THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Isolation and Characterization of Novel Bacteriophages KPP2018 and KPP2020 infecting *Klebsiella pneumoniae* and Development of a Phage Cocktail as a Biocontrol Agent in Food

클렙시엘라 뉴모니애를 저해하는
박테리오파지 KPP2018과 KPP2020의 분리 및
특성 분석과 칵테일을 이용한
식품에서의 생물학적 제어

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ABSTRACT

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Klebsiella pneumoniae is a well-known opportunistic human pathogen causing chronic pulmonary obstruction and primarily infects the immunocompromised individuals in nosocomial environment. This pathogen can produce extended-spectrum beta-lactamases (ESBL), which are resistant to almost all beta-lactam antibiotics, and nowadays carbapenem-resistant strains are increasing. Recently, *Klebsiella pneumoniae* is detected in food samples, especially in poultry products or in raw vegetables. Therefore, the development of new agent is urgently needed and to control this pathogen, 12 *Klebsiella*-infecting phages were isolated from sewage samples. The analysis of host range revealed that the isolated phage KPP2020 has high host specificity among them, inhibiting only *K. pneumoniae*. The phage KPP2018 infects *K. pneumoniae* mainly, also infects *Shigella* spp., and *Salmonella* serovars. Morphological observation using TEM showed that both phages belong to the family *Siphoviridae*. The stability of KPP2020 and KPP2018 was maintained for 12 h under stress conditions (-20~60℃ and pH 3~11 for KPP2020 and -20~65℃ and pH 3~12 for KPP2018). Bacterial challenge assay of KPP2020 showed 3.51 log reduction of *K. pneumoniae* KCTC 2242 within 2 h. The complete genomes of KPP2020 and KPP2018 were analyzed and revealed that

KPP2020 consists of 49,044 bp containing 95 ORFs with a GC content 51.33%, while KPP2018 consists of 137,988 bp DNA with 228 ORFs. Subsequent bioinformatics analysis revealed no toxin genes or virulence factor, suggesting the safety for human applications. Comparative genome analysis about tail gene cluster was conducted and there was no identity between KPP2020 and KPP2018 tail-related genes, indicating that differences in host range results may related to this. Application of KPP2020 using cutting board showed about 4 log reduction for at least 7 h, indicating that KPP2020 has potential to control *K. pneumoniae* effectively. Food application of the phage cocktail consisting of KPP2020 and KPP2018 in a 1:1 ratio using chicken meat showed higher lytic activity (4.35 log reduction within 2 h) and phage resistance of indicator strain developed slower than that of single phages. KPP2020 can lower the secretion of pro-inflammatory cytokines of *K. pneumoniae* infected RAW 264.7 cells, suggesting that KPP2020 has therapeutic effect against bacterial infection. KPP2020 does not induced inflammatory response of RAW 264.7 cells and not involved in the response induced by LPS, suggesting that KPP2020 can be an effective therapeutic agent against bacterial infection. Therefore, these two novel bacteriophages KPP2020 and KPP2018 can be used as natural food preservatives for food safety, and KPP2020 can be a therapeutic agent against *K. pneumoniae* infection.

Keywords: *Klebsiella pneumoniae*, Bacteriophage, Phage therapy, Phage cocktail

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1. INTRODUCTION

Klebsiella can be found ubiquitously in the environment like surface water or soil (Podschun, Pietsch, Höller, & Ullmann, 2001), (Podschun & Ullmann, 1998), and in the mucosal surfaces of mammals. *Klebsiella* is a well-known opportunistic human pathogen which can cause severe infections to respiratory tract, urinary tract and blood stream, and primarily infects immunocompromised individuals who are hospitalized or suffering from underlying diseases (Podschun & Ullmann, 1998). It has been estimated that it causes about 10% of all nosocomial bacterial infections in the United States and Europe (Struve & Krogfelt, 2004). Nowadays *Klebsiella pneumoniae* is gaining attention due to the increasing number of strains resistant to antibiotics, *Klebsiella* has the ability to produce extended-spectrum beta-lactamases (ESBL) which are resistant to almost all beta-lactam antibiotics (Nathisuwan, Burgess, & Lewis Ii, 2001). The Centers for Disease Control and Prevention (CDC) reported 8% of all *Klebsiella* isolates were carbapenem-resistant *K. pneumoniae* (Hirsch & Tam, 2010), and the pooled mortality was 42.145 among the 2,462 patients infected with carbapenem-resistant *K. pneumoniae* (Xu, Sun, & Ma, 2017). The increase in multidrug resistant *K. pneumoniae* strains is due to not only the outer membrane structure which protects them from many antibiotics, also the abuse of various antibiotics for therapy. In 2017, the World Health Organization (WHO) published a list of bacteria where new antibiotics to tackle them are needed urgently and grouped them according to their priority as critical, high, and medium (Breijyeh,

Jubeh, & Karaman, 2020). Among various drug resistant strain, carbapenem-resistant *Klebsiella pneumoniae* and 3rd generation cephalosporin-resistant strains are classified in critical group, which are urgently needed to develop new antibiotics or alternatives.

According to the U.S. Centers for Disease Control (CDC) and the U.S. Food and Drug Administration (FDA), *Klebsiella pneumoniae* is not a foodborne pathogen, however, in USA market, 14% of 316 raw chicken samples were positive for *Klebsiella*. In 2018, 47% of 508 meat products were contaminated with *Klebsiella*, and among the broiler samples, 25.8% were positive for *K. penumoniae*, with many strains being antibiotic-resistant (Franklin-Alming et al., 2021). Some strains were nearly identical to the isolates from the patients with urinary tract or blood infections, suggesting that *K. pneumoniae* can be picked up from the food. Due to emergence and prevalence of multidrug resistant strains in the nosocomial environment and food samples, it is vital to develop alternatives to control *K. pneumoniae* in nosocomial environment or food product.

Bacteriophage (phage) has been proposed as a novel biocontrol agent to control bacteria. Phages are self-replicating viruses that infect only specific host bacteria and do not infect mammalian cells. Phages are ubiquitous in the environment, and humans are routinely exposed to them at high levels through food and water without any adverse effects, indicating that they are safe for treatment of food products without additional treatments (Bai, Jeon, & Ryu, 2019). Moreover, phages do not affect properties of food products such as flavor, color and taste,

making phages an attractive alternative biocontrol agents in foods (Pietracha & Misiewicz, 2016). In deed, it has been already used to control various pathogens in Europe for several decades (Duckworth & Gulig, 2002), and the FDA approved phages as GRAS (Generally Recognized as Safe), indicating that phages are suitable biocontrol agent in food product. In 2006, various phage products are developed and approved as a novel natural preservative by FDA (Lang, 2006). Many commercial products were developed, for example the ListShield™ (Intralytix, USA) targeting *Listeria monocytogenes* contamination in foods and food processing facility, the EcoShield™ (Intralytix, USA) targeting *Escherichia coli* O157:H7, and the SALMONELEX™ (MICREOS Food Safety) targeting *Salmonella* (Bai, Kim, Ryu, & Lee, 2016). Also, the PowerPhage (BioChimPharm, Georgia) is developed as a feed additive to eliminates the pathogenic bacteria in poultry.

Although phage has the advantages mentioned, bacteria may rapidly evolve the resistance against phage infection. To overcome the phage resistance of bacteria, phage cocktails composed of more than two phages are used to improve the impact of phages on bacteria (Townsend et al., 2021). A phage cocktail composed of two phages targeting different receptors successfully reduced the emergence of phage-resistant *Escherichia coli* O157:H7 (Y. Tanji et al., 2004), and the cocktail containing three phages without knowing receptors showed a significant reduction in *E. coli* O157:H7 (Yasunori Tanji et al., 2005).

Although the concept of using phage as an anti-infective agent has been around for over a century, the number of patients with refractory bacterial infections

who have been treated with phage has increased in recent years. Positive clinical outcomes have been observed in multiple experimental cases, suggesting that phages can confer therapeutic benefits (Mathur, Vidhani, & Mehndiratta, 2003). The phage was injected at MOI of 1 into mice that had previously been infected intraperitoneally with multidrug-resistant *K. pneumoniae* ST28 (5×10^8 CFU/mouse). This treatment resulted in a significant improvement in the survival rate of the infected mice (Hesse et al., 2021).

In this study, 12 phages targeting *Klebsiella pneumoniae* were isolated. Among them, KPP2018 and KPP2020 were characterized and genomes were completely sequenced and analyzed using bioinformatic tools, showing that both phages are virulent phages and have all required components for reconstruction of the phages in the host cells. Furthermore, therapeutic effect of KPP2020 was evaluated by quantifying secretion of cytokines using murine macrophage, RAW 264.7 cells. In addition, application tests of KPP2020 and phage cocktail were carried out to evaluate the lytic activity on food sample and food processing facility. Both phages would be useful for the development of novel biocontrol agents against *K. pneumoniae*.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

All bacterial strains used in this study and their growth conditions are listed in Table 1. These bacteria were grown aerobically at 37℃, and as an indicator strain, *K. pneumoniae* KCTC 2242 was selected for phage isolation and characterization.

Table 1. Bacterial strains used in this study

Bacterial strain	Medium[a]	Reference or Source[b]
Gram-negative strains		
Klebsiella pneumoniae KCTC 2242	LB	KCTC
Klebsiella pneumoniae KCTC 2690	LB	KCTC
Klebsiella oxytoca ATCC 43863	LB	ATCC
Klebsiella oxytoca KCTC 1686	LB	KCTC
Shigella flexneri 2a strain 2457T	TSB	IVI
Shigella flexneri KCTC 2517	TSB	KCTC
Shigella flexneri KCTC 2993	TSB	KCTC
Shigella boydii KCTC 22528	TSB	KCTC
Shigella sonnei KCTC 22530	TSB	KCTC
Escherichia coli O157:H7 ATCC 43890	LB	ATCC
Escherichia coli MG1655	LB	(Hayashi et al., 2006)
Pseudomonas aeruginosa ATCC 27853	LB	ATCC
Cronobacter sakazakii ATCC 29544	TSB	ATCC
Yersinia enterocolitica ATCC 55075	TSB	ATCC
Salmonella Typhimurium LT2	LB	(McClelland et al., 2001)
Salmonella Typhimurium DT 104	LB	(Ribot, Wierzba, Angulo, & Barrett, 2002)
Salmonella Typhimurium ATCC 14028	LB	ATCC
Salmonella Typhimurium SL1344	LB	(Hoiseth & Stocker, 1981)
Salmonella Enteritidis ATCC 13076	LB	ATCC
Gram-positive strains		
Bacillus cereus ATCC 13061	BHI	ATCC
Bacillus cereus ATCC 14579	BHI	ATCC
Bacillus subtilis ATCC 23857	BHI	ATCC
Listeria monocytogenes ATCC 15313	BHI	ATCC
Staphylococcus aureus ATCC 29213	TSB	ATCC
Staphylococcus epidermidis ATCC 35983	TSB	ATCC

[a], LB, Luria-Bertani medium; TSB, Tryptic Soy Broth medium; BHI, Brain Heart Infusion medium.
[b], KCTC, Korean Collection for Type Cultures; ATCC, American Type Culture Collection; IVI, International Vaccine Institute.

