The Journal of Veterinary Medical Science

FULL PAPER

Bacteriology

Evaluation of the broad-spectrum lytic capability of bacteriophage cocktails against various *Salmonella* serovars and their effects on weaned pigs infected with *Salmonella* Typhimurium

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ABSTRACT. The broad-spectrum lytic capability of *Salmonella* bacteriophages against various *Salmonella* species was evaluated to determine their potential as an alternative for antibiotics, and the safety and preventive effects of the bacteriophages were assessed on mice and pigs. Four bacteriophage cocktails were prepared using 13 bacteriophages, and the lytic capability of the four bacteriophage cocktails was tested using *Salmonella* reference strains and field isolates. Bacteriophage cocktail C (SEP-1, SGP-1, STP-1, SS3eP-1, STP-2, SChP-1, SAP-1, SAP-2; $\geq 10^9$ pfu/ml) showed the best lytic activity against the *Salmonella* reference strains (100% of 34) and field isolates (92.5% of 107). Fifty mice were then orally inoculated with bacteriophage cocktail C to determine the distribution of bacteriophages in various organs, blood and feces. The effects of bacteriophages on *Salmonella* infection in weaned pigs (n=15) were also evaluated through an experimental challenge with *Salmonella* Typhimurium after treatment with bacteriophage cocktail C. All mice exhibited distribution of the bacteriophages in all organs, blood and feces until 15 days post infection (dpi). After 35 dpi, bacteriophages were not detected in any of these specimens. As demonstrated in a pig challenge study, treatment with bacteriophage cocktail C reduced the level of *Salmonella* shedding in feces. The metagenomic analyses of these pig feces also revealed that bacteriophage treatment decreased the number of species of the Enterobacteriaceae family without significant disturbance to the normal fecal flora. This study showed that bacteriophages effectively controlled *Salmonella* in a pig challenge model and could be a good alternative for antibiotics to control *Salmonella* infection.

KEY WORDS: bacteriophage, *Salmonella*, distribution of bacteriophage, pig intestinal bacteria flora, next-generation sequencing

J. Vet. Med. Sci.
80(6): 851–860, 2018
doi: 10.1292/jvms.17-0501

Received: 5 September 2017
Accepted: 19 March 2018
Published online in J-STAGE: 4 April 2018

Salmonella is a common food-borne pathogen worldwide [3, 33, 35], and its major route of transmission to humans is the consumption of *Salmonella*-contaminated food products, such as pork and poultry products. *Salmonella* causes acute enteric infections, and the intestinal contents of pigs infected with *Salmonella* can contaminate their carcasses [9, 27]. Subclinically infected pigs with *Salmonella* can also act as super-shedders of *Salmonella* in lairage and slaughterhouses because the shedding can be increased by stress, such as changes in environment, transport, and other factors [39]. Effective public health measures for the control of *Salmonella* contamination in farms are needed to prevent transmission to the carcasses and thus to humans. A recent study demonstrated that the prevalence of *Salmonella* in 1,194 samples from slaughterhouses and swine farms (977 fecal contents and 67 organ samples) was 5.3% [29]. In another study, *Salmonell*a was isolated from 15.7 to 21.3% of animals over 30 days of age in Korean swine farms [31].

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Antibiotics have been widely used in pig and poultry farms, but multidrug-resistant (MDR) bacteria are a serious emerging concern in the fields of food safety and animal disease control [6]. Antibiotic resistance that originated from pigs and poultry threatens human health due to the transferability of antibiotic-resistance genes to zoonotic pathogens [47], and the frequency of antibiotic resistance among food-borne pathogens has increased [37, 38]. Therefore, the use of antibiotics as growth promoters in animal feed was banned in most developed countries [12, 17]. The difficulties in controlling the disease caused by various pathogenic bacteria without antibiotics, particularly during the weaning period, have reduced product quality and livestock yield [45, 46]. Therefore, an available and inexpensive method for the prevention and treatment of *Salmonella* is urgently needed.

