail raw chickens. *E. coli* phages ELT2 and ELT4 were isolated from duck feces and pork, respectively. Also, *E. coli* phages ELSA1 and ELSA2 were isolated from sewage in Jungnang water treatment center. To isolate phages, the samples were homogenized by vortexing in sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 8 mM $MgSO_4 \cdot H_2O$). The homogenized samples were transferred to 50 ml tubes and centrifuged at 10,000 x g for 5 min at 4°C, the supernatant was filtered through a 0.22 μm polyethersulfone (PES) membrane filter. 5 ml of the filtered

samples were mixed with the equal volume of 2 x LB broth and 100 μl overnight culture of host strains at 37°C with aeration at 220 of revolutions/min (rpm) overnight. After incubation, the culture was centrifuged and filtered as described to remove bacterial cells. To confirm the presence of phages, the phage lysates were spotted on 0.4% LB soft top agar containing 100 μl of overnight culture of host strains. After incubation at 37°C overnight, individual plaques were picked and eluted with 1 ml SM buffer. This step was repeated at least three times.

For phage propagation, the incubation time was determined based on the lysis activity of each phage. Overnight culture of the propagation host *E. coli* strains (ELT1, ELT2, ELT3, and ELT4: *E. coli* MG1655, ELSA1: *E. coli* EC12, ELSA2: *E. coli* EC9) were inoculated to LB and incubated with aeration at 220 rpm for 2 h. Subsequently, purified phage lysates were added to the culture and the mixtures were incubated at 37°C for 4 h. Phage propagation was performed with three different culture volumes (4, 40, and 250-ml LB broth), and the culture was centrifuged and filtered. To obtain the phage at a high titer, 40 ml of filtered phage lysates were mixed with 5 g of polyethylene glycol (PEG) 6000 and 10 ml of 5M NaCl. Also, CsCl density gradient ultracentrifugation was conducted at 25,000 x g for 2 h at 4°C. After centrifugation, collected phage was dialyzed using dialysis buffer (50 mM Tris-HCl, pH 8.0, 10mM NaCl, and 10 mM $MgCl_2$). The concentrated phage stocks were stored at 4°C.

2.3 Host range determination of phages

Total 21 MDR *E. coli* clinical isolates listed in Table 1 were used in determination of host ranges of nine *E. coli* phage infections. Phages SSP1, JEP6, and JEP7 were obtained from laboratory stocks and used in host range determination and comparison. Each strain was incubated at 37°C overnight at 220 rpm. 100 µl of each bacterial culture was added to 5 ml of 0.4% LB soft agar and mixture was overlaid on LB agar plate. Subsequently, 10 µl of 10-fold serially diluted phage lysates with SM buffer were spotted onto prepared plates and incubated at 37°C for 12 h. After incubation the phage sensitivity of each strain was determined by recording the single plaque formations. The efficiency of phage infection against each strain was compared to that against host strain.

2.4 Transmission electron microscopy (TEM) analysis

Morphology of purified *E. coli* phages ELSA1, ELSA2, and ELT4 was analyzed using transmission electron microscopy (TEM). 10 µl of diluted phage sample [10^{10} plaque-forming units (PFUs)/ml] was placed on carbon-coated copper grids and negatively stained with 2% uranyl-acetate (pH 4.0). Phages were observed with an energy-filtering TEM at operating voltage of 120-Kv (Kwiatek et al., 2012). Phages were identified and classified using the guidelines of the International Committee on Taxonomy of Viruses (Fauquet, et al., 2005).

2.5 Phage stability under various temperature and pH

To determine phage stability under different temperature conditions, phages ELSA1, ELSA2, and ELT4 (final concentration, 10^8 PFU/ml) were added to SM buffer and incubated at 4, 25, 37, 45, 55, 60, 65, 75, and 80°C for 1 h. After incubation, phage titers were determined with plaque assays using host strains. To determine phage stability under different pH conditions, pH of SM buffer was adjusted with hydrogen chloride or sodium hydroxide over pH range of 2 to 11, and phages ELSA1, ELSA2, and ELT4 (final concentration, 10^8 PFU/ml) were added to pH adjusted SM buffer. After incubation at 37°C for 1 h, the phage suspensions were neutralized, and phage titers were determined using plaque assays with host strains.

2.6 Phage receptor analysis

To identify the receptor of *E. coli* phages ELSA1 and ELT4, *E. coli* MG1655 were used. *E. coli* MG1655 strains with deletions of *ompA*, *waaG*, or *tsx* genes were constructed using the lambda red recombination system (Datsenko and Wanner. 2000). Briefly, the kanamycin resistance (Kan^R) cassette from plasmid pKD13 was amplified using primers specific for each gene. The resulting polymerase chain reaction (PCR) products were transformed to the wild type strain harboring pKD46 and integrated into the chromosomal *ompA*, *waaG*, or *tsx* genes. The Km^R cassette

was removed by pCP20 plasmid. Each 10-fold serially diluted lysate of phage ELSA1 or ELT4 was spotted onto 0.4% LB soft agar containing *E. coli* MG1655 △*ompA*, △*waaG*, or △*tsx*, and its complemented strain harboring pUC19 or pBAD18 followed by incubation at 37°C (Yanisch-Perron et al., 1985).

2.7 Phage in vitro adsorption assays

E. coli host strains (*E. coli* EC12, EC9, and MG1655) of phages ELSA1, ELSA2, and ELT4 were grown in LB broth overnight. The cells were inoculated into 20 ml LB medium and incubated at 37°C at 220 rpm. When the optical density at 600 nm (OD_{600}) of the host culture reach to 1.0, the bacterial culture was harvested, resuspended, and 10-fold diluted with LB broth. Phages ELSA1, ELSA2, or ELT4 were added at an MOI of 0.01, and incubated at 37°C for 10 to 30 min. Then, 1 ml samples were collected at intervals of 2 or 5 min, centrifuged at 16,000 x g for 1 min, and filtrated using 0.22 μm polyethersulfone (PES) membrane filters. The collected supernatant samples were 10-fold serially diluted with SM buffer and spotted onto 0.4% LB soft top agar containing host strains. Based on the ratio between initial titer and tested titer of each phage, adsorption rates of three phages to the host strains were determined.

2.8 Phage one-step growth curve analysis

Same *E. coli* host strains (*E. coli* EC12, EC9, and MG1655) used in the phage adsorption assays were incubated until an OD_{600} of 1.0 reached, 50 ml of bacterial culture was harvested. Phages ELSA1, ELSA2, or ELT4 were added at an MOI of 0.01. After incubation at 37°C for 10 min to enable adsorption of phages, the culture was centrifuged at 10,000 x g for 5 min, and the pellet containing phage-infected cells was resuspended with 50 ml of LB broth. The resuspended culture was incubated at 37°C with shaking at 220 rpm, and two sets of samples were collected every 5 min for 60 to 100 min. 1% chloroform (final concentration) was added to the second of the two sets of samples to release intracellular phage. Subsequently, two samples were 10-fold serially diluted and spotted for phage titration. Based on the comparison of PFU/ml between chloroform-treated and non-treated sets of the samples, eclipse periods, latent periods, and burst sizes were analyzed.

2.9 Phage inhibition assays

Host strains of phages ELSA1, ELSA2, or ELT4 (*E. coli* EC12, EC9, and MG1655) were incubated at 37°C overnight, sub-inoculated in LB broth, and incubated at 37°C until the early exponential growth phase. Subsequently, phage lysates at MOIs of 0.1, 1, and 10 were added to host cultures to determine the bacterial growth inhibitory abilities of phages. OD_{600} was measured with the

SpectraMax i3 multimode microplate reader (Molecular Devices, Sunnyvale, CA, United States) at 37°C for 24 h. An uninfected culture was used as a positive control, and LB broth was used as a negative control. All experiments were performed in triplicates.

2.10 Phage DNA purification and whole-genome sequencing

To extract genomic DNA from phages, previously described method was used (Wilcox et al., 1996). In the first step, DNase I and RNase A (1 μg/ml) was used to remove bacterial nucleic acids. Subsequently, to lyse the phage capsid 20 mM ethylenediaminetetraacetic acid (EDTA), 50 μg/ml proteinase K, and 0.5% sodium dodecyl sulfate (SDS) were treated and incubated for 1 h at 56°C. After incubation, phage DNA was purified by treating phenol solution, phenol-chloroform-isoamyl solution (24:24:1), and chloroform solution at 1:1 ratio and centrifuged at 5,000 rpm for 5 min. Then, phage DNA was precipitated by ethanol, and pellet of DNA was resuspended with TE buffer.