2.2. Bacteriophage isolation, purification, and propagation

K. pneumoniae-infecting bacteriophages were isolated from the sewage treatment plants, including Seongnam, Opo, Gyeongan and Gwangju, South Korea. The phage isolation was performed by modifying the previously described procedures (M. Kim & Ryu, 2011). To isolate the pure individual phage, single plaque was picked and resuspended in 200 μl of sodium chloride-magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris·HCl, pH 7.5). These steps were repeated five times to obtain the single phage. For phage propagation, *K. pneumoniae* KCTC 2242 was inoculated (1%) and cultured until optical density (OD) reached 0.5 at 600 nm wavelength. Subsequently, the phage lysate was added and incubated at 37℃ with shaking at 180 rpm. After 3 h, the culture was centrifuged at 8,000 × g for 10 min and the supernatant was filtered with 0.22 μm filters (Pall Corporation, USA) to remove host cells. To obtain more phages, this propagation method was performed five different culture volumes (3, 8, 20, 200, and 800 ml). After propagation, the phage particles were precipitated with polyethylene glycol (PEG) 6000 at 4℃ for overnight and concentrated using CsCl density gradient ultracentrifugation (Optima XE; Beckman Coulter, USA) at 78,500 × g for 2 h at 4℃. The band was extracted and dialyzed in SM buffer for overnight. The purified phage was stored at 4℃ for further experiments.

2.3. Transmission Electron Microscopy (TEM)

To observe the morphology of the phage, phage stock was diluted approximately 10^8 to 10^9 PFU/ml. The phage was dropped on a glow discharged carbon-coated copper grid, and negatively stained with 2% uranyl acetate (pH 4.0). Excessive uranyl acetate was removed carefully by filter paper. The morphology was examined using BIO-TEM (Talos L120C, Czech) at 80 kV. Phage was identified and classified according to the guidelines for the International Committee on Taxonomy of Viruses.

2.4. Host range test

All bacterial strains listed in Table 1 were tested for phage host range identification. Cultured strains were added to 6 ml of soft top agar respectively and the mixture was overlaid. Then, 7 μl of serially diluted phages were spotted respectively on the overlaid plate and incubated at 37℃ for overnight. After the dotting assay, titer of each phages was determined by overlay assay, and efficiency of plating (EOP) was measured to compare the sensitivity of each bacterial strain to the phage. All experiments were performed in triplicate.

2.5. Stability test under various stress conditions

To investigate stability of the phage under various stress conditions, the phage at final concentration of 10^9 PFU/ml was incubated at different temperature and pH conditions. For determination of phage stability under temperature, the phage

was stationary cultured at -20, 4, 20, 30, 37, 40, 50, 60, 65, and 70℃ for 12 h. After incubation, dotting assay and overlay assay was carried out with *K. pneumoniae* KCTC 2242 as an indicator strain to measure the phage titers. To determine stability of the phage under various pH conditions, SM buffer was treated with HCl or NaOH to form pH values range of 1 to 13. Then the phage which was diluted to final concentration 10^9 PFU/ml was added respectively. After incubation at 37℃ for 12 h, the phage titers were measured by dotting assay and triplicated overlay assay.

2.6. Bacterial challenge assay

To determine the bacterial reduction of KPP2020 phage, *K. pneumoniae* KCTC 2242 was incubated at 37℃ for 12 h with shaking, and 1% was sub-cultured to 100 ml of fresh LB broth with shaking at 180 rpm until the OD_{600} reached at 1.0. Then, the culture was divided into two sets of 50 ml, and the phage was added to one set at 10 of multiplicity of infection (MOI). Phage added set and non-added set were incubated at 37℃ with shaking, and samples were collected every hour, serially diluted samples were spread on LB agar plates. After incubation at 37℃ overnight, viable cells were counted (CFU/ml). This challenge assay was performed in triplicate.

2.7. Genome sequencing and bioinformatics analysis

Genomic DNA of the phage was extracted using Viral Gene-Spin™ Viral DNA/RNA extraction kit (iNtRON Biotechnology, Korea). The phage KPP2020 was sequenced by Illumina Hiseq DNA sequencer (USA) by LabGenomics (Korea). The

qualified sequence reads were assembled using CLC Genomics Workbench v.10.0.1 (QIAGEN, Germany). The phage KPP2018 was sequenced by MinION (Oxford Nanopore Technologies, USA), and the qualified sequence reads were assembled by Canu (Koren et al., 2017). The open reading frames (ORFs) were predicted by Glimmer3 (Altermann & Klaenhammer, 2003), genesV (Softberry, USA), and GeneMarkS (Besemer, Lomsadze, & Borodovsky, 2001) and to confirm the ORF predictions the ribosomal binding sites (RBSs) were predicted by RBS finder program (J. Craig Venter Institute, USA). The predicted ORFs were annotated to specific functions using BLASTP (Altschul et al., 1997) and InterProScan (Quevillon et al., 2005) programs with protein domain databases. Comparative analysis about tail protein with other phages was carried out using BLAST genome alignment programs.

2.8. Cutting board application

To verify the ability of phage KPP2020 to inhibit host bacteria in nosocomial environment, especially kitchen, a cutting board was prepared. The cutting board was cut in size 10 cm^2 (2 cm × 5 cm) and sterilized using autoclave before use. The host strain was incubated in LB broth at 37℃ with shaking until the OD_{600} value reached at 1.0 (10^8 CFU/ml). 10^5 CFU of *K. pneumoniae* KCTC 2242 was spotted on the surface of cutting board and the phage KPP2020 was added to each cutting board sample at MOI of 10^2 or 10^3. The cutting boards were incubated in a static state at room temperature up to 7 h. Every hour, sample was

collected and homogenized for 2 min with 45 ml of 0.1% peptone water using stomacher (Interscience MiniMix® 100; HOMOGENIZERS, France). After stomaching, cutting board was removed using stomacher filter bag (3M, USA) and the filtrate was transferred to new centrifuge tube. To separate the bacterial cells from phage KPP2020, the filtrate was centrifuged at 8,000 rpm for 10 min and the supernatant containing KPP2020 was removed. The bacterial cell pellet was resuspended with 1 ml of 0.1% peptone water and serially diluted via 10-fold dilutions. Each diluted sample was spread on LB agar plate and incubated at 37℃ for 24 h. Viable cell counting was conducted in triplicate.

2.9. Food application

To estimate the ability of the phage to *K. pneumoniae* KCTC 2242 in food environments, food application procedure was performed by modifying the previously described procedures (Bai et al., 2019). To verify the best ratio of phages for the highest lytic activity, KPP2020 and KPP2018 were mixed at various ratio. The plaque assay was performed for all mixtures, and revealed that mixture in 1 : 1 ratio inhibits the indicator strain strongly. Phage cocktail was prepared in a 1 : 1 ratio and applied to food samples to inhibit *K. pneumoniae* more effectively. The raw chicken meat was purchased and cut in approximately 5 g and sterilized using autoclave before use. The host strain *K. pneumoniae* KCTC 2242 (10^5 CFU/ml) was added to sterilized chicken meat, and after inoculation of the host strain, each phage and prepared phage cocktail were sprayed respectively to the chicken meat at MOI

of 10^3. The samples were incubated at 37°C and collected at the indicated time points. The treated samples were collected at every 2 h and transferred into sterile stomacher bags containing 45 ml of peptone water and homogenized for 1 min with stomacher. And then, centrifuge at 8,000 rpm for 10 min, and the supernatants were removed. The bacterial cell pellets were resuspended with 1 ml of peptone water for viable cell counting. Viable cell counting was conducted in the same way as the application with cutting board. All the tests were carried out in triplicate.

2.10. Secretion of cytokines with RAW 264.7 cell

RAW 264.7 cells (ATCC TIB-71™), macrophage cells of *Mus musculus* were purchased from the Korean Collection for Type Cultures (KCTC). The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium; WELGENE, Korea), supplemented with 10% FBS (Fetal Bovine Serum; GW Vitex, Korea) and 100 U/ml penicillin and streptomycin (WELGENE, Korea) at 37°C containing 5% CO_2. To evaluate the secretion of cytokines, 2×10^5 cells/ml of cells were cultured in 12 well cell culture plates (SPL Life Sciences, Korea) for 24 h. Before experiments, supernatants of the cultured cells were removed and incubated with fresh medium without antibiotics for culturing bacteria. For evaluating therapeutic effects of the phage, 2×10^5 cells/ml of cells were infected with 10^6 CFU of cultured bacteria, and 10^7 PFU of phage was added. LPS (Lipopolysaccharide from *E. coli* O55:B5; Sigma, USA) was used as positive control and PBS (WELGENE, Korea) was used as negative control. Cells were incubated 37°C for 24 h and the

supernatants were collected after centrifugation (8,000 × g, 7 min, 4℃) and stored at -80℃ until used.

Secretion of cytokines was quantified by quantitative real-time PCR and mouse ELISA kits (Enzyme-Linked Immunosorbent Assay; Komabiotech, Korea). Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA was quantified and cDNA was synthesized by PrimeScript™ RT Reagent Kit (TaKaRa, Japan). The quantitative real-time PCR was conducted with SYBR green under the following conditions: 1 cycle at 95℃ for 3 min, and 40 cycles containing 95℃ for 5 sec and 58℃ for 30 sec. The mRNA expression levels of cytokines were calculated by $2^{-\Delta\Delta Ct}$ method, normalized with that of house-keeping gene encoding GAPDH. The primer sequences are listed in Table 6. The secreted cytokines were also quantified using ELISA kits with the cultured cells supernatants. All experiments were repeated three times.