Bacteriophages are viruses that can specifically infect and lyse bacteria. Bacteriophages are natural, nontoxic, and inexpensive and replicate only in specific bacteria, allowing avoidance of the gut flora imbalance caused by broad-spectrum antibiotics. Bacteriophages have also been used successfully to treat various bacterial infections in animals [5, 18, 22, 36, 42]. Furthermore, bacteriophages can also be effective against MDR pathogens [1, 16, 32, 44]. Notably, several studies have reported that some bacteriophages can reduce the enteric *Salmonella* levels in pigs [10, 48]. Additionally, a cocktail of bacteriophages is more effective than the use of one bacteriophage to control bacterial disease in pigs [44], and Yoichi *et al.* [51] reported that the use of a bacteriophage mixture delayed bacteriophage-resistant bacterial growth.

In the present study, we evaluated the efficiency of four bacteriophage cocktails against *Salmonella* isolates from pig farms and slaughterhouses in Korea. Additionally, we evaluated the distribution of bacteriophages in mouse organs and the changes in the pig intestinal bacterial flora induced by bacteriophage cocktail treatment.

MATERIALS AND METHODS

Bacterial strains

One hundred seven *Salmonella* isolates from fecal samples or carcasses collected from slaughterhouses or farms during 2009 to 2011 were used assessing the lytic spectra of bacteriophages and antimicrobial susceptibility. Additionally, 34 reference strains representing 34 serotypes were used for assessment of the bacteriophage lytic spectrum. All the isolates were streaked on xylose lactose deoxycholate agar to assess their purity and were genetically confirmed by polymerase chain reaction (PCR) [11]. All the isolates were serotyped by a slide agglutination test according to the Kauffmann-White scheme at the Salmonella reference center RIVM (National Institute of Public Health and the Environment) at Bilthoven, The Netherlands.

Antibiotic susceptibility tests

One hundred seven *Salmonella* isolates were subjected to an antibiotic susceptibility test. The antibiotic susceptibility test was performed using the agar disk diffusion test on Mueller Hinton agar plate [23]. The concentrations of antibiotics on BBL Sensi-Disk (BD, Franklin Lakes, NJ, U.S.A.) were as follows; ampicillin (AM, 10 μg), streptomycin (S, 10 μg), gentamicin (GM, 10 μg), neomycin (N, 30 μg), tetracycline (Te, 30 μg), nalidixic acid (NA, 30 μg), enrofloxacin (ENR, 5 μg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg), and chloramphenicol (C, 30 μg). MDR isolates were defined as isolates resistant to three or more antibiotics belonging to different antibiotic classes [in the following order, one penicillin (Pe), three aminoglycosides (Ag), one tetracycline (Te), two quinolones (Qu), one sulfonamide (Su) and one phenicol (Ph)]. Each isolate was plated in Mueller Hinton agar, and each antibiotic disk was placed over the bacteria layer. The plates were incubated at 37°C for 18 hr according to the method described by CLSI guidelines [15]. The antibiotic susceptibilities of the isolates were then classified as susceptible, intermediate, and resistant according to the zone diameter on each antibiotic disk.

Isolation of bacteriophages and preparation of bacteriophage cocktails

Isolation of bacteriophages was carried out as previously described [43]. Briefly, six *Salmonella* isolates (*Salmonella enterica* Enteritidis SE30, *Salmonella enterica* Gallinarum SG40, *Salmonella enterica* Typhimurium ST11, *Salmonella enterica* Typhimurium ST2, *Salmonella enterica* Enteritidis SE5, and *Salmonella enterica* Choleraesuis SC1) were used as the host strains for bacteriophage isolation. To isolate bacteriophages that had lytic activity against the *Salmonella* isolates, various samples were collected from the sewage and feces in five swine farms at Paju, Asan and Yesan in Korea (Table S1). All the isolates were cultured at 37°C in TSB (tryptic soy broth, BD) and then co-cultivated with each farm specimen overnight at 37°C. Following overnight growth, the culture was centrifuged for 20 min at 10,000 × g. The resultant supernatant was filtered with 0.45-μm syringe filter. This procedure, from co-cultivation to filtration, was repeated twice to enhance the bacteriophage titer. A conventional double-layered agar method was used to examine whether the filtrates contained lytic bacteriophages for the *Salmonella* strains, respectively. A series of purification steps (plaque isolation, co-cultivation, centrifugation and filtration) were performed at least three times to obtain pure bacteriophages.