Purified phage genomic DNA of phage ELSA1, ELSA2, and ELT4 was sequenced using Illumina MiSeq platform and assembled with De novo Assembly of High-Quality Reads (SPAdes v3.15.2) at Sanigen Inc., South Korea. Open reading frames (ORFs) were predicted using GeneMarkS software (Besemer et al., 2001), and annotated using the Rapid Annotation using Subsystem Technology

(RAST) pipeline (McNair et al., 2018). The complete genome sequences of *E. coli* phages ELSA1, ELSA2, and ELT4 were deposited in GenBank under the accession number OL9618120, ON557615, and OM803178, respectively.

2.11 Bacterial inhibition assays using phage cocktail

The antibacterial efficiency of the phage cocktail prepared by phages ELSA1, ELSA2, and ELT4 with a ratio of 1:1:1 was assessed with MDR *E. coli* strains EC9, EC12 and EC16, and mixed culture of them. Each strain was incubated at 37°C overnight, sub-inoculated in LB broth, and incubated at 37°C until the early exponential growth phase. The mixed culture of three *E. coli* strains were prepared by inoculating 1% (v/v) of each bacterial culture to fresh LB broth. Subsequently, lysates of single phage or phage cocktail at MOIs of 0.1, 1, and 10 were added to bacterial suspension of single host or mixture. OD_{600} was measured with the SpectraMax i3 multimode microplate reader (Molecular Devices, Sunnyvale, CA, United States) at 37°C for 24 h. An uninfected culture was used as a positive control, and LB broth was used as a negative control. All experiments were performed in triplicates.

2.12 Inhibition of MDR *E. coli* on romaine lettuce by phage cocktail

Organic romaine lettuce was purchased from retail stores. The lettuce samples were cut aseptically into small pieces (2 x 2 cm^2) with sterilized razor. For decontamination, the surface of samples was rinsed with 70% ethanol and UV-treated on both sides for 30 min in a biosafety cabinet. After treatment, the surface of lettuce pieces was spotted with 20 µl of the mixed culture (*E. coli* EC9, EC12, and EC16) in the final inoculum concentration of 5 log CFU/cm^2. The same volume of PBS was inoculated as a negative control. Samples were dried in a 6-well plate for 30 min to allow the attachment of bacteria. Then, 100 µl of phage cocktail at an MOI of 10 to 10^4 or SM buffer as a control were spotted onto lettuce surfaces and incubated at 4 and 25°C. At 3, 6, 9, and 24 h of incubation, each sample was collected and mixed with 10 ml 0.1% buffered peptone water (BPW) and vortexed for 1 min in a 50 ml tube. Subsequently, samples were removed, and the mixture was centrifuged at 10,000 x g for 5 min. The pellets were resuspended with 1 ml 0.1% BPW. Viable cell counts were performed by 10-fold serial dilution with PBS and plating on LB agar plates. All experiments were performed in triplicates.

2.13 Checkerboard titration assays

To evaluate synergy effect between phage cocktail and antibiotic treatment, checkerboard titration assay was performed as described previously (Hsieh MH. et

al., 1993). Following antibiotics were used in this study: ciprofloxacin hydrochloride (Thermo Fisher Scientific, USA), levofloxacin (Sigma Aldrich, USA), Meropenem (Sigma Aldrich, USA), Imipenem (Sigma Aldrich, USA), Ceftazidime hydrate (Sigma Aldrich, USA), Ceftriaxone, Ampicillin sodium (Duchefa), Piperacillin sodium salt (Sigma Aldrich, USA), Tazobactam sodium salt (Cayman).

Minimum inhibitory concentration (MIC) values for MDR *E. coli* strains and eight antibiotics were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, P.A., 2019) with *E. coli* ATCC 25922. In the checkerboard titration assay, eight antibiotics (ciprofloxacin, levofloxacin, meropenem, imipenem, ceftazidime, ceftriaxone, ampicillin, piperacillin with tazobactam) were serially diluted 2-fold on each column in a 96-well plate, and the phage cocktail was diluted 10-fold on each column. The mixed culture of MDR *E. coli* strains (EC9, EC12, EC16) was added to the 96-well plate (10^5 CFU/well). The plate was incubated at 37°C for 18 h, and OD_{600} was measured using SpectraMax i3 multimode microplate reader (Molecular Devices, Sunnyvale, CA, United States). All experiments were performed in triplicates.

III. RESULTS

3.1 Isolation and host range determination of phages

For phage isolation, MDR *E. coli* clinical isolates (Table 1) and *E. coli* MG1655 were used. Total six *E. coli* phages were isolated form retail raw chicken, duck feces, pork, and sewage samples (Table 2). Based on host range determination of phages with twenty-one MDR *E. coli* strains using six isolated phages and three *E. coli* phages from laboratory stocks, three phages ELSA1, ELSA2, and ELT4 were selected for phage cocktail construction because of broad host range against MDR strains by combining these three phages (Table 3). Phage ELSA1, ELSA2, and ELT4 infected 71.4% (15/21), 9.5% (2/21), and 28.6% (6/21) of MDR *E. coli* strains, respectively. The phage cocktail composed of three phages controlled 85.7% (18/21) of MDR *E. coli* strains.

The morphologies of phages ELSA1, ELSA2, and ELT4 were analyzed using transmission electron microscopy (TEM). Based on big heads and contractile inflexible tails, all three phages were classified as the *Myoviridae* family (Figure 1).

Table 1. List of *E. coli* clinical isolates

	MDR *E. coli* clinical isolate																				
Strain number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Source	Urine	Hand	Urine	Blood	Sputum	Stool	Stool	Stool	Blood	Urine	Stool	Stool	Bile	Blood	Sputum	Sputum	Ascites	Stool	Stool	Sputum	Blood
Carbapenemase type	-	KPC	OXA	-	KPC	-	KPC	KPC	KPC	OXA	-	NDM	-	OXA	NDM	-	-	-	-	-	KPC
Antibiotic resistance[a]	FEP CIP CAZ PIP GM ATM TZP	FEP CIP CAZ PIP IPM ATM TZP MEM	FEP CIP CAZ PIP ATM TZP	FEP CIP CAZ PIP GM AN ATM TZP	FEP CIP CAZ PIP GM IPM ATM TZP MEM	FEP CIP CAZ PIP GM ATM	FEP CIP CAZ PIP IPM ATM TZP MEM	CIP PIP GM AN IPM ATM TZP MEM	FEP CIP CAZ PIP IPM ATM TZP MEM	FEP CIP PIP IPM ATM TZP MEM	FEP CIP CAZ PIP GM AN IPM ATM TZP MEM	FEP CIP CAZ PIP IPM ATM TZP MEM	FEP CIP CAZ PIP IPM ATM TZP MEM	CIP PIP GM TZP MEM	FEP CIP CAZ PIP IPM ATM TZP MEM	FEP CIP CAZ PIP IPM ATM TZP MEM	FEP CIP PIP ATM	FEP CIP CAZ PIP AN ATM MEM	FEP CIP CAZ PIP ATM TZP MEM	FEP CIP CAZ PIP GM AN ATM TZP MEM	FEP CIP CAZ PIP IPM ATM TZP MEM

[a]FEP, cefepime; CIP, ciprofloxacin; CAZ, ceftazidime; PIP, piperacillin; GM, gentamicin; AN, amikacin; IPM, imipenem; ATM, aztreonam; TZP, tazobactam; MEM, meropenem.