Table 2. Primers used for quantificaiton of secreted cytokines

Primer	Sequence (5'→3')		Reference
GAPDH	Forward	GAA GGT CGG TGT GAA CGG AT	(Dai et al., 2017)
	Reverse	GAC AAG CTT CCC ATT CTC GG	
TNF-α	Forward	GAA GAG GCA CTC CCC CAA AA	In this study[a]
	Reverse	TGG GCC ATA GAA CTG ATG AGA	
IL-6	Forward	GTA CTC CAG AAG ACC AGA GG	(Yang et al., 2019)
	Reverse	TGC TGG TGA CAA CCA CGG CC	
IL-1β	Forward	GGC AAC TGT TCC TGA ACT CAA	In this study[a]
	Reverse	GAG TGA TAC TGC CTG CCT GA	

[a], Primer designed by Molecular Food Microbiology Laboratory of Seoul National University

2.11. Nucleotide sequence accession number

The complete genome sequences of KPP2020 and KPP2018 are available at the GenBank database of the National Center for Biotechnology Information (NCBI) database under accession numbers OQ031071 and OQ031075, respectively.

3. RESULTS

3.1. Host range and morphological observation

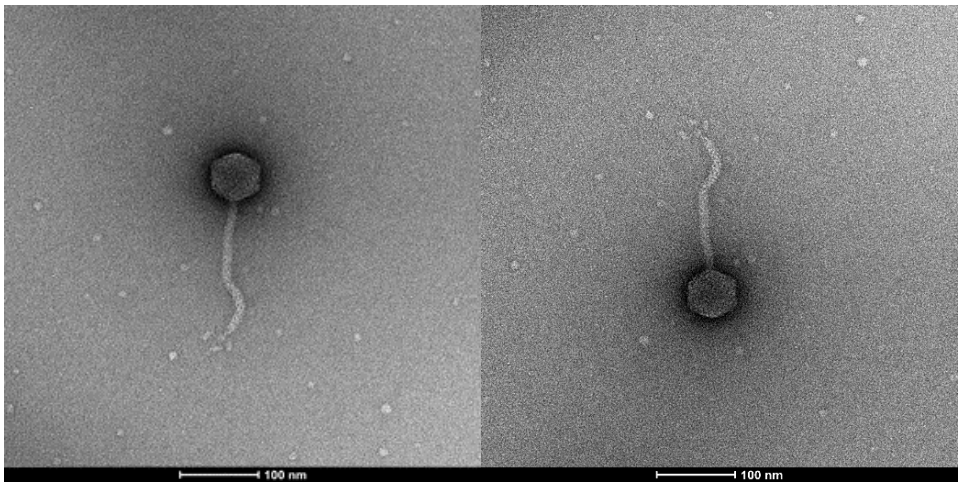
Twelve novel bacteriophages infecting *Klebsiella pneumoniae* were isolated from sewage plants in Seongnam, Opo, Gyeongan, and Gwangju, South Korea, using the indicator strain *K. penumoniae* KCTC 2242. The host range analysis was carried out about all isolated phages, and KPP2020 specifically infects *K. pneumoniae* and not infects any other bacteria, suggesting that this phage has high specificity to the host strain compared to other phages. Morphology observation by TEM showed that the phage KPP2020 has an icosahedral head, non-contractile and flexible tail with tail fibers, suggesting that it belongs to the family *Siphoviridae* (Fig. 1A). The length and width of head were measured about 63 ± 2 nm, and the tail was measured about 154 ± 5 nm (n=5). KPP2018 has a broad host range, mainly infecting *K. pneumoniae*, also *Shigella flexneri*, *S. sonnei*, and various *Salmonella* serovars. TEM analysis showed that KPP2018 also belongs to the family *Siphoviridae* (Fig. 1B). The length and width of head were measured about 71 ± 3 nm, and the tail was measured about 232 ± 2 nm (n=5). The phage PKP126 which belongs to *Siphoviridae* family and inhibits *K. pneumoniae* but only partially inhibits *K. oxytoca* and *Cronobacter sakazkii* was used to compare phages (Park, Kim, Cho, Ryu, & Lee, 2017).

Table 3. Host range of phages infecting *K. pneumoniae*

Bacterial strain	EOP[a] of phage												
	KPP2020	KPP2018	KPP01	KPP02	KPP03	KPP04	KPP05	KPP06	KPP07	KPP08	KPP09	KPP10	PKP126
Gram-negative strains													
Klebsiella pneumoniae KCTC 2242	+++	+++	+++	++	++	+	+++	+	+++	+	+++	+++	+++
Klebsiella pneumoniae KCTC 2690	+++	-	-	-	-	-	-	-	-	-	-	-	-
Klebsiella oxytoca ATCC 43863	-	-	+	-	+++	-	+	+++	-	+++	-	-	++
Klebsiella oxytoca KCTC 1686	-	-	+	-	+	-	-	++	-	-	-	-	-
Shigella flexneri 2a strain 2457T	-	+	-	-	-	-	-	-	-	-	-	-	-
Shigella flexneri KCTC 2517	-	+++	-	-	-	-	-	-	-	-	-	-	-
Shigella flexneri KCTC 2993	-	-	-	-	-	-	-	-	-	-	-	-	-
Shigella boydii KCTC 22528	-	-	-	+++	-	+++	-	-	-	-	-	-	-
Shigella sonnei KCTC 22530	-	+	-	+	-	+	-	-	-	-	-	-	-
Escherichia coli O157:H7 ATCC 43890	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli MG1655	-	+++	-	-	-	-	+	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853	-	-	-	-	-	-	-	-	-	-	-	-	-
Cronobacter sakazakii ATCC 29544	-	-	-	-	-	-	++	+	-	+	+	-	+
Yersinia enterocolitica ATCC 55075	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium LT2	-	+++	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium DT 104	-	+	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium ATCC 14028	-	+++	-	-	-	-	-	-	-	-	-	-	-
Salmonella Typhimurium SL1344	-	+++	-	-	-	-	-	-	-	-	-	-	-
Salmonella Enteritidis ATCC 13076	-	+	-	-	-	-	-	-	-	-	-	-	-
Gram-positive strains													
Bacillus cereus ATCC 13061	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus cereus ATCC 14579	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacillus subtilis ATCC 23857	-	-	-	-	-	-	-	-	-	-	-	-	-
Listeria monocytogenes ATCC 15313	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus ATCC 29213	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus epidermidis ATCC 35983	-	-	-	-	-	-	-	-	-	-	-	-	-

[a], +++, EOP of 1 to 0.5; ++, EOP of 0.5 to 0.2; +, EOP less than 0.2; -, no susceptibility.

(A)



(B)

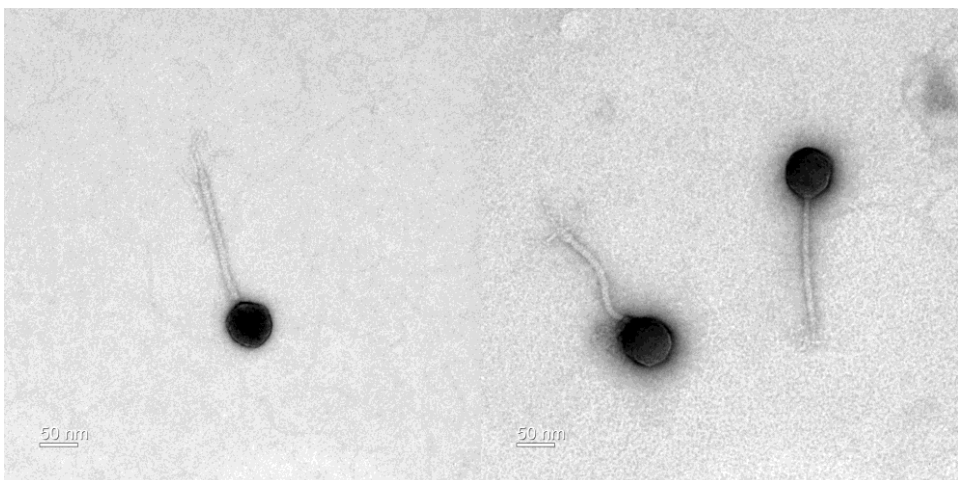


Figure 1. Morphological observation by Transmission Electron Microscopy.

(A) KPP2020, scale bar 100 nm. (B) KPP2018, scale bar 50 nm.

3.2. Phage stability under various stress conditions

The infectivity of phage KPP2020 and KPP2018 should be maintained under various stress conditions for food processing. To determine the stability of two phages, the test was conducted under various temperature (-20 to 70℃) and pH (1 to 13). Interestingly, the phage KPP2020 was stable for 12 h under wide temperature range (-20 to 60℃) and pH range (3 to 11) (Fig. 2). The phage KPP2018 also was stable under temperature range of -20 to 65℃ and pH range of 3 to 12 (Fig. 3). According to this, KPP2020 and KPP2018 can be a useful natural preservative to withstand various food application conditions.