Thirteen lytic bacteriophages (SEP-1, SGP-1, STP-1, SS3eP-1, EK99P-1, SalTP-2, SChP-1, SAP-1, SAP-2, E41P-1, EK88P-1, CPP-3 and CPP-5) were isolated using the procedures described above. The lytic activity of 13 bacteriophages against *Salmonella* reference strains was summarized in Table S1. Based on the lytic spectrum and final titer of each bacteriophage, 4 bacteriophage cocktails were prepared with a mixture selected from the 13 individual bacteriophages: cocktail A (SEP-1, SGP-1, STP-1, SS3eP-1 and EK99P-1; $\geq 10^9$ plaque forming units (pfu)/m*l*), cocktail B (SEP-1, SGP-1, STP-1, SS3eP-1 and EK99P-1; $\geq 10^{11}$ pfu/m*l*), cocktail C (SEP-1, SGP-1, STP-1, SS3eP-1, SalTP-2, SChP-1, SAP-1 and SAP-2; $\geq 10^9$ pfu/m*l*), and cocktail D (SalTP-2, SChP-1, SAP-1, SAP-2, E41P-1, EK88P-1, EK99P-1, CPP-3 and CPP-5; $\geq 10^9$ pfu/m*l*). Each cocktail was prepared with equal numbers of the selected bacteriophages.

Assessment of bacteriophage host spectrum

Bacteriolytic activity of 13 bacteriophages was preferentially examined for 141 *Salmonella* isolates, respectively. The bacterial susceptibilities of all the *Salmonella* isolates to bacteriophage cocktails were assayed. One hundred microliters of bacterial culture were spread on a TSA (tryptic soy agar, BD) plate with five squares and allowed to dry. Ten microliters of each bacteriophage cocktail suspension were dropped into the center of each square and allowed to dry. A drop of saline, as the negative control, was used on one square area per plate. Following overnight incubation at 37°C for 18 hr, the plates were examined for the following: a clear zone of complete lysis, incomplete lysis (not all the infected cell underwent lysis), and no lysis in the bacterial lawn.

Distribution of bacteriophages in mice after oral administration

To determine the residual period of bacteriophages after oral administration, 50 four-week-old female BALB/c mice (DBL, Eumseong, Korea) were purchased and randomly divided into 10 groups: nine groups of five mice were orally administered 200 μl of bacteriophage cocktail C at 1×10^8 PFU/m*l* in sodium chloride-magnesium sulfate (SM) buffer with gelatin (100 mM NaCl, 10 mM $MgSO_4$ [heptahydrate], 50 mM Tris-HCl [pH 7.5], and 0.01% gelatin), and one group was the non-treatment group and administered 200 μl of SM buffer. The mice were housed in a temperature-controlled animal room with a 12-hr light-dark cycle. The reuptake of bacteriophages through the feces, respiratory system, body fluids, feed, and water was minimized by providing fresh water and feed and changing the bedding every day until the end of the experiment. The mice in the nine bacteriophage-treated groups were humanly euthanized 0, 3, 6, 9, 12, 15, 21, 28 or 35 days after bacteriophage treatment. Samples of blood, feces, and eight organs (liver, lung, spleen, kidney, small intestine, large intestine, brain, and heart) were collected at each time point.

The bacteriophage titer was measured by calculating the weight of each organ and feces in the mice. The bacteriophage titer was measured through a double agar overlay plaque assay, which was performed as follows: each organ, blood and feces sample from mice was suspended in PBS, homogenized and centrifuged at 10,000 × g for 5 min. The bacteriophage was then purified using a 0.45-μm syringe filter. The filtered bacteriophage was serially diluted 10-fold with PBS, and the diluted solution was mixed with the *Salmonella* Typhimurium culture broth at 1×10^8 colony-forming units (cfu)/m*l*. The mixture of bacteriophage and *Salmonella* was suspended in Top agar (TSB with 0.6% agar and 0.2% magnesium chloride), poured onto Bottom agar (TSB with 1.5% agar) and incubated at 37°C for 24 hr, and the bacteriophage titer was then calculated by assessing the clear zone of complete lysis. The animal experimental protocol was approved by the Chonbuk National University Institutional Animal Care and Use Committee (Approval Number: CBNU 2016-0041).