Table 2. Phages used in host range determination

Phage	Isolation source	Propagation host	Reference
ELT1	Retail raw chicken	*E. coli* MG1655	This study
ELT2	Duck feces	*E. coli* MG1655	This study
ELT3	Retail raw chicken	*E. coli* MG1655	This study
ELT4	Pork	*E. coli* MG1655	This study
ELSA1	Sewage	*E. coli* EC12	This study
ELSA2	Sewage	*E. coli* EC9	This study
SSP1	Chicken feces	*E. coli* MG1655	Laboratory stock
JEP6	Retail raw chicken	*E. coli* E79	Laboratory stock
JEP7	Retail raw chicken	*E. coli* E45	Laboratory stock

Table 3. Host ranges of *E. coli* phages against MDR *E. coli* clinical isolates

Phage	MDR *E. coli* clinical isolate[a]																					
	EC1	EC2	EC3	EC4	EC5	EC6	EC7	EC8	EC9	EC10	EC11	EC12	EC13	EC14	EC15	EC16	EC17	EC18	EC19	EC20	EC21	Total (%)
ELT1	++	+	-	I	+++	I	-	-	-	I	-	+++	+	I	I	++	I	I	I	I	+	7/21 (33%)
ELT2	+++	I	-	I	+++	I	-	-	-	I	-	-	-	-	I	++	I	I	I	I	I	3/21 (14%)
ELT3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0/21 (0%)
ELT4	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	I	+++	++	+	+	-	++	6/21 (29%)
ELSA1	+++	+++	-	++	+++	+	-	-	-	++	-	+++	-	+++	+++	++	+++	+	+	+++	+++	15/21 (71%)
ELSA2	-	-	-	-	-	-	-	-	+++	-	++	-	-	-	-	-	-	-	-	-	-	2/21 (9%)
SSP1	I	+	-	I	-	-	-	-	-	I	-	-	-	-	I	++	I	+++	+++	I	I	4/21 (19%)
JEP6	+++	+++	-	+	++	++	-	-	-	++	-	+++	-	I	I	+	I	++	++	+	I	11/21 (52%)
JEP7	++	I	-	I	I	I	-	++	-	+	-	I	-	I	I	+	I	I	I	+++	I	5/21 (24%)

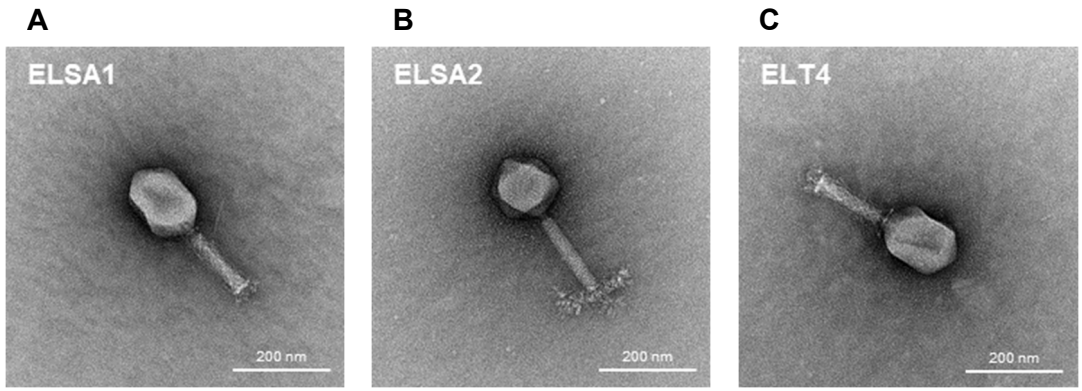


Figure 1. Transmission electron microscopy (TEM) images of isolates phages. Based on morphologies, *E. coli* phages ELSA1 (A), ELSA2 (B), and ELT4 (C) were classified as the *Myoviridae* family.

3.2 Phage stability under various stress conditions

Viable stabilities of phages ELSA1, ELSA2, ELT4 under a broad range of temperatures (4 to 80℃) and pH values (2 to 11) were determined. In results, viabilities of three phages were stably maintained between 4 to 55℃ which is indicated as optimal temperature for phage activities. However, viabilities of three phages decreased at 60℃, and were completely lost above 65℃. In the pH stability test, activities of three phages were stable between pH 3 to 11. However, all phages completely lost their viabilities under pH 2 which indicates strong acidic condition.

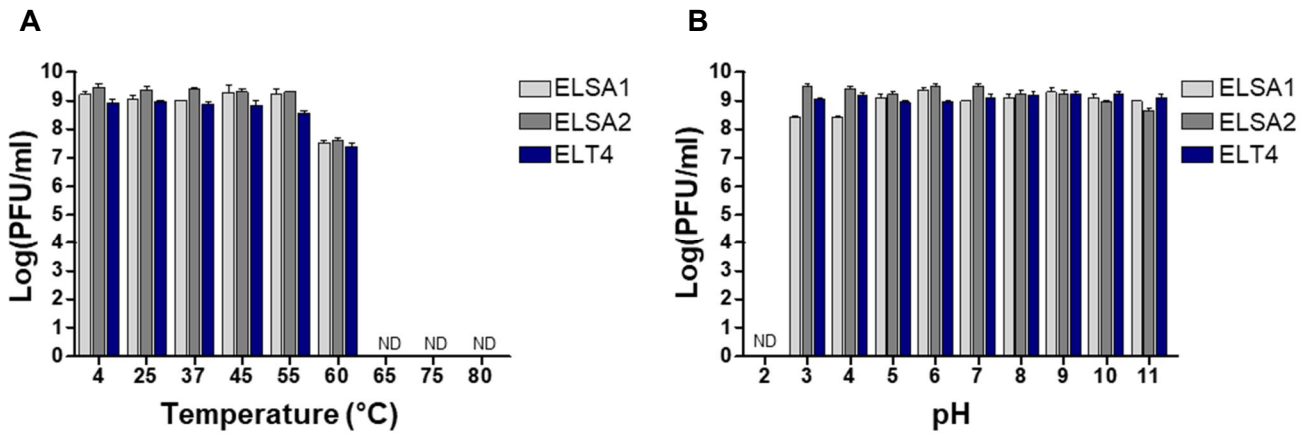


Figure 2. Stabilities of phages after under various temperatures (A) and pH (B). Error bar indicates standard deviation in triplicate experiments. ND, not detected

3.3 Receptor analysis of phages

It is known that *E. coli* phages from *myoviridae* family usually use lipopolysaccharide (LPS) or outer membrane proteins such as OmpA and Tsx of host bacteria (Silva et al., 2016). In phage receptor analysis, *E. coli* MG1655 derivative mutant strains with deletions of *ompA*, *waaG*, or *tsx* genes using lambda red recombination system which encode outer membrane protein A, lipopolysaccharide glucosyltransferase I, and nucleoside-specific channel-forming protein Tsx were used. In the results, MG1655 derivative △*ompA* and △*waaG* mutant strains were resistant to phage ELSA1, and complemented mutants restored their susceptibilities against ELSA1. Also, △*tsx* deletion mutant strain showed resistance to phage ELT4, however infectivity of ELT4 was restored when mutant strain was complemented with *tsx* gene. The results indicate that phage ELSA1 uses LPS and OmpA, but ELT4 uses Tsx to infect the hosts (Table 4).

Table 4. Receptors of phages ELSA1 and ELT4 identified in the study

Phage	Receptor
ELSA1	OmpA (Outer membrane protein A) and Lipopolysaccharide
ELSA2	Unknown
ELT4	Tsx (Nucleoside specific channel forming protein)

3.4 Phage one-step growth curve

In analysis of phage one-step growth curve, phages were inoculated to host bacterial culture and incubation time was determined using results from adsorption assay of each phage. The eclipse periods, latent periods, and burst sizes of three phages were determined by one-step growth curve analysis (Figure 3). Phages ELSA1, ELSA2, and ELT4 showed 5, 15, and 10 min of eclipse periods with 10, 40, and 20 min of latent periods, respectively. The bust sizes of phages ELSA1, ELSA2, and ELT4 after cell lysis were 33, 160, and 45 PFU per cell. For 60 min in the experiments, cell lysis by ELT4 occurred twice (Shkoporov et al., 2018). Among three phages tested, ELSA2 showed longer eclipse and latent period followed by a bigger bust size than those of phages ELSA1 and ELT4. On the other hand, phage showed short eclipse and latent period, and relatively smaller burst size than that of two other phages.