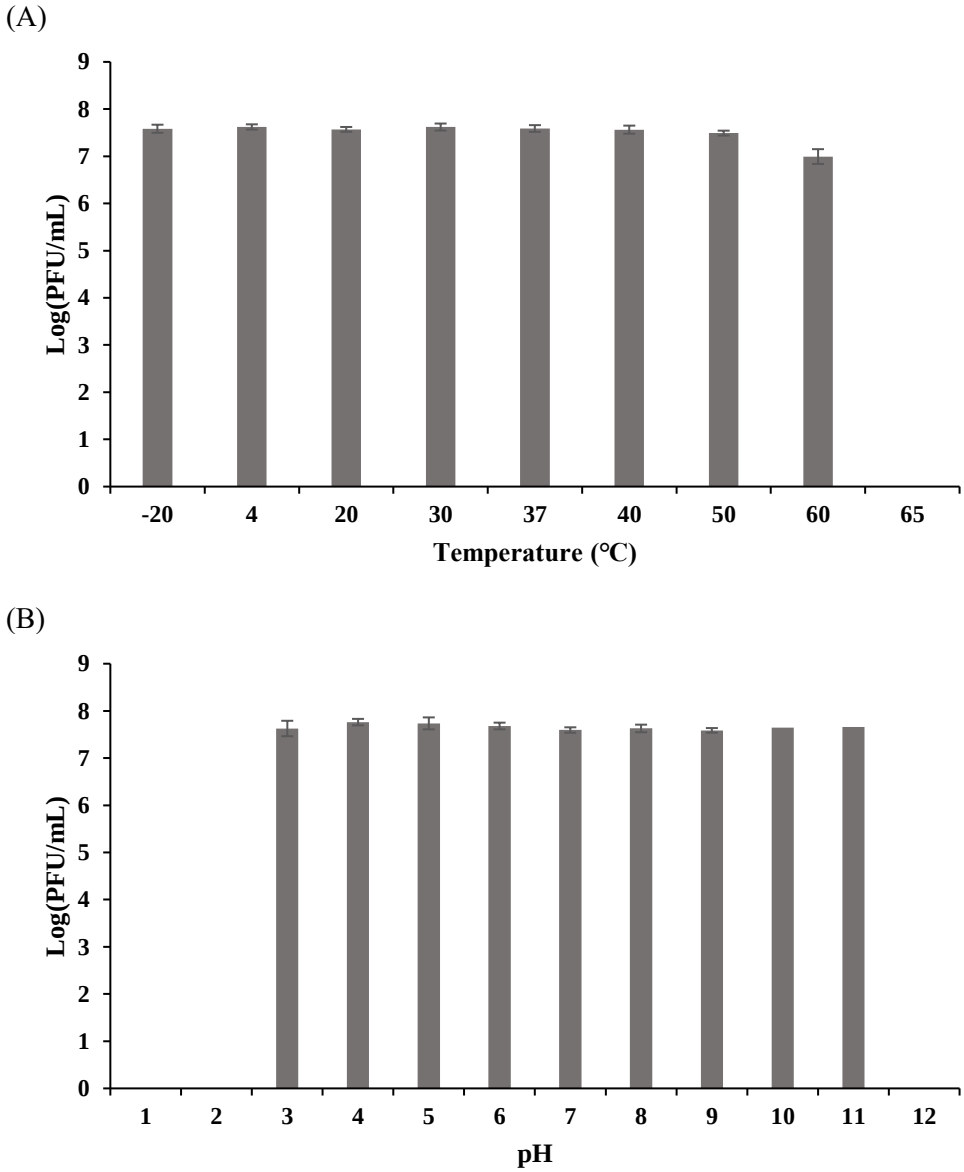
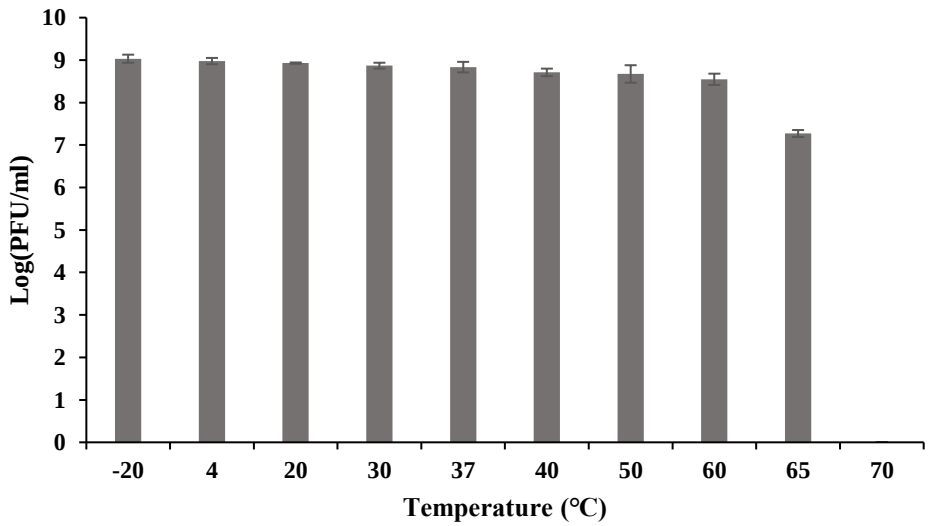


Figure 2. Stability of KPP2020 under stress conditions.

(A) Temperature and (B) pH stability.

(A)



(B)

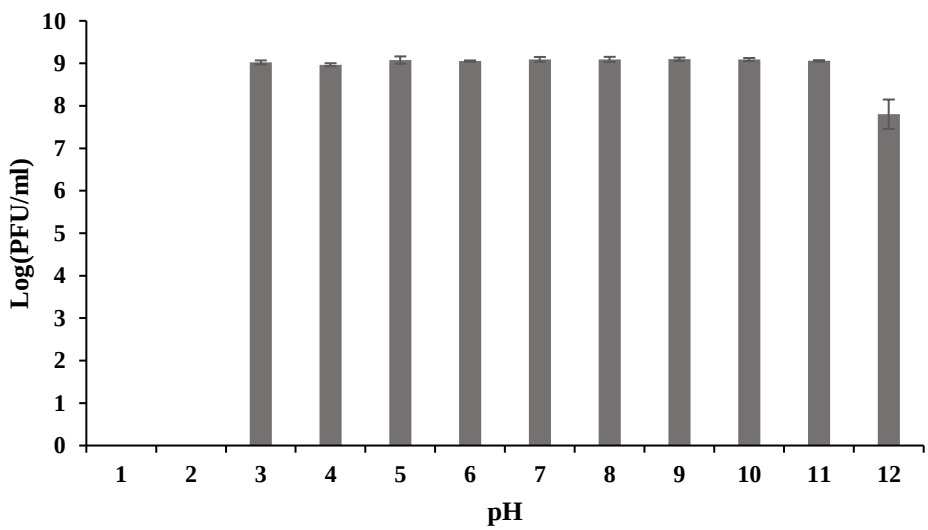


Figure 3. Stability of KPP2018 under stress conditions.

(A) Temperature and (B) pH stability.

3.3. Bacterial challenge assay

To examine the host lysis activity of phage KPP2020, the viable cells of the host strain was monitored after KPP2020 treatment. After 2 h incubation with KPP2020, the host strain showed 3.51 log CFU/ml reduction, indicating that we can apply the phage KPP2020 to infect the host strain rapidly (Fig. 4). The indicator strain completely recovered in 10 h, because phage-insensitive mutant was generated temporarily as seen in other phages.

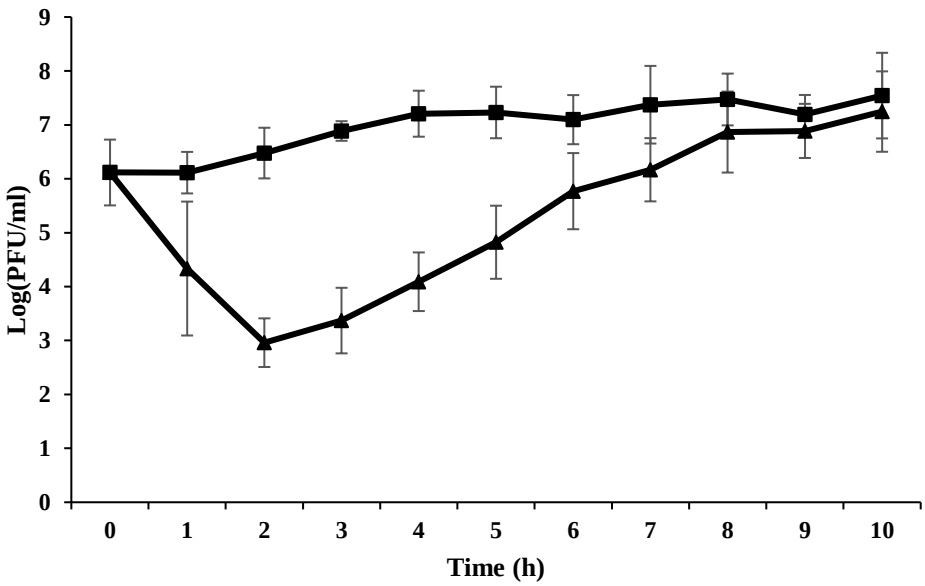


Figure 4. Bacterial challenge assay of KPP2020 with *Klebsiella pneumoniae* KCTC 2242. Triangles, phage-infected samples; squares, non-phage-infected samples.

3.4. Phage genome characterization

To understand the genomic characteristics of KPP2020 and KPP2018 at molecular levels, and to verify these are safe for application, the genomes of KPP2020 and KPP2018 were completely sequenced and analyzed using various bioinformatics tools. The genome of KPP2020 consists of 49,044 bp of DNA with a GC content of 54.33%, and contains 95 predicted open reading frames (ORFs). The phage KPP2018 genome consists of 137,988 bp of DNA with a GC content of 39.23%, and containing 228 predicted ORFs. Based on the functional annotation results, 77 ORFs for KPP2020 and 71 ORFs for KPP2018 were predicted to have specific functions and these functional ORFs were categorized into six functional groups : DNA replication/modification (DNA polymerase, DNA terminase, DNA endonuclease, and DNA primase/helicase), host lysis (endolysin, and holin), structure and packaging (major capsid protein, and membrane protein), tail (tail fiber protein, and tail assembly protein), and additional function (recombination protein and transposase). The genome annotation showed that each phage has own ability for DNA replication and host lysis because it contains all required genes. In addition, there is no toxin and virulence factors, indicating that the phage KPP2020 and KPP2018 can be used for many applications.

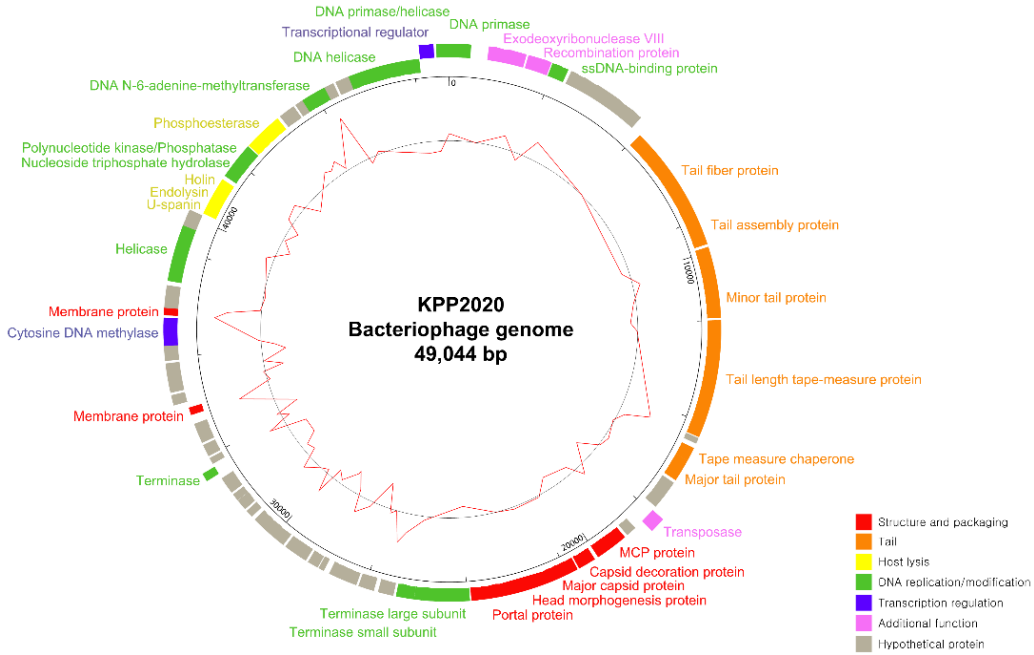


Figure 5. Genome map of KPP2020.