Evaluation of bacteriophage treatment against Salmonella infection in weaned pigs

To evaluate the preventive effects of bacteriophage treatment against *Salmonella* infection, 15 four-week-old pigs were purchased and divided into three groups. The bacteriophage control (PC) group and bacteriophage treatment (PT) group were fed 5 m*l* of the *Salmonella*-specific bacteriophage cocktail C at 10^9 pfu/m*l* until the end of the study. One week after initiation of the bacteriophage treatment in the PT group, the PT and challenge control (CC) groups were simultaneously challenged with 10 m*l* of *Salmonella* Typhimurium (ATCC 14028) culture at 10^8 cfu/m*l*. Fecal swabs were collected daily from all the groups at 2 and 8 days post infection (dpi) and tested for bacterial shedding. The weight of the pigs was also measured on a weekly basis. All the pigs were sacrificed for pathological evaluation two weeks after infection. The animal experimental protocol was approved by the Chonbuk National University Institutional Animal Care and Use Committee (Approval Number: CBNU 2016-0041).

Salmonella PCR

Nucleic acids were extracted from fecal samples using a MagMax™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster city, CA, U.S.A.) according to the manufacturer's instructions. Briefly, 0.01 M PBS, pH 7.4, was added to each sample to generate 30% fecal homogenates. After centrifugation for 1 min at 100 × g to pellet the larger-size particles, 175 μl of the supernatant of each sample was added to a bead tube containing zirconia beads and 235 μl of the lysis/binding solution. The bead tube was beaten at maximum speed for 5 min with a Bullet Blender® (Next Advance, Inc., Troy, NY, U.S.A.). After the beating process, the bead tubes were centrifuged at 16,000 × g for 3 min, and the supernatant was carefully transferred to clean microcentrifuge tubes. After another centrifugation at 16,000 × g for 6 min, 115 μl of the supernatant, as well as washing and elution buffers, was transferred to a 96-well microplate, and the plate was placed in a MagMax™ Express magnetic particle processor (Applied Biosystems) for automated extraction, which consisted of a 5-min lysis/binding step, two 90-sec wash steps, two 150-min wash steps, a 1-min drying step, and elution for 3 min. The extracted total nucleic acids in the elution plate were stored at −80°C until used for PCR.

The sequence information of the primers and probes used for real-time PCR is as follows: forward primer, 5′-GCCATGCTGTTCGATGAT-3′; reverse primer, 5′-GTTACCGATAGCGGGAAAGG-3′; and probe primer, 5′-Cy5-TTTTGCACCACMGCCAGCCC-BHQ-3′ [14]. The PCR was optimized with an AgPath-ID™ Multiplex RT-PCR Kit (Applied Biosystems) following the manufacturer's recommended protocol in a 25-μl reaction volume using 8 μl of extracted template. The final concentration of each primer or probe was 0.2 μM. The PCR amplification was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: a) reverse transcription for 10 min at 45°C (this step was omitted for bacterial/protozoan PCR), b) a 10-min activation step at 95°C, and c) 35 cycles of 15 sec at 95°C and 60 sec at 60°C. Samples with a threshold cycle (Ct) of 35 cycles or less were considered positive.

Table 1. Lytic spectra of four bacteriophage cocktails determined on 34 serotypes of *Salmonella* reference strains

Groups	Serotypes	No. of completely lysed isolates with the indicated phage solution			
		A	B	C	D
B	Derby, Eko, Haifa, Paratyphi B, Typhimurium (n=5)	5	5	5	5
C_1	Bareilly, Braenderup, Choleraesuis, Colindale, Oranienburg, Tennessee, Virchow (n=7)	0	7	7	0
C_2–C_3	Glostrup, Kentucky, Litchfield, Muenchen, Tallahassee, Wippra (n=6)	0	6	6	0
D_1	Berta, Dublin, Enteritidis (n=3)	3	3	3	3
D_2	Hillingdon (n=1)	0	1	1	0
E_1	Amsterdam, Give, Illinois, Muenster, Onireke, Westhampton (n=6)	0	6	6	0
E_4	Liverpool, Senftenberg (n=2)	0	2	2	0
F	Abaetetuba, Aberdeen (n=2)	0	2	2	2
G	Kedougou (n=1)	0	1	1	0
H	Sundsvall (n=1)	0	1	1	0