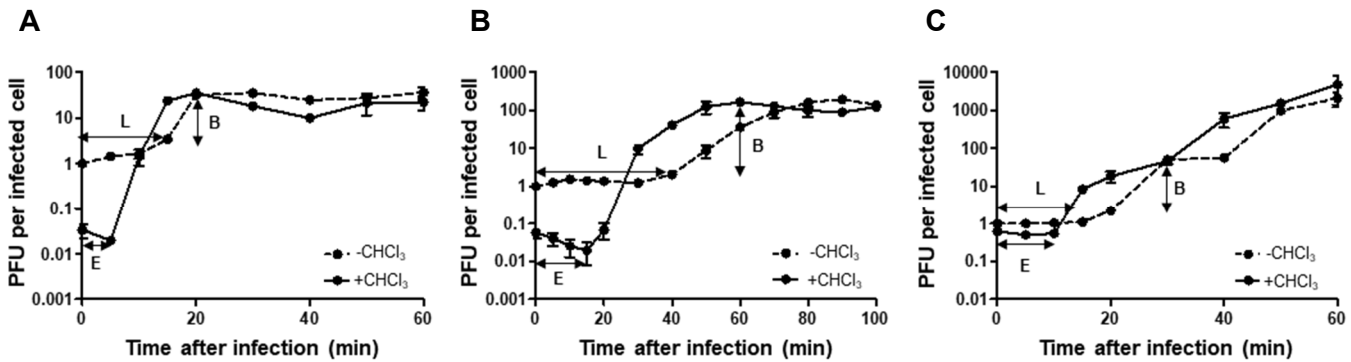


Figure 3. Analysis of one-step growth curves of phages ELSA1 (A), ELSA2 (B), and ELT4 (C) against hosts. Each bacterial host was infected by phages at a MOI of 0.01. Bacterial culture was incubated in LB broth at 37°C. E, eclipse period; L, latent period; B, burst size. Error bar indicates standard deviation in triplicate experiments.

3.5 Inhibition of host strains with phages

Bacterial inhibition analysis using single phage were performed to evaluate the lytic activities of phages ELSA1, ELSA2, and ELT4 against *E. coli* EC12, EC9, and MG1655, respectively. ELSA1 completely reduced the growth of *E. coli* EC12 at MOIs of 0.1, 1, and 10 for 6h after inoculation, and regrowth of host bacteria was observed. ELSA2 showed the best lytic ability to suppress bacterial growth of *E. coli* EC9 at a MOI of 10. When MOI of 0.1 or 1 were inoculated, ELSA2 didn't show efficient antibacterial activities compared to the phage non-treated negative control. At MOIs of 0.1, 1, and 10, ELT4 showed antibacterial activities against *E. coli* MG1655 for 10 h after infection. Results of bacterial inhibition assay using ELT4 showed that the higher the MOI used, the faster the bacterial regrowth was observed.

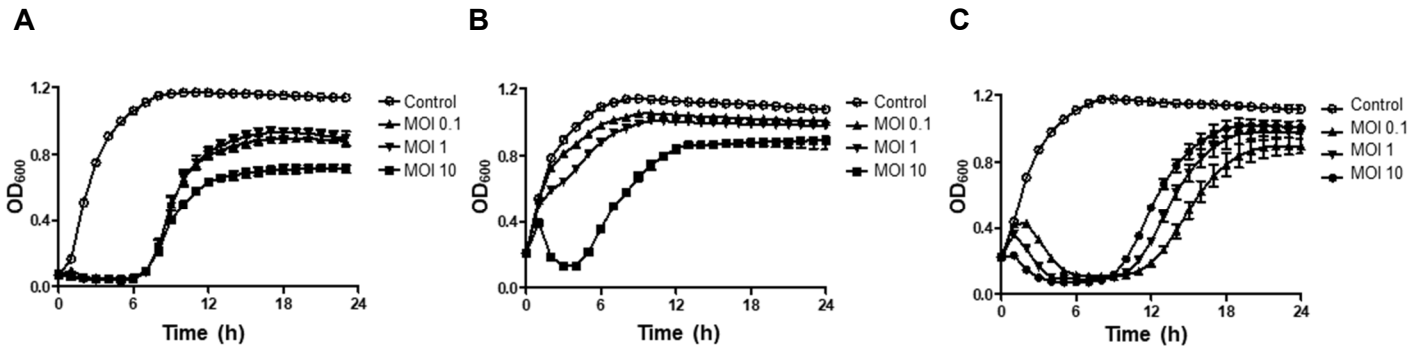


Figure 4. Growth inhibition assays of phages against hosts. Antibacterial activities of phages ELSA1 (A), ELSA2 (B), and ELT4 (C) were evaluated at MOIs of 0.1, 1, and 10 against *E. coli* EC12, EC9, and MG1655, respectively. Error bar indicates standard deviation in triplicate experiments.

3.6 Whole genome analysis of phages

Genome sequences of phages ELSA1, ELSA2, and ELT4 were determined by whole-genome analysis (Figure 5). Three phages had genome sizes of 169 kb (ELSA1), 110 kb (ELSA2), and 165 kb (ELT4) with predicted genes of 299, 154, and 281 open reading frames (ORFs). Genes related with lysogeny functions such as integrases, recombinases, transposases, excisionases, and repressors were not detected from genome sequences of three phages, which indicates that phages used in this study are lytic phages (Farlow et al., 2020). Also, genes encoding bacterial antimicrobial resistance or toxicity were not detected from the phage genomes. From genome sequences of phages ELSA1 and ELT4, genes encoding phage spanin and holin, which are required for lysis of phage in bacterial hosts (Ry Young, 2013) were identified and arranged on phage genomes.

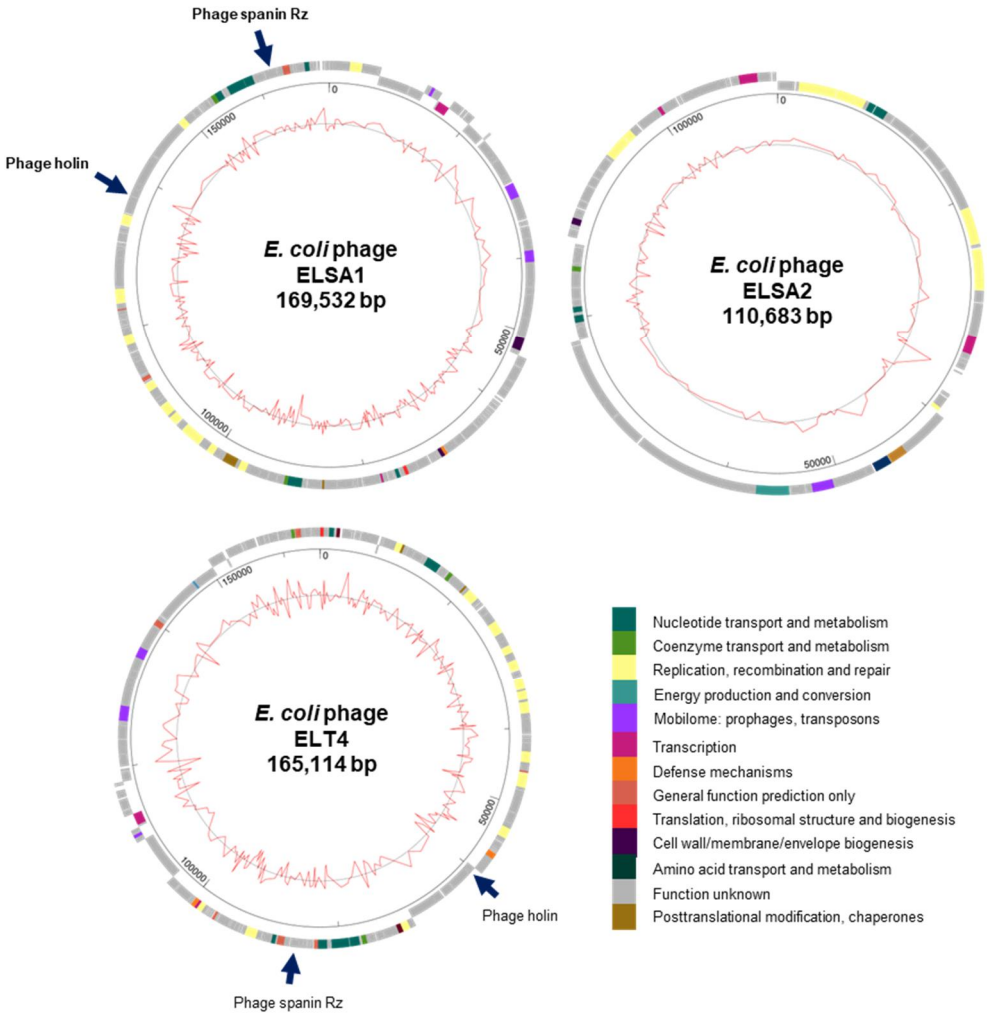
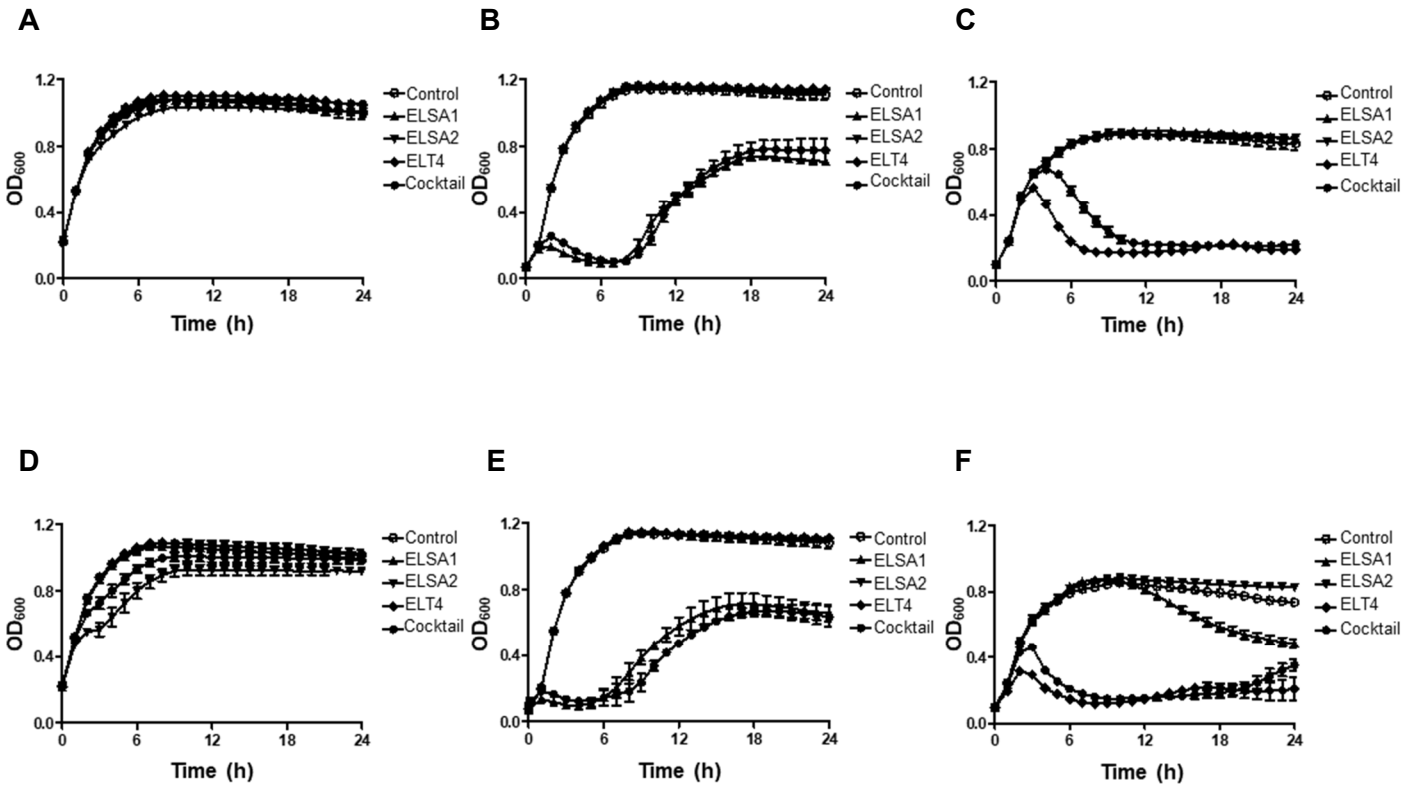