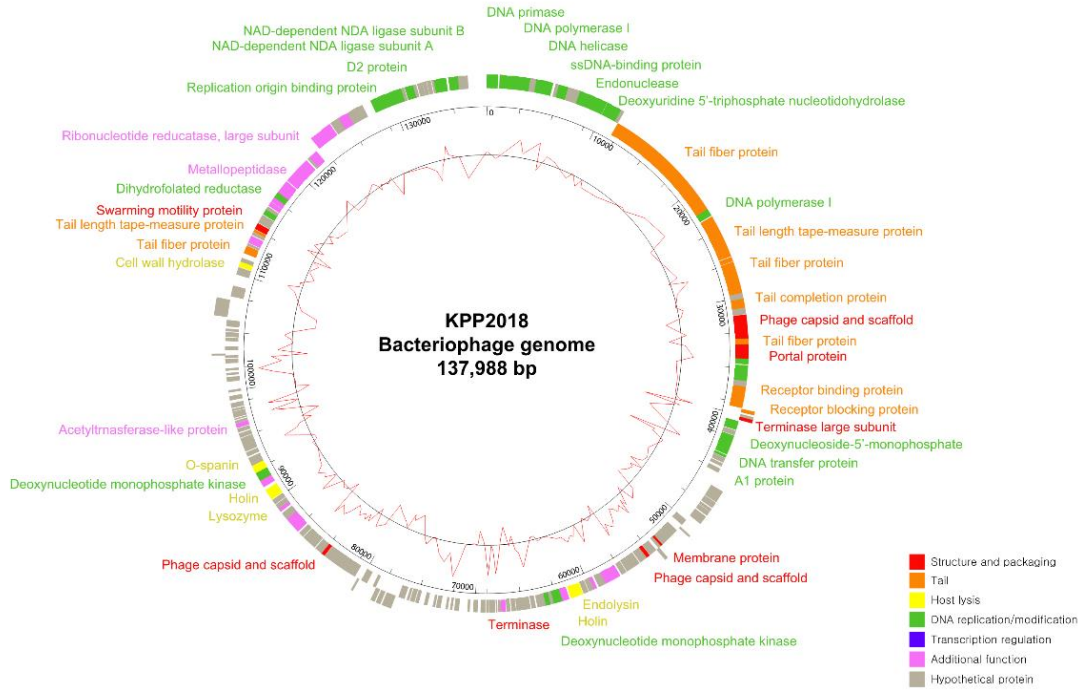


Figure 6. Genome map of KPP2018.

3.5. Comparative genome analysis

Based on the complete genome sequencing of KPP2020, comparative phylogenetic tree analysis was performed about major capsid proteins (MCPs), showing that KPP2020 was not grouped with other phages. The phage KPP2020 was located near *K. pneumoniae* phage PKP126, comparative genome analysis was performed among KPP2020, KPP2018, and PKP126 (Table 2).

The phage KPP2020 infects only *K. pneumoniae*, while PKP126 infects *K. pneumoniae*, *K. oxytoca*, and *C. sakazakii*. The phage KPP2018 infects *K. pneumoniae* mainly, *Shigella* spp., *S. enterica* serovar Typhimurium and Enteritidis, suggesting that KPP2018 has broad host range comparing to the phage KPP2020 and PKP126. To further understand the difference in the host specificities, comparative genome analysis about tail-related genes using BLASTP program was conducted (Fig. 7). Between KPP2020 and PKP126, the identity of tail related genes was quite high, however, the tail gene cluster of KPP2018 has no identity with other two phages. Therefore, for each phage, the best matches of tail gene clusters were identified by using BLASTP program. The gene clusters of KPP2020 and PKP126 which have narrow host range compared to KPP2018 were matched with other tail-related genes of *Klebsiella*-infecting phages (Table 3 and Table 4). The gene cluster of KPP2018 has high identity with that of *Salmonella* or *E. coli* infecting phages, supporting the host range of the phage KPP2018 (Table 5).

Table 4. General genome characteristics of phage KPP2020, KPP2018, and PKP126

Characteristics	KPP2020	KPP2018	PKP126
Infection host	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* *Shigella flexneri* *Escherichia coli* *Salmonella* Typhimurium	*Klebsiella pneumoniae* *K. oxytoca* *Cronobacter sakazakii*
Morphology	*Siphoviridae*	*Siphoviridae*	*Siphoviridae*
Genome size (bp)	49,044	137,988	50,934
G+C contents (%)	51.33	39.23	50.37
Predicted ORFs	95	228	78
Tail related genes	7	16	9

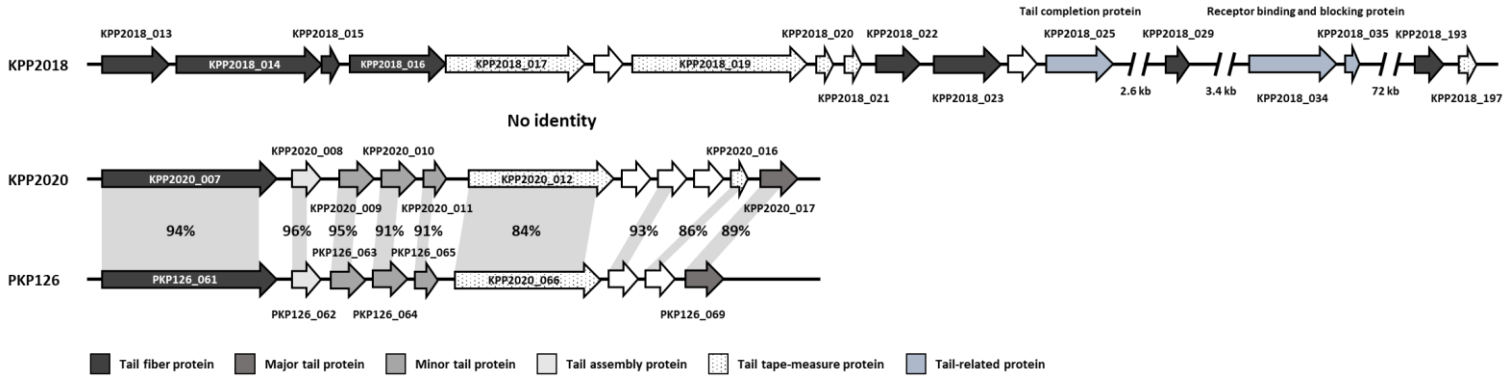


Figure 7. Comparative analysis of tail region gene clusters between three phages. Percentages indicates the identity score of amino acid sequences between homologous ORFs.

Table 5. Comparative analysis of KPP2020 ORFs in the tail regions by BLASTP program

Locus tag	Predicted function	BLASTP best matches	Identity (%)	References
KPP2020_007	Tail fiber protein	Tail fiber protein [*Klebsiella* phage NPat]	99	UPW42648.1
KPP2020_008	Tail assembly protein	Tail assembly protein [*Klebsiella* phage KPN N141]	100	YP_009791644.1
KPP2020_009	Minor tail protein	Tail tip assembly protein [*Klebsiella* phage vB_KpnS_MUC-5.2]	100	UMW87906.1
KPP2020_010	Minor tail protein	Minor tail protein [*Klebsiella* phage KPN N141]	99	YP_009791642.1
KPP2020_011	Minor tail protein	Tail protein [*Klebsiella* phage KPN N141]	99	YP_009791641.1
KPP2020_012	Tail tape-measure protein	Tail measure protein [*Klebsiella* phage KPN N141]	99	UPW42643.1
KPP2020_016	Tape measure protein	Tape measure chaperone [*Klebsiella* phage NPat]	100	UPW42642.1
KPP2020_017	Major tail protein	Major tail protein [*Klebsiella* phage NPat]	99	UPW42641.1

Table 6. Comparative analysis of PKP126 ORFs in the tail regions by BLASTP program

Locus tag	Predicted function	BLASTP best matches	Identity (%)	References
PKP126_061	Putative tail fiber protein	Tail fiber protein [*Klebsiella* phge KLPN1]	92	AKS10681.1
PKP126_062	Putative tail assembly protein	Tail assembly protein [*Klebsiella* phage KLPN1]	100	AKS10680.1
PKP126_063	Putative minor tail protein	Minor tail protein [*Klebsiella* phage KLPN1]	99	AKS10679.1
PKP126_064	Putative minor tail protein	Minor tail protein [*Klebsiella* phage KLPN1]	97	AKS10678.1
PKP126_065	Putative minor tail protein	Minor tail protein [*Klebsiella* phage KLPN1]	95	AKS10677.1
PKP126_066	Putative tail length tape-measure protein	Tail length tape-measure protein [*Klebsiella* phage KLPN1]	96	AKS10676.1
PKP126_067	Tape measure chaperone	Tail measure chaperone [*Klebsiella* phage Sushi]	91	AKQ07486.1
PKP126_068	Tape measure chaperone	Tail measure chaperone [*Klebsiella* phage Sushi]	88	AKQ07485.1
PKP126_069	Putative major tail protein	Major tail protein [*Klebsiella* phage KP36]	91	AEX26798.1

Table 7. Comparative analysis of KPP2018 ORFs in the tail regions by BLASTP program

Locus tag	Predicted function	BLASTP best matches	Identity (%)	References
KPP2018_013	Tail fiber protein	Tail fiber protein [*Salmonella* phage vB_SenS_PHB06]	97.25	AVQ09853.1
		[*Escherichia* phage chee24]	88.84	YP_009795127.1
KPP2018_014	Tail fiber protein	Tail fiber protein [*Salmonella* phage S130]	94.38	AXC41492.1
		[*Salmonella* phage smaug]	90.07	QIO00967.1
KPP2018_015	Tail protein	Putative phage tail protein [*Salmonella* phage SPC35]	100	YP_004306607.1
		[*Salmonella* phage NR01]	99.29	YP_009283409.1
KPP2018_016	Tail fiber protein	Putative tail protein [*Salmonella* phage L6jm]	97.61	YP_009856532.1
		Tail fiber protein [*Escherichia* phage EC148]	96.79	URF97871.1
KPP2018_022	Tail fiber protein	Tail fiber protein [*Salmonella* phage vB_SenS_PHB06]	99.67	AVQ09844.1
		[*Escherichia* phage fp01]	99.67	YP_009841475.1
KPP2018_023	Tail fiber protein	Major tail protein [*Salmonella* phage vB_SalS_ABTNLsp4]	99.14	QPI13175.1
		[*Escherichia* phage PNJ1902]	98.28	UIS65934.1
KPP2018_029	Tail fiber protein	Tail fiber protein [*Salmonella* phage SP01]	98.77	YP_009792481.1
		[*Salmonella* phage vB_SalS_ABTNLsp9]	96.93	QPI13626.1
KPP2018_193	Tail fiber protein	Tail fiber protein [*Escherichia* phage IME178]	100	QYC97208.1
		[*Escherichia* phage H8]	98.11	YUP_009966025.1

3.6. Cutting board application

By comparing the number of viable cells of *K. pneumoniae* on the cutting board, we determined the potential of KPP2020 as a practical agent for safety. The phage KPP2020 inhibited the indicator strain for longer than 7 h at MOI of 10^2 and 10^3, suggesting that KPP2020 successfully controlled *K. pneumoniae* KCTC 2242 with long duration. After 6 h incubation of KPP2020, the number of CFUs showed 4.9 log CFU/ml reduction at MOI = 10^2 and 5.3 log CFU/ml reduction at MOI = 10^3 (Fig. 8).