Fecal metagenomic analysis

To evaluate the negative effects of bacteriophage treatment on the normal intestinal flora, fecal swabs collected from the above-described pig challenge study was subjected to a fecal metagenomic analysis. The DNA in each fecal sample was extracted using a stool DNA extraction kit (PowerFecal® DNA Isolation Kit, Qiagen, Hilden, Germany), and each extracted fecal DNA was pooled to obtain each corresponding group for library construction and sequenced using a NGS system. The microflora analysis was performed as follows: Bacterial 16S rRNA genes were amplified by PCR from DNA samples using the V1-V12 variable regions (Ion 16S™ Metagenomics Kit, Life Technologies, Carlsbad, CA, U.S.A.). The library preparation and 16S metagenomic sequencing were analyzed using the Ion Torrent Personal Genome Machine (PGM, Life Technologies) according to the manufacturers' specifications and as described previously [40, 49]. All sequencing reactions were performed using 316 chips and the Ion Torrent 400-bp sequencing kit (Life Technologies) with an approximate runtime of 4.5 hr per chip. Primary base calling was performed using Torrent Suite v5.0 software (Life Technologies), and sequences were exported in FastQ format. FastQ files were used for all subsequent analyses.

Data analysis

Graphs were constructed using GraphPad Prism 5.0.2 (GraphPad, San Diego, CA, U.S.A.), and the statistical analyses were performed using SPSS Advanced Statistics 17.0 software (SPSS, Inc., Chicago, IL, U.S.A.). The levels of *Salmonella* shedding were analyzed by ANOVA with repeated measurements. A contrast was constructed when direct comparisons between the groups were necessary.

RESULTS

Host spectra of bacteriophage cocktails

The abilities of four bacteriophage cocktails to lyse 34 reference strains of 34 serotypes and 107 field isolates of 12 serotypes isolated from swine farms and slaughterhouses in Korea were evaluated. As summarized in Table 1, all the reference strains were clearly lysed by cocktails B and C, whereas eight (23.5%) serotypes of reference strains showed complete lysis by cocktails A and D. Among 107 *Salmonella* isolates, 99 (92.5%) isolates were lysed by cocktails B and C, but 63 (58.9%) and 57 (55.3%) of the isolates were lysed by cocktails A and D, respectively. All the isolates of *Salmonella* Typhimurium (n=64), the most prevalent serotype in Korean swine farms, were completely lysed with cocktails B and C. Serotypes such as Schwarzengrund (n=7), Derby (n=3), London (n=2) and Hadar (n=2) were also clearly lysed by cocktails B and C. However, the testing of 23 isolates belonging to serotype Rissen with cocktails A and D revealed no lysis, but 16 (69.6%) of the isolates were completely lysed with cocktails B and C. Essen (n=1), one of the least frequently isolated serotypes in Korea, was not sensitive to any of the tested bacteriophage cocktails (Table 2).

Antibacterial properties of bacteriophage cocktails against antibiotic-resistant Salmonella isolates

One hundred seven *Salmonella* isolates were tested for antibiotic susceptibility (Table 3). Among 107 *Salmonella* isolates, all isolates were resistant to one or more of the antibiotics tested in this study. The antibiotic susceptibility of the isolates varied as follows: N (80.4%), SXT (76.7%), ENR (61.7%), C (60.7%), GM (60.7%), AM (48.6%), S (34.6%), Te (18.7%) and NA (0%). Of the 107 resistant isolates, 80 (74.8%) were MDR isolates showing resistance to at least three antibiotics belonging to different antibiotic classes. Most of the MDR isolates (65%, 52/80) were *Salmonella* Typhimurium, and only *Salmonella* Typhimurium isolates were resistant to all eight antibiotics (Table 3). Among the 80 MDR isolates, 74 (92.5%) isolates were clearly lysed with cocktails B and C (Table 4). As described above, five isolates of serotype Rissen and one isolate of serotype Essen (n=1) were not sensitive to any of the tested bacteriophage cocktails (Table 2).