Figure 5. Circular genome maps of *E. coli* phages. Predicted ORFs are arranged on genome maps of phages ELSA1, ELSA2, and ELT4. Functional groups are categorized into colors as shown in the legend.

3.7 Inhibition of MDR *E. coli* with the phage cocktail

Bacterial inhibition analysis using MDR *E. coli* strains were performed. The antibacterial activities of the single phages and the phage cocktail were evaluated and compared. Three MDR *E. coli* strains (EC9, EC12, and EC16) isolated from different sources were selected for the study. Lytic activities of phages against single hosts were evaluated at MOIs of 0.1, 1, 10 (Figure 6). Phage cocktail composed of phages ELSA1, ELSA2, and ELT4 reduced bacterial growth of all hosts tested. The results suggest that antibacterial activities of phage cocktail against MDR strains were comparable to that of single phage against hosts at three different MOIs.

Also, antibacterial activities of the single phages and the phage cocktail were evaluated using mixed culture of three MDR *E. coli* strains were determined (Figure 7) of phage cocktail reduced bacterial growth of mixed cultures of MDR strains, whereas single phage treatment did not at three different MOIs. And the phage cocktail showed the greatest antibacterial activities against bacterial mixture when a MOI of 10 was used.


A
B
C
D
E
F
OD600
Time (h)
0.0
0.4
0.8
1.2
0
6
12
18
24
Control
ELSA1
ELSA2
ELT4
Cocktail

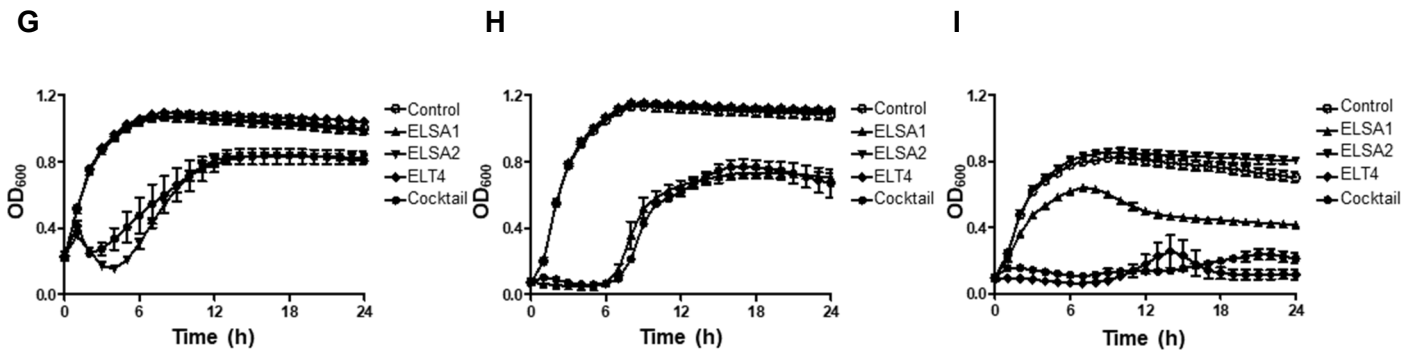


Figure 6. Phage inhibition of MDR *E. coli* strains. The phage inhibitions against three MDR *E. coli* strains at three different MOIs in the assay included: (A) inhibition of EC9 at a MOI of 0.1, (B) inhibition of EC12 at a MOI of 0.1, (C) inhibition of EC16 at a MOI of 0.1, (D) inhibition of EC9 at a MOI of 1, (E) inhibition of EC12 at a MOI of 1, (F) inhibition of EC16 at a MOI of 1, (G) inhibition of EC9 at a MOI of 10, (H) inhibition of EC12 at a MOI of 10, and (I) inhibition of EC16 at a MOI of 10. Error bar indicates standard deviation in triplicate experiments.

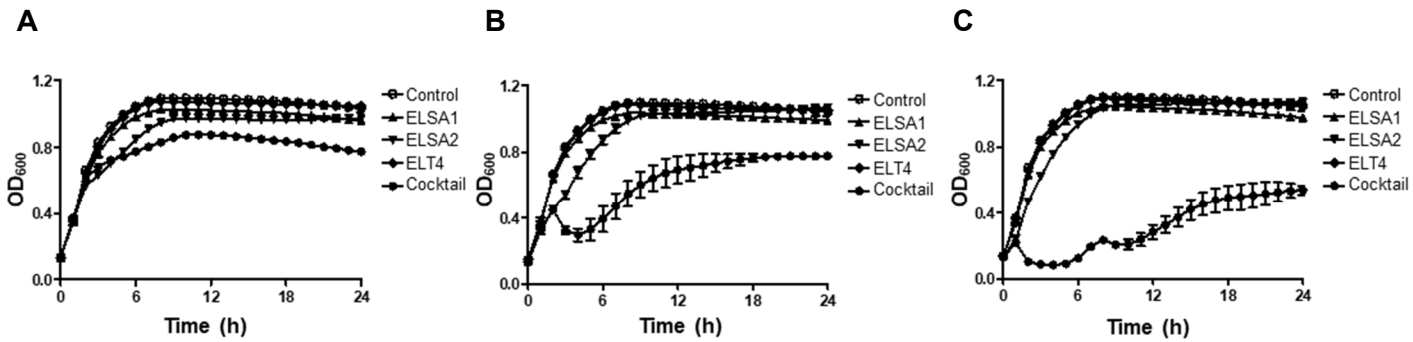


Figure 7. Phage inhibition of mixed cultures of MDR *E. coli* strains. Antibacterial activities of phages ELSA1, ELSA2, ELT4, and phage cocktail were evaluated at MOIs of 0.1 (A), 1 (B), and 10 (C) against mixed culture of *E. coli* EC9, EC12, and EC16. Error bar indicates standard deviation in triplicate experiments.