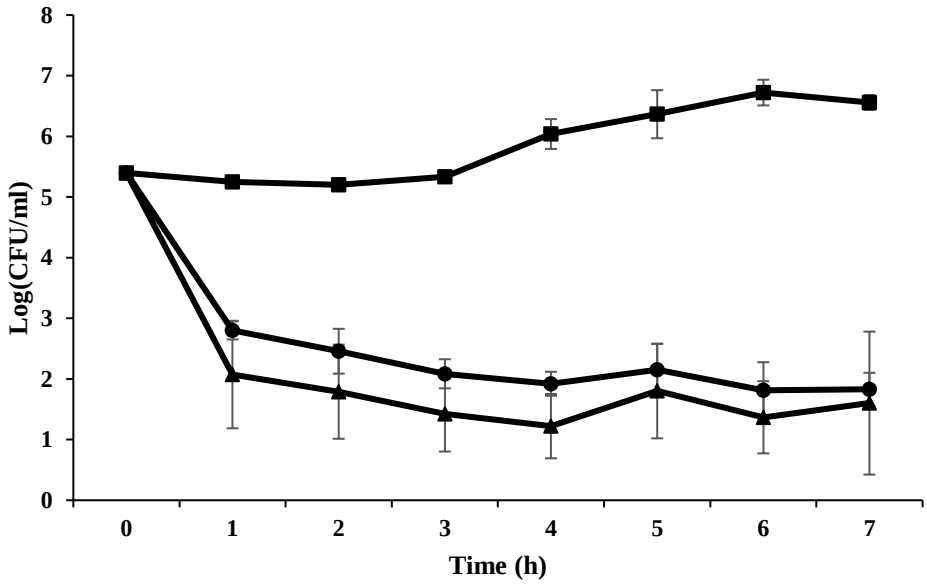


Figure 8. Application of phage KPP2020 with *K. pneumoniae* KCTC 2242 in cutting board. Closed squares, control sample without phage KPP2020; closed circles, KPP2020 treated samples at MOI of 10^2; closed triangles, KPP2020 treated samples of MOI at 10^3.

3.7. Food application with phage cocktail

To overcome the rapid development of bacterial resistance to phage infection, we developed a novel phage cocktail consisting of the two phages KPP2020 and KPP2018. The phage KPP2020 and KPP2018 (10^{10} PFU/ml) were mixed in 1:1, 1:2, 2:1, 1:4, and 4:1 ratio. Each mixture was used at performing plaque assay, and the titer of mixture with 1:1 ratio of KPP2020 and KPP2018 was the highest (data not shown). The phage cocktail consisting of 1:1 ratio was applied to *K. pneumoniae* contaminated food, and by monitoring viable cells of *K. pneumoniae* in chicken meat, we identified the possibility of phage KPP2020 and KPP2018 as natural food preservatives. After 1 h incubation with phage, KPP2020 showed 2.85 log reduction of the indicator strain, while KPP2018 showed 1.71 log reduction. The bacterial resistance against KPP2020 infection was developed after 4 h incubation, while KPP2018 maintained its lytic activity for at least 12 h. Each phage has advantages to inhibit the bacterial strain, the phage cocktail resulted 4.35 log reduction and its lytic activity was sustained longer than 12 h (Fig. 9), so the cocktail holds promise as a novel, effective agent that can improve the safety of poultry products.

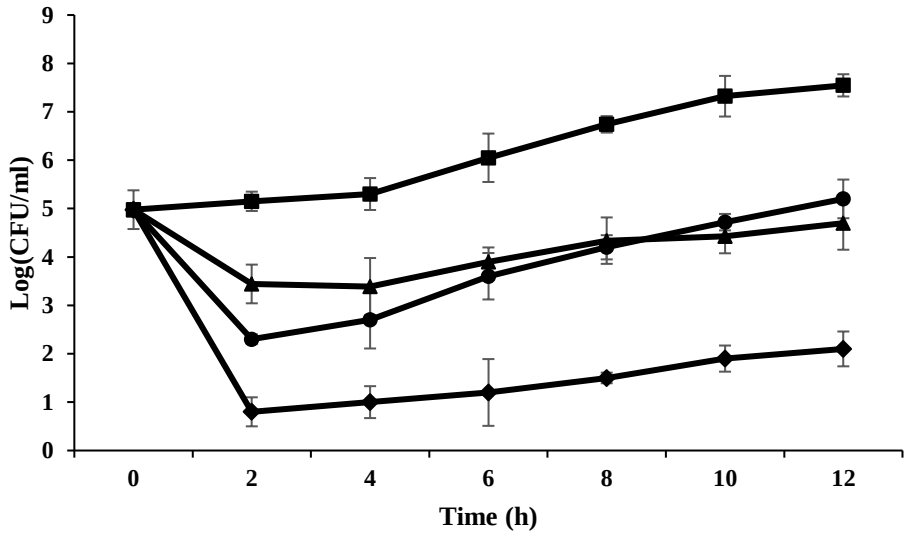


Figure 9. Application of phage cocktail with *K. pneumoniae* KCTC 2242 in chicken. Closed squares, control sample without any phage treatment; closed circles, KPP2020 treated sample; closed triangles, KPP2018 treated sample; closed diamonds, phage cocktail treated sample. Phage was treated at MOI of 10^3.

3.8. Inflammatory alleviation of RAW 264.7 cells

To check the therapeutic effect of KPP2020 against the bacterial infection, secretions of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and IL-1β were tested in mRNA and protein level. The primers used in this study are listed in Table 6. RAW 264.7 cells can be activated and induce inflammation by LPS. The RAW 264.7 cells infected with *K. pneumoniae* secreted pro-inflammatory cytokines as much as cells activated by LPS, indicating that *K. pneumoniae* can stimulate the macrophage and induce the inflammation. The phage KPP2020 significantly reduced the secretions of TNF-α, IL-6, and IL-1β in mRNA level and protein level, indicating that KPP2020 has therapeutic effect against the bacterial infection (Fig. 10, Fig. 11, and Fig. 12). Because the KPP2020 is not an immune-boosting agent, the secretion of IL-10, an anti-inflammatory cytokine, also was decreased by the treatment of KPP2020 (data not shown)

To identify the interaction between phage and macrophage, the phage KPP2020 was treated to RAW 264.7 cells and LPS-stimulated cells, then secretions of the pro-inflammatory cytokines were measured as previously described. When the phage was treated to RAW 264.7 cells, TNF-α, IL-6, and IL-1β were expressed as much as PBS, negative control, indicating that KPP2020 does not induce inflammatory response. Also when the phage was treated to LPS-stimulated cells, the secretions of cytokines did not reduced meaningfully (Fig. 13, Fig. 14, and Fig. 15). According to these results, KPP2020 does not involve in the inflammatory response induced by LPS, while has therapeutic effect against bacterial infection.

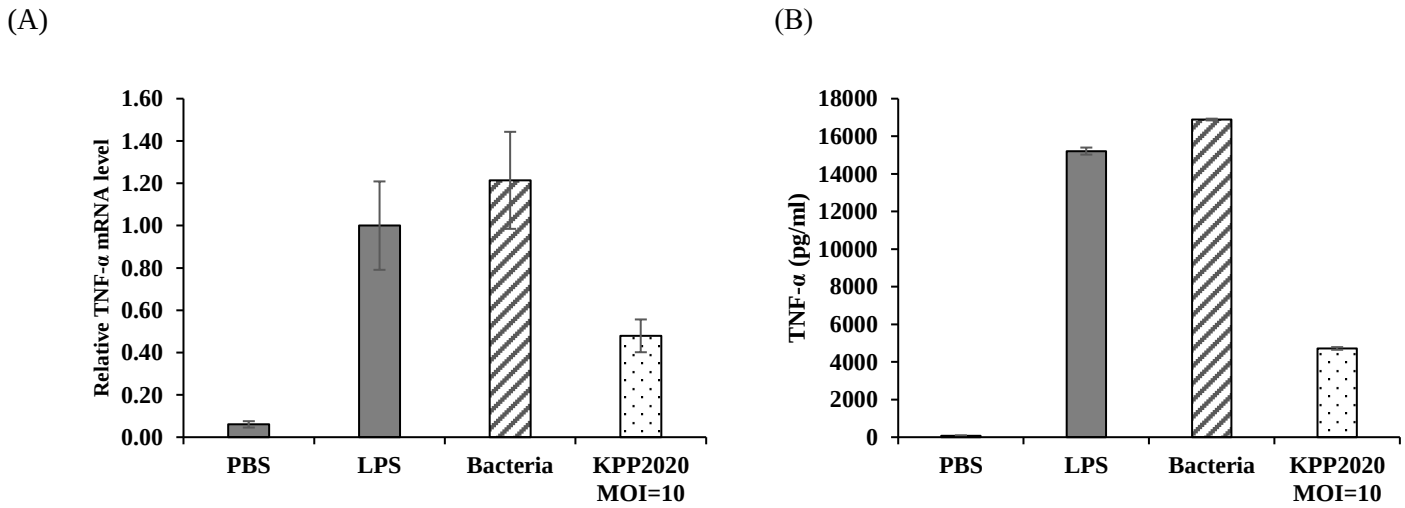


Figure 10. Effects of KPP2020 on cytokine-production in *K. pneumoniae*-infected RAW 264.7 cells such as TNF-α. (A) Relative mRNA expression using Real-Time RT-PCR and (B) Protein production using ELISA. Error bars present the standard deviations of three replicates (n=3).