Table 2. Lytic spectra of four bacteriophage cocktails determined on 107 *Salmonella* field isolates

Serotypes	No. of completely lysed isolates with the indicated phage solution (%)			
	A	B	C	D
Typhimurium (n=64)	57	64	64	54
Teddington (n=1)	0	1	1	0
Derby (n=3)	3	3	3	0
Othmarshen (n=1)	0	1	1	0
Rissen (n=23)	0	16	16	0
Essen (n=1)	0	0	0	0
London (n=2)	0	2	2	0
Hadar (n=2)	2	2	2	2
Schwarzengrund (n=7)	0	7	7	0
Virchow (n=1)	0	1	1	0
Newport (n=1)	0	1	1	0
Mendoxa (n=1)	1	1	1	1
Total (n=107)	63 (58.9)	99 (92.5)	99 (92.5)	57 (53.3)

Table 3. Rate of host lysis in cocktail C observed among antibiotic-resistant *Salmonella* serotypes

Serotypes	No. of isolates to complete lysis in cocktail C/ No. of isolates resistant to indicated number of antibiotics classes						
	1[a)]	2[a)]	3[a)]	4[a)]	5[a)]	6[a)]	MDR[b)] (≥3)
Rissen (n=23)	1/1	5/7	2/5	6/7	2/3		10/15
Schwarzengrund (n=7)	1/1		6/6				6/6
Typhimurium (n=64)	5/5	7/7	5/5	23/23	14/14	10/10	52/52
Other serotypes (n=13)	4/4	2/2	6/6		0/1[c)]		6/7
Total (n=107)	11/11	14/16	19/22	29/30	16/18	10/10	74/80

a) Number of resistant to indicated number of antibiotics classes, b) Multi antibiotics-resistance to more than three antibiotics classes, c) Isolates of *Salmonella* Essen.

Table 4. Percentage of multidrug-resistant *Salmonella* isolates lysed by the bacteriophage cocktails

No. of classes of resistant antibiotics[a)]	Patterns of resistant antibiotic classes	No. of isolates completely lysed by the indicated phage solution (%)			
		A	B	C	D
3 (n=22)	PeAgTe[b)] (n=1) PeTeQu (n=1) AgTeQu (n=16) AgQuPh (n=2) TeQuPh (n=2)	10	19	19	3
4 (n=30)	PeAgQuPh (n=1) PeTeQuSu (n=3) PeAgTeQu (n=18) PeTeQuPh (n=2) AgTeQuPh (n=5) AgTeQuSu (n=1)	20	29	29	20
5 (n=18)	PeAgTeQuPh (n=8) PeAgTeSuPh (n=2) PeAgTeQuSu (n=5) AgTeQuSuPh (n=3)	11	16	16	12
6 (n=10)	PeAgTeQuSuPh (n=10)	10	10	10	10
Total (n=80)		51 (63.8)	74 (92.5)	74 (92.5)	45 (56.3)

a) Number of isolates resistant to the indicated number of antimicrobials classes; b) penicillins (Pe), aminoglycosides (Ag), tetracyclines (Te), quinolones (Qu), sulfonamides (Su) and phenicols (Ph).

Evaluation of the distribution and stability of bacteriophages in mice

Three days post-bacteriophage inoculation (dpi), bacteriophages were detected in all the organs examined in the current study at the range of $10^{4.544}$(liver) to $10^{8.301}$(lung) pfu/m*l*. At 3 dpi, a high bacteriophage titer ($10^{8.041}$ pfu/m*l*) was also detected in feces, whereas a relatively low bacteriophage titer was observed in blood ($10^{3.041}$ pfu/m*l*). At 15 dpi, higher titers of bacteriophages ($10^{3.342}$–$10^{4.462}$ pfu/m*l*) were detected in the digestive system (small and large intestine, respectively) and feces than in any other specimen, and low titers of bacteriophages ($10^{0.602}$ and $10^{0.699}$ pfu/m*l*) were continuously detected in the digestive system until 28 dpi, whereas no bacteriophage was detected elsewhere. Although high titers of bacteriophages ($10^{2.362}$–$10^{3.322}$ pfu/m*l*) were maintained in the lung, spleen, brain and heart until 15 dpi, a low bacteriophage titer was detected in blood and other organs only up to 21 dpi. No bacteriophages were detected in any specimen at 35 dpi (Table 5).