3.8 Food application using the phage cocktail

The efficacy of the phage cocktail to control the mixed culture of MDR *E. coli* strains (EC9, EC12, and EC16) on the surface of romaine lettuce was evaluated at MOIs of 10 to 10^4. As romaine lettuce products are usually stored at refrigeration conditions or room temperatures for handling, antibacterial efficacy of phage treatment was determined at 4 and 25°C. No bacterial count was measured from samples after washing with 70% ethanol and UV treatment. At 4°C, the treatment phage reduced bacterial levels at four inoculation levels. Phage cocktail showed the best efficacy of infection at a MOI of 10^4, which reduced the mixed culture of MDR *E. coli* strains by 1.97 log CFU/cm^2 after 3 h. At 25°C, the phage cocktail treatments at four inoculation levels showed reduction of bacterial counts of mixed culture after 3 h, however after 3 h MDR *E. coli* strains continued to grow on the surface of romaine lettuce. Phage treatment at a MOI of 10^4 showed the greatest reduction of mixed culture by 2.29 log CFU/cm^2 and 3.63 CFU/cm^2 after 3 h and 24 h, respectively. The results indicate that the phage cocktail used in the study showed antibacterial activities against MDR *E. coli* mixed culture on the romaine lettuce, and phage cocktail treatment at a MOI of 10^4 is highly effective in inhibition of bacterial hosts at 4°C and 25°C.

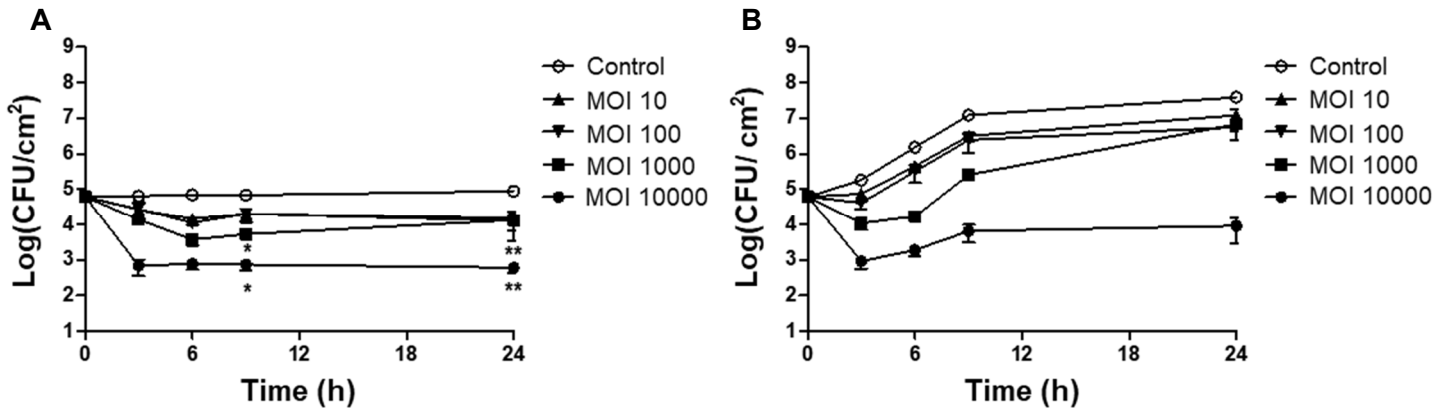


Figure 8. Phage inhibition of bacterial mixture using *E. coli* clinical isolates on romaine lettuce. The levels of MDR *E. coli* strains on the surface of romaine lettuce without or with the phage cocktail treatment at 4°C (A) and 25°C (B). Error bar indicates standard deviation in triplicate experiment. Statistical analysis was performed using a Student's *t*-test compared to the non-treated control with GraphPad Prism (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

3.9 Evaluation of phage-antibiotic synergy effect

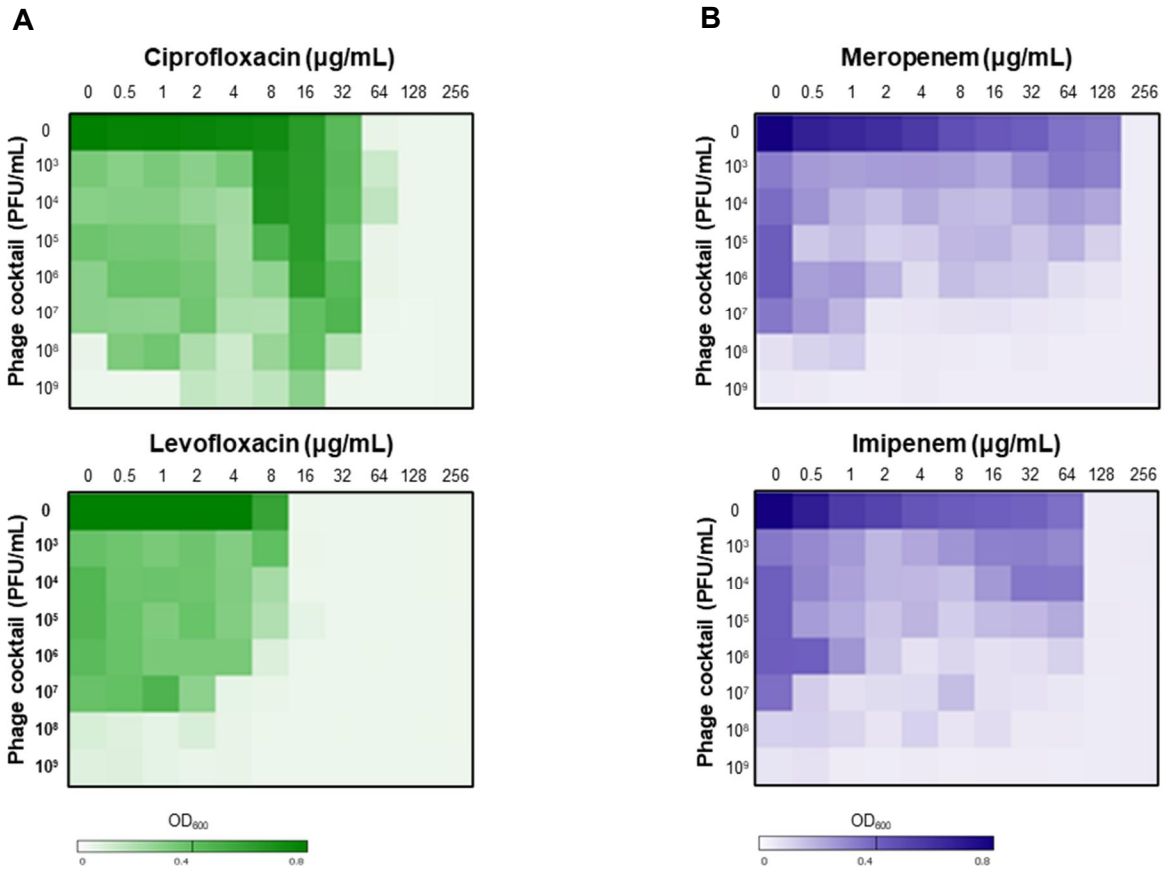
Minimum inhibitory concentrations (MICs) of eight antibiotics (ciprofloxacin, levofloxacin, ceftriaxone, ceftazidime, meropenem, imipenem, ampicillin, and piperacillin-tazobactam) for MDR *E. coli* strains EC9, EC12, EC16 were determined by the broth microdilution method (Table 5). The results indicate that three *E. coli* strains are resistance to eight antibiotics tested, which shows the multidrug resistance. The breakpoints of three strains for ceftriaxone, ampicillin, and piperacillin-tazobactam were over 256 μg/ml.