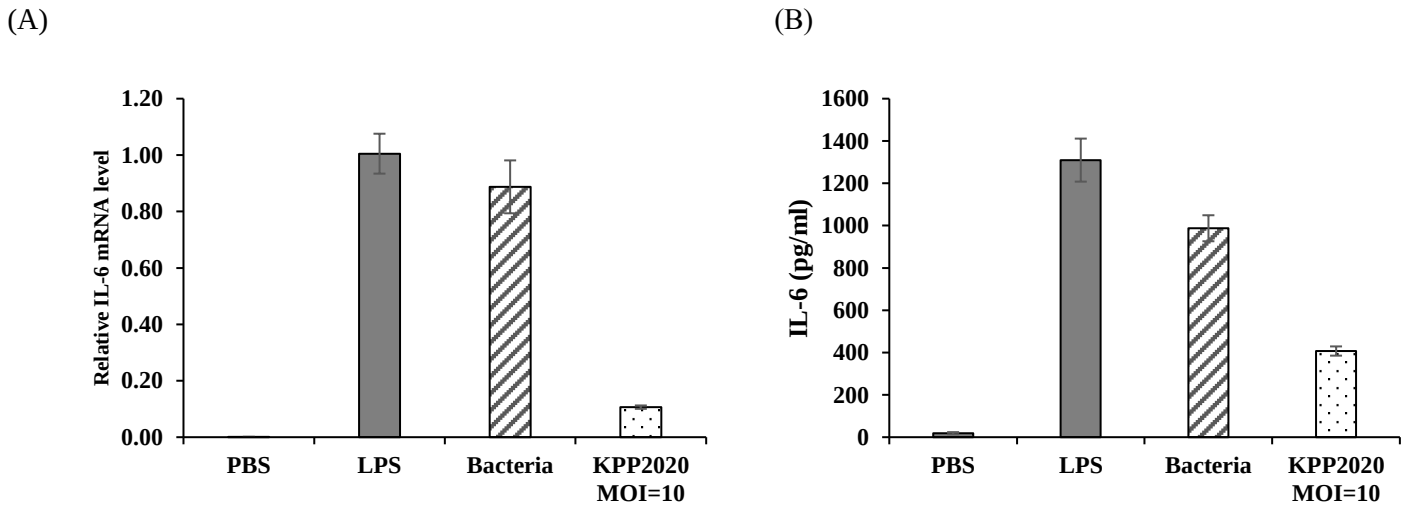


Figure 11. Effects of KPP2020 on cytokine-production in *K. pneumoniae*-infected RAW 264.7 cells such as IL-6. (A) Relative mRNA expression using Real-Time RT-PCR and (B) Protein production using ELISA. Error bars present the standard deviations of three replicates (n=3).

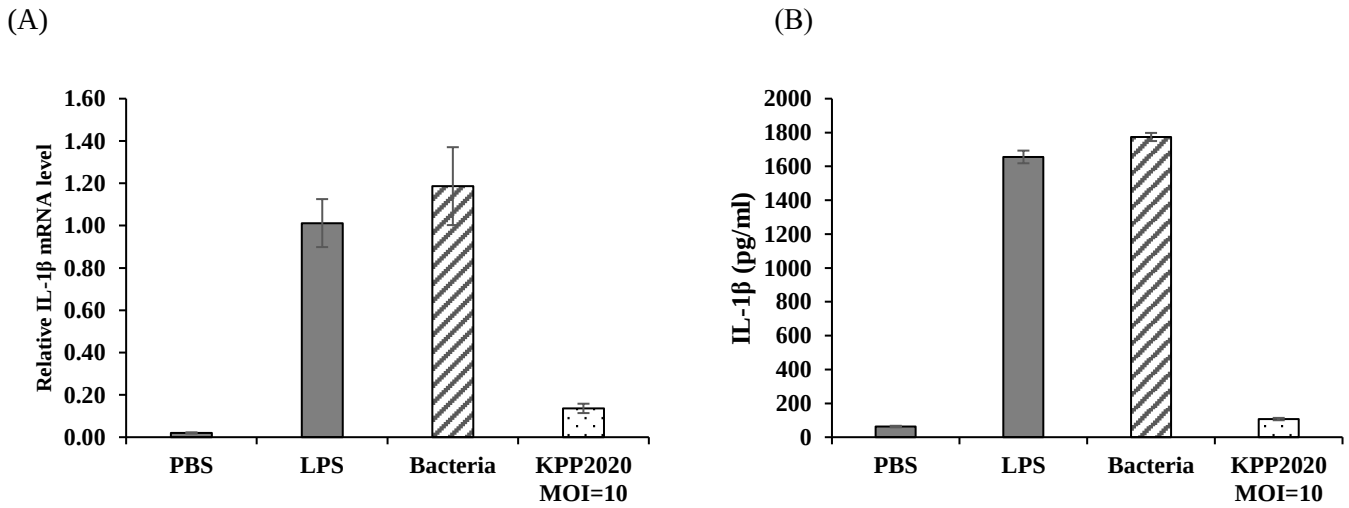


Figure 12. Effects of KPP2020 on cytokine-production in *K. pneumoniae*-infected RAW 264.7 cells such as IL-1β. (A) Relative mRNA expression using Real-Time RT-PCR and (B) Protein production using ELISA. Error bars present the standard deviations of three replicates (n=3).

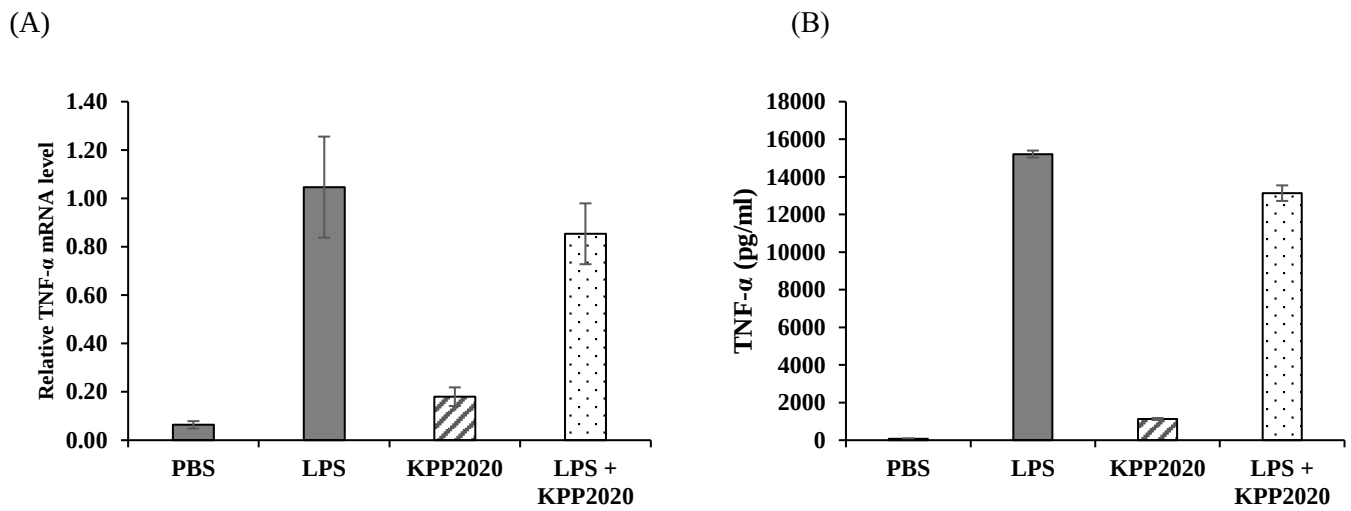


Figure 13. Effects of KPP2020 on cytokine-production in RAW 264.7 cells such as TNF-α. (A) Relative mRNA expression using Real-Time RT-PCR and (B) Protein production using ELISA. Error bars present the standard deviations of three replicates (n=3).

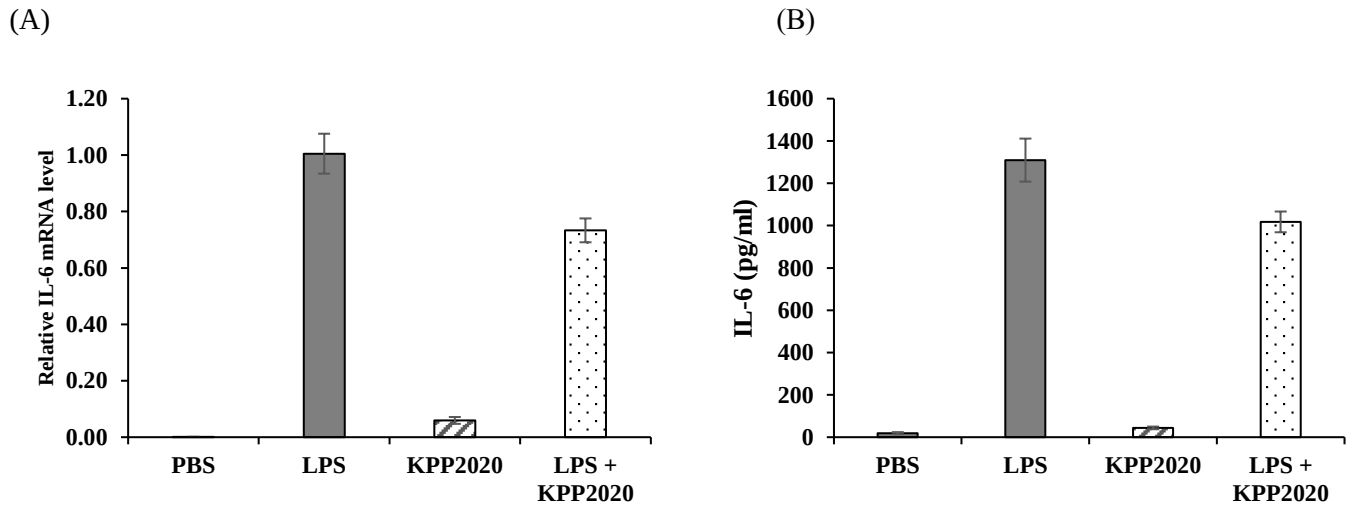


Figure 14. Effects of KPP2020 on cytokine-production in RAW 264.7 cells such as IL-6. (A) Relative mRNA expression using Real-Time RT-PCR and (B) Protein production using ELISA. Error bars present the standard deviations of three replicates (n=3).