Table 5. Distribution of bacteriophages in the organs, blood and feces of mice treated with the bacteriophage cocktail C

DPI	Blood	Liver	Lung	Spleen	Kidney	Small intestine	Large intestine	Brain	Heart	Feces
3	3.041[a)] ± 2.7	4.544 ± 4.4	8.301 ± 8.5	7.230 ± 6.8	8.041 ± 7.1	7.380 ± 7.2	6.792 ± 5.5	6.000 ± 6.0	6.447 ± 6.1	8.041 ± 7.7
6	2.176 ± 1.2	5.230 ± 4.4	5.398 ± 4.4	6.863 ± 6.6	7.279 ± 6.0	6.114 ± 5.6	5.740 ± 5.1	6.519 ± 4.3	6.301 ± 6.4	7.580 ± 5.1
9	1.204 ± 0.7	5.146 ± 5.1	4.908 ± 5.1	6.799 ± 6.6	6.000 ± 7.8	6.230 ± 6.0	5.477 ± 6.7	5.613 ± 6.7	6.079 ± 6.9	7.591 ± 7.0
12	0.322 ± 0.6	0.934 ± 0.4	4.903 ± 4.7	4.934 ± 4.6	1.643 ± 1.0	3.643 ± 3.1	5.491 ± 5.4	4.699 ± 5.3	5.633 ± 5.0	6.987 ± 7.5
15	0.342 ± 0.1	0.672 ± 0.1	3.079 ± 2.9	3.322 ± 3.0	1.663 ± 1.1	3.342 ± 3.0	4.462 ± 4.2	2.690 ± 2.0	2.362 ± 1.9	3.633 ± 3.1
21	0.079 ± 0.1	0.255 ± 0.0	1.041 ± 0.8	ND	1.000 ± 0.9	2.301 ± 1.9	3.000 ± 3.3	ND	1.771 ± 1.5	2.301 ± 2.6
28	ND	ND	ND	ND	ND	0.602 ± 0.8	0.699 ± 1.4	ND	ND	ND
35	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

a) (PFU/m*l*), Log_{10} ± standard deviation. ND: no detection.

Effects of Salmonella-specific bacteriophages on weaned pigs infected with Salmonella Typhimurium

To evaluate the effects of *Salmonella*-specific bacteriophage cocktail C, we measured *Salmonella* shedding in pig feces after experimental challenge with *Salmonella* Typhimurium in the PT and CC groups until 8 dpi. The PT group showed the highest level of *Salmonella* shedding at 2 dpi ($10^{1.8}$ cfu/m*l*), and thereafter, a continuous decrease in *Salmonella* shedding was observed until 7 dpi. The CC group showed similar levels of *Salmonella* shedding compared with the PT group until 6 dpi and a continuous increase in *Salmonella* shedding until at the end of the experiment (8 dpi, Fig. 1).

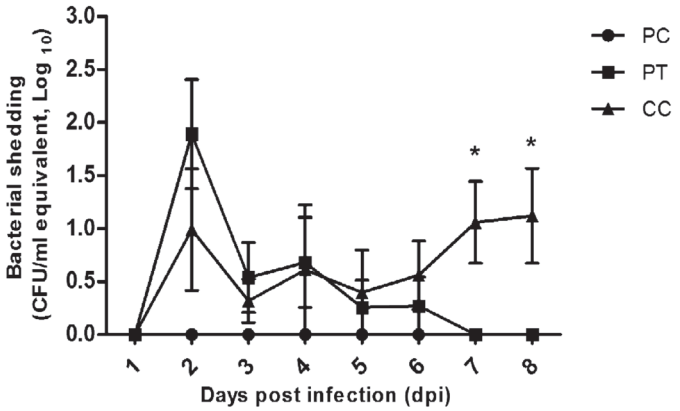

Fig. 1. Levels of *Salmonella* shedding in feces (means ± SEMs) collected from pigs treated or not treated with *Salmonella*-specific bacteriophages after challenge with *Salmonella* Typhimurium. PC, Phage control; PT, Phage treatment; CC, Challenge control.

Evaluation of changes in the pig intestinal bacterial flora after Salmonella bacteriophage cocktail C treatment

To compare the fecal bacterial flora in the pigs belonging to the three experimental groups (PC, PT and CC) after bacteriophage treatment and/or *Salmonella* challenge, we analyzed the relative ratio of the phylum- and family-level assignments through metagenomics. As shown in Table 6 and Fig. 2, the unique phylum- and family-level compositions of fecal bacterial flora in each pig group challenged with *Salmonella* or treated with bacteriophage cocktail C were observed.

To investigate the changes in fecal bacterial flora during the experimental period, the pig fecal bacterial flora before