The checkerboard titration assays in presence of eight antibiotics used in MIC determination and phage cocktail were conducted to evaluate the synergy effect in inhibition efficacy of mixed culture including three MDR *E. coli* strains by combining two other antimicrobial agents. The most remarkable synergy effects were observed when the phage cocktail was treated with meropenem and imipenem, which are included in carbapenems class (Figure 3B). Although the MIC of meropenem and imipenem were 256 μg/ml and 128 μg/ml for bacterial mixture, growth inhibition of bacteria was observed under MIC of antibiotics in presence of the phage cocktail at high MOIs. Ciprofloxacin and levofloxacin, which are in quinolone class showed conflicting effects in antibacterial ability when treated with the phage cocktail (Figure 3A). It was confirmed that there were sections in which the antibacterial activity of phage cocktail decreased when treated with

ciprofloxacin, whereas levofloxacin showed synergy effects in growth inhibition when combined with phage cocktail at high concentration (10^6 to 10^7 PFU/ml). Four antibiotics which are included in cephalosporins or penicillins class didn't make enhancement or reduction of antibacterial activities when treated with the phage cocktail under concentrations of 256 μg/ml. These results suggest that treatment of the phage cocktail could lead to synergism or antagonism of antibacterial activity, or no change when used with antibiotics.

Table 5. Minimum inhibitory concentrations (MICs) of antibiotics for *E. coli* EC9, EC12, and EC16

Antibiotics class	Antibiotics	MIC (μg/ml)		
		E. coli EC9	*E. coli* EC12	*E. coli* EC16
Fluoroquinolones	Ciprofloxacin	32	64	64
	Levofloxacin	8	16	16
Cephalosporins	Ceftriaxone	>256	>256	>256
	Ceftazidime	>256	>256	32
Carbapenems	Meropenem	8	32	256
	Imipenem	4	8	128
Penicillins or Penicillin/Beta-lactamase-inhibitor	Ampicillin	>256	>256	>256
	Piperacillin-tazobactam	>256/4	>256/4	>256/4


A
Ciprofloxacin (µg/mL)
0 0.5 1 2 4 8 16 32 64 128 256
Phage cocktail (PFU/mL)
0
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
Levofloxacin (µg/mL)
0 0.5 1 2 4 8 16 32 64 128 256
Phage cocktail (PFU/mL)
0
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
OD600
0
0.4
0.8
B
Meropenem (µg/mL)
0 0.5 1 2 4 8 16 32 64 128 256
Phage cocktail (PFU/mL)
0
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
Imipenem (µg/mL)
0 0.5 1 2 4 8 16 32 64 128 256
Phage cocktail (PFU/mL)
0
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
OD600
0
0.4
0.8

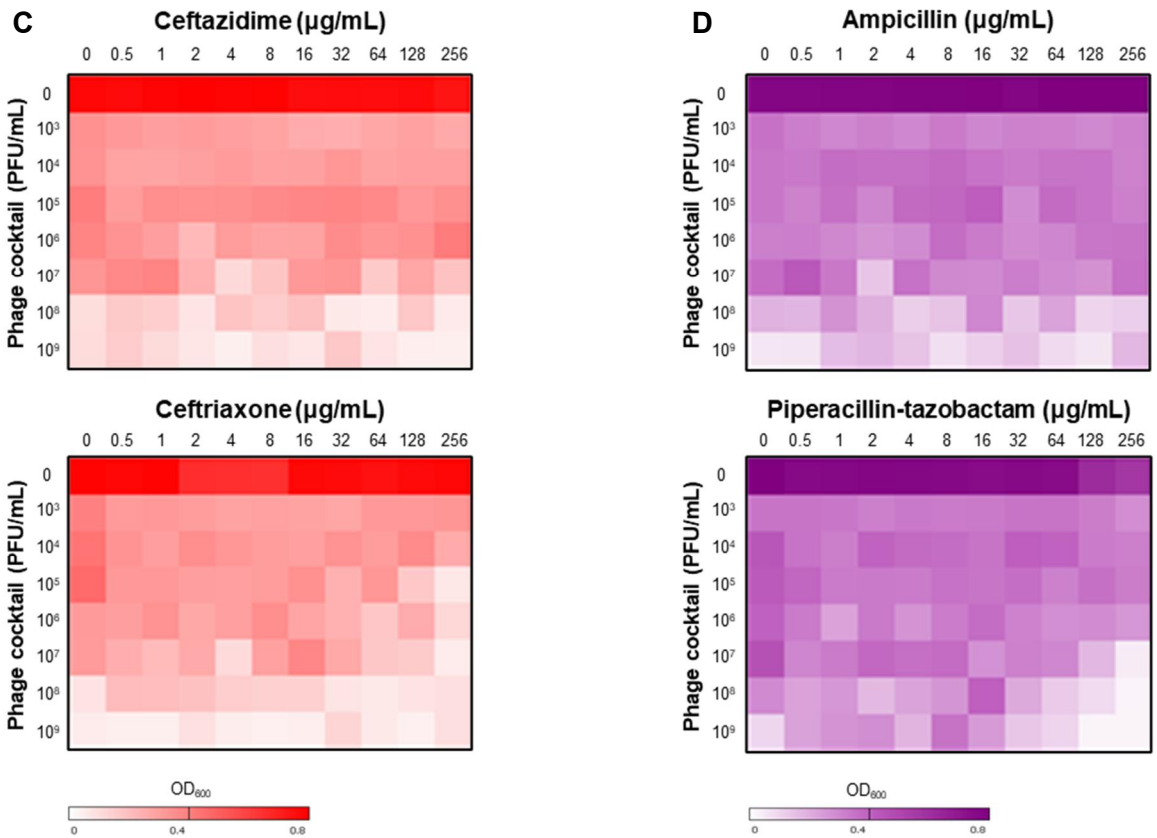

C
Ceftazidime (µg/mL)
0 0.5 1 2 4 8 16 32 64 128 256
Phage cocktail (PFU/mL)
0
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
D
Ampicillin (µg/mL)
Ceftriaxone (µg/mL)
Piperacillin-tazobactam (µg/mL)
OD600
0
0.4
0.8

Figure 9. Checkerboard titration assays using the phage cocktail. Antibacterial synergy between the phage cocktail and antibiotics from four quinolones (A), carbapenems (B), cephalosporins (C), and penicillins (D) classes were evaluated. The experiments were repeated at least three times.

IV. DISCUSSION

In this study, a total of twenty-one MDR *E. coli* clinical isolates were obtained and used. *E. coli* strains were isolated from various sources including urine, hand, blood, sputum, stool, bile, and ascites of patients. Antibiotic resistance patterns of strains were determined using antibiotics from cephalosporins, quinolones, penicillins, aminoglycosides, carbapenems, and monobactams classes. The results show that all isolates are multidrug-resistant.

To control a range of MDR *E. coli* strains, I isolated six different *E. coli* phages from retail raw chicken, duck feces, pork, and sewage samples. In phage host range determination, a total of nine phages were used including three different *E. coli* phages SSP1, JEP6, and JEP7 from laboratory stocks. Among those phages, ELSA1 showed the broadest host range by infecting 71.4% (15/21) of MDR *E. coli* strains. The phage cocktail composed of phages ELSA1, ELSA2, and ELT4 were designed because the cocktail can control 85.7% (18/21) of MDR *E. coli* strains, which is identified as optimal combination of phages. By mixing phages infecting various hosts, the limitations of single phage treatment can be solved (Nilsson, 2014).

Biochemical and genomic characterization of three phages composing the cocktail were conducted. In morphological analysis, all phages were classified as *myoviridae* family based on the morphologies with big heads and contractile tails. Based on stability test under a range of stress condition, phages ELSA1, ELSA2,

and ELT4 stably maintained their viabilities between 4 to 55°C and pH 3 to 11, which indicates that three phages used in this study are not thermophilic.

In phage receptor analysis, results showed that phage ELSA1 uses OmpA and LPS, whereas ELT4 uses Tsx as receptors for host infection. It is unknown whether OmpA and LPS form as types of a coreceptor or primary-secondary receptor complex for the phage infection. However, it is known that LPS plays a major role in the folding and conformation of outer membrane proteins of bacteria including OmpA (Morona R et al., 1984; Bogdanov M, Dowhan W., 1999). Also, it is known that LPS and Skp participate in placing OmpA in the outer membrane and supports development of its secondary and tertiary structures (Levin BR, 2010; Mutalik VK, 2020). Therefore, a hypothesis can be suggested in which improper conformation or placement of OmpA by damage of LPS may result in the resistance to phage of bacteria with deletions of genes related to LPS. Further research for relationships between receptors OmpA and LPS is needed.