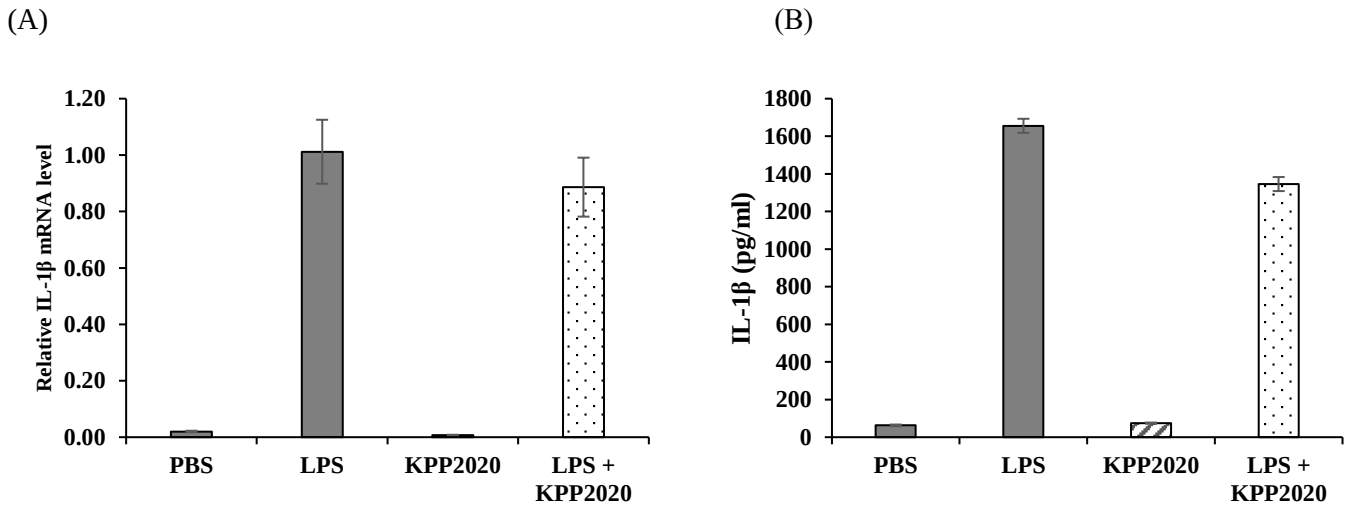


Figure 15. Effects of KPP2020 on cytokine-production in RAW 264.7 cells such as IL-1β. (A) Relative mRNA expression using Real-Time RT-PCR and (B) Protein production using ELISA. Error bars present the standard deviations of three replicates (n=3).

4. DISCUSSION

Klebsiella pneumoniae is the main cause of pneumonia and other diseases, the infections from human to human through contaminated hands or in environment are occurred easily and frequently. Nowadays detection rate of *K. pneumoniae* is increasing significantly in food samples, especially in poultry, raw vegetables, and ready-to-eat products (Rodrigues et al., 2022), (H.-S. Kim et al., 2015). As a way to control this pathogen effectively, various antibiotics were used for a long time, however, other alternatives should be developed because of the emergence of antibiotic resistant bacteria. Recently, multidrug-resistant *K. pneumoniae* is increasing, a new biocontrol agent is needed to replace antibiotics. Virulent bacteriophages have strong bactericidal activity, causing host cell lysis, and therefore they have potential as novel agents to inhibit specific pathogens.

In this study, phage KPP2020 and KPP2018 were isolated and characterized. Both phages are stable under various stress conditions like temperature and pH. The phage KPP2020 has been proved that it has sufficient lytic activity by challenge assay showing 3.51 log reduction of *K. pneumoniae* KCTC 2242. Host range test showed that KPP2020 infects only *K. pneumoniae*, while KPP2018 infects *K. pneumoniae* mainly and slightly *Shigella* spp., and *Salmonella* serovars. To understand the difference of host range between two phages, both phage genomes were sequenced and analyzed. The whole genomes were compared using BLAST, and revealed that KPP2020 and PKP126 which have narrow host range were similar to *Klebsiella*-infecting phages. KPP2018 which has broad host range was matched

to *Salmonella* and *E. coli*-infecting phages with high identity. To compare especially between the tail-related genes, 7 genes of KPP2020 and 16 genes of KPP2018 were analyzed. BLASTP analysis showed that there was no identity between tail gene clusters of KPP2020 and KPP2018, showing that difference in tail cluster genes made different host range of two phages. The tail proteins of KPP2018 were matched with tail-related proteins of *Salmonella* phage or *E. coli* phage. KPP2018 can infects *Salmonella* and *E. coli* with high EOP, suggesting that host range of phage and its tail protein are associated (Chaturongakul & Ounjai, 2014).

To apply KPP2020 and KPP2018 to food industry, a new strategy to control *K. pneumoniae* and temporary phage-resistant *K. pneumoniae* should be developed. Previous studies have indicated that the phage cocktail can delay the appearance of phage-resistant variants and enhance treatment efficacy (Kudva Indira, Jelacic, Tarr Phillip, Youderian, & Hovde Carolyn, 1999), (Y. Tanji et al., 2004). Phage cocktail consisting of KPP2020 and KPP2018 was applied to contaminated chicken meat, and its lytic activity and maintenance of hindrance were compared to them of single phages. KPP2020 can inhibits host bacteria rapidly but recovery of bacteria starts quickly, while KPP2018 inhibits the host bacteria slighter than KPP2020, but maintenance of its effect is continued longer than KPP2020. Interestingly, the phage cocktail inhibits the host bacteria most effectively and the maintains its effect for the longest time. There was the difference of host range between two phages, KPP2018 can inhibits various foodborne pathogens, we expect that the cocktail can be applied to other food samples contaminated with foodborne pathogens and may inhibit the

pathogens.

Klebsiella pneumoniae is highly related to clinical environment, *Klebsiella*-infecting phages are candidates for an effective therapeutic agent for inflammation. Previously, the *K. pneumoniae* phage Kpn5 was evaluated for therapeutic application (Seema, Kusum, & Sanjay, 2010). Kpn5 was used to treat a clinical burn wound infection with *K. pneumoniae* B5055, showing complete elimination of *K. pneumoniae* seven days after treatment. This indicated that phage therapy using *Klebsiella* phages could be an alternative treatment for bacterial infections. To verify the therapeutic effect of KPP2020, the murine macrophages RAW 264.7 cells were infected with *K. pneumoniae*, then KPP2020 was treated to lower the inflammatory response. The pro-inflammatory cytokines that secreted by macrophages were quantified by Real-Time RT-PCR and ELISA. The secretion of TNF-α, IL-6, and IL-1β was reduced significantly by the treatment of KPP2020, indicating that KPP2020 can be used as a therapeutic agent for bacterial infections. KPP2020 does not induce the inflammation of RAW 264.7 cells and does not lower the secretion of pro-inflammatory cytokines from LPS-stimulated cells, indicating that KPP2020 does not involve in the inflammatory response induced by LPS. *In vivo* study should be performed to prove the therapeutic effect of KPP2020.

Characterization and genome analysis of the *K. pneumoniae* phages KPP2020 and KPP2018 showed that they have high host specificity, high bactericidal activity, and high stability under wide range of stress conditions. KPP2020 also has therapeutic effect for bacterial infection, both phages may be good

candidates for the development of novel biocontrol agents to control *K. pneumoniae* in nosocomial environments and food industry. This study might be meaningful to provide information of phage KPP2020 and KPP2018, and potential usefulness for further applications.

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국문초록

클렙시엘라 뉴모니애는 만성 폐 장애를 일으키는 균주로, 선택적으로 병원성을 가지는데 주로 병원 환경에서 면역이 약화된 개인을 감염한다. 이 병원균은 대부분의 베타-락탐 항생제에 내성이 있는 ESBL을 생성할 수 있고, 최근에는 식품, 특히 가금류나 야채에서 클렙시엘라 뉴모니애가 검출되는 사례가 증가하고 있어서 이를 제어하기 위한 새로운 제제의 개발이 시급하다. 이를 제어하기 위해서 클렙시엘라를 감염하는 파지를 하수 처리장에서 분리했다. 각 파지의 감염 범위를 분석한 결과, KPP2020은 클렙시엘라 뉴모니애만을 억제하는 아주 높은 숙주 특이성을 보이는 것을 확인했고, KPP2018은 주로 클렙시엘라 뉴모니애를 감염하며, 시겔라 속, 살모넬라도 감염하는 것을 확인하였다. 투과 전자 현미경을 사용한 형태학적 관찰을 통해, 두 파지 모두 *Siphoviridae* 과에 속한다는 것을 알 수 있었다. 다양한 스트레스 조건에서 KPP2020과 KPP2018의 안정성을 확인해본 결과, KPP2020의 경우, −20℃에서 60℃ 그리고 pH 3에서 11까지, KPP2018의 경우, −20℃에서 65℃ 그리고 pH 3에서 12까지 범위 안에서 12시간 동안 안정적이었다. KPP2020의 숙주 제어 능력을 확인한 결과, 클렙시엘라 뉴모니애를 2시간 이내에 3.51 로그 감소시키는 것을 확인하였다. KPP2020 및 KPP2018의 유전체를 분석하였고, 파지 꼬리 부분에 해당하는 유전자군을 비교 분석한 결과, KPP2020과 KPP2018 사이에는 상동성이 없었으며, 이는 두 파지의 감염 범위의 차이와 연관이 있음을 확인하였다. 클렙시엘라 뉴모니애가 오염된

도마에 KPP2020을 적용하여 균주 저해 효과를 확인한 결과, 최소 7시간 동안 지속적으로 4 로그 이상의 균을 감소시키는 것을 확인하였다. 식품에서 파지를 더 효과적으로 적용하기 위해 KPP2020과 KPP2018을 1:1 비율로 혼합하여 파지 칵테일을 제작하였고, 이를 닭고기에 적용해 보았을 때, 단일 파지보다 더 높은 용균 활성을 보였고 (2시간 내 4.35 로그 감소), 파지에 대한 균주의 저항성이 더 느리게 생성되었다. KPP2020은 클렙시엘라 뉴모니애가 감염된 RAW 264.7 세포에서 전 염증성 사이토카인의 분비를 낮출 수 있어 KPP2020이 세균 감염에 대한 치료 효과가 있음을 시사한다. KPP2020을 RAW 264.7 세포에 처리하였을 때 전 염증성 사이토카인이 발현되지 않았고, LPS로 인해 염증 반응이 활성화된 RAW 264.7 세포에 파지를 처리하였을 때 사이토카인의 발현에 관여하지 않기 때문에 KPP2020은 더 효과적인 치료제로서 사용될 수 있다. 본 연구를 통해 새로운 박테리오파지 KPP2018과 KPP2020은 식품 안전을 위한 천연 식품 첨가제로 사용될 수 있으며, KPP2020은 세균 감염에 대한 치료제가 될 수 있음을 확인하였다.