In whole genome analysis of phages ELSA1, ELSA2, and ELT4, no genes were detected which are related to lysogenic life cycle of phage including integrase, recombinases, excisionases, and repressors. These results indicate three phages have lytic cycles (Farlow et al., 2020). It is known that lytic phages are more optimal for therapy because lysogenic phages without any genetic modifications usually can acquire toxic genes in progress of phage genome integration into host chromosomes

(Matsuzaki et al., 2005). Therefore, phages ELSA1, ELSA2, and ELT4 are thought to be suitable candidates for phage therapy.

In phage inhibition analysis using MDR *E. coli* strains with three phages, antibacterial activities of the single phages and the phage cocktail were evaluated and compared. The phage cocktail showed comparable antibacterial activities with that of single phages against MDR *E. coli* EC9, EC12, and EC16. These results suggest that use of the phage cocktail can be more efficient than single phages in phage treatment. Also, the mixed culture of three MDR *E. coli* strains were used in inhibition assays to assess and compare antibacterial activities of single phage and the phage cocktail treatments. In the results, single phage treatments did not reduce the growth of MDR *E. coli* mixture, whereas the phage cocktail treatment reduced the growth of mixed culture, especially effectively at a MOI of 10. These results indicate that the phage cocktail can show effective inhibition of a range of hosts in the conditions including various bacterial strains which single phages cannot (Legesse Garedew Kifelew, 2020). Also, it is known that the phage cocktail composed of phages using different receptor can enhance the antibacterial activity and prevent the appearance of phage-resistant strains (Levin BR, 2004). Therefore, there can be a synergy effect in controlling bacterial hosts when treating the phage cocktail constructed in this study.

It is known that phage treatment can be an optimal option for inhibition of specific foodborne pathogens not controlling beneficial bacterial strains. And to reduce pathogens effectively, treatment using high numbers of phage are suggested (Coffey, Mills et al. 2010). In food application using the phage cocktail, the pieces of romaine lettuce were used for bacterial contamination of MDR *E. coli* strains. The phage cocktail was treated at MOIs of 10 to 10^4. In results, numbers of MDR *E. coli* reduced after the phage cocktail treatment at all MOIs tested at 4°C and 25°C. The phage cocktail treatment at a MOI of 10^4 showed the highest effectivity in inhibition of bacterial hosts both at 4°C and 25°C, which indicates high numbers of phages are needed for improved efficacy of infection.

To evaluate the phage-antibiotic synergy (PAS) effect using the phage cocktail and antibiotics, the checkerboard titration assays were conducted. Total eight antibiotics (ciprofloxacin, levofloxacin, ceftriaxone, ceftazidime, meropenem, imipenem, ampicillin, and piperacillin-tazobactam) from four classes were selected based on the World Health Organization's (WHO) Essential Medicine List and results of MIC tests using MDR *E. coli* EC9, EC12, and EC16 (Budd, 2019). The phage cocktail showed noticeable synergy effects in inhibitions of MDR *E. coli* strains when inoculated with meropenem and imipenem from carbapenems class. On the other hand, the phage cocktail didn't show any enhancement or decrease of antibacterial activities when combined with four antibiotics included in cephalosporins or penicillins. It is known that beta-lactam antibiotics inhibit the

penicillin binding proteins (PBPs) of bacterial cell and can increase phage activity by accelerating cell lysis (Chengxi Liu, 2022). Therefore, it is thought that because of high MIC values of those four antibiotics against MDR *E. coli* strains, PAS effects of antibiotics and the phage cocktail were not detected. In the results using quinolones and the phage cocktail, ciprofloxacin showed antagonism effects in antibacterial activities by decreasing the activities of the phage cocktail. Related research showed that quinolones can inhibit DNA replication of the phage by preventing the host cell DNA gyrase regardless of phage's own DNA gyrase. It was thought that ciprofloxacin and levofloxacin showed conflicting effects in bacterial inhibition because of differences in MICs against MDR *E. coli* strains. Further studies showed that ciprofloxacin also showed antagonism when treated with the phage cocktail under concentrations of 10^3 PFU/ml. These results suggest the phage cocktail with antibiotics from carbapenems can be ideal candidate for phage therapy.

In conclusion, we designed the phage cocktail with broad host range against MDR *E. coli* strains and aimed to evaluate the efficiency of inhibition of the phage cocktail in various conditions. The phage cocktail showed effective antibacterial activities when used in food application and showed synergy effect when treated with specific antibiotics in checkerboard titration assays. Overall, this study suggests the phage cocktail designed in the study is highly utilized as a biocontrol agent in controlling various MDR *E. coli* strains.

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국문 초록

다제내성 세균은 전세계적으로 증가하고 있으며 새로운 치료제의 개발이 요구된다. 대장균은 그람 음성균의 하나로서 다양한 질병의 원인이 되며, 다양한 국가에서 다제내성 대장균의 출현이 보고되고 있다. 박테리오파지(파지)는 항생제의 유망한 대안으로 고려되며, 파지 칵테일은 파지 처리 중 숙주 범위를 넓히기 위해 주로 이용된다. 본 연구에서는 다양한 다제내성 대장균 임상 균주들을 제어할 수 있는 파지들을 분리하였다. 파지들의 생화학 및 유전학적 특성 분석을 진행하였고, 넓은 숙주 범위 기반의 파지 칵테일을 이용하여 여러 조건에서 감염 효과를 평가했다. 다양한 종류의 항생제에 내성을 갖는 총 21 주의 다제내성 대장균 임상 분리 균주들이 본 연구에서 이용되었다. 그 결과, 총 여섯 개의 대장균 파지들을 분리하였다. 파지의 숙주 범위 평가를 통해 ELSA1, ELSA2, 그리고 ELT4 로 구성된 파지 칵테일을 동일 비율로 구축하였고, 이는 다제내성 대장균 균주들에 대하여 넓은 숙주 범위를 갖는다. 해당 파지 칵테일은 다제내성 대장균 균주의 85.7%(18/21)를 제어하였다. 투과전자현미경 분석을 통해 세 개 파지 모두 *Myoviridae* family 에 속함을 확인하였다. 파지들의 활성은 4-55°C 와 pH 3-11 범위에서 각각 안정성을 보였다. 파지의 성장 곡선

분석 실험에서, 파지 ELSA1, ELSA2, 그리고 ELT4 는 각각 세포 당 33, 160, 45 플라크 형성 단위만큼의 증폭량을 가짐을 확인하였다. 파지의 전유전체 서열 분석 결과 ELSA1, ELSA2, 그리고 ELT4 는 각각 약 170, 110, 그리고 165 kb 의 유전체 크기를 가짐을 확인하였다. 파지의 수용체 분석 결과 ELSA1 은 지질다당류와 외막단백질 A 를, ELT4 는 Tsx 단백질을 숙주 감염시에 수용체로 이용하는 것을 확인하였다. 3 주의 다제내성 대장균 균주 각각과 혼합 균주에 대한 단일 파지 및 파지 칵테일의 균 성장 억제 실험 수행 결과, 세 가지 다른 MOI 조건에서 단일 균주에 대한 파지 칵테일의 항균 능력은 단일 파지의 능력과 유사하였으며, 파지 칵테일은 단일 파지에 비해 다제내성 대장균 혼합 균주의 증식을 더 효과적으로 제어하였다. 식품 적용 실험에서 파지 칵테일은 10^4 의 MOI 조건에서 로메인 양상추 표면의 다제내성 대장균을 4℃ 와 25℃ 에서 효과적으로 감소하였다. 파지 칵테일과 8 개 항생제 (시프로플록사신, 레보플록사신, 메로페넴, 이미페넴, 세프타지딤, 세프트리악손, 암피실린, 타조신)를 이용한 checkerboard 적정 실험에서, 파지 칵테일은 메로페넴 혹은 이미페넴과 함께 투여되었을 때 세 다제내성 대장균 혼합 균주의 증식을 억제함에 있어 synergy effect 를 보였다. 종합하면, 본 연구에서 구축된 파지 칵테일은 다양한 다제내성 대장균에 대하여 단일 파지에 비해 개선된

항균 효과를 보였다. 더 나아가, 식품 및 checkerboard 실험 결과를 바탕으로 본 연구에서 이용된 파지 칵테일은 생물학적 방제제로서 활용도가 높은 것으로 사료된다.

주요어: 다제내성, 대장균, 박테리오파지, 박테리오파지 칵테일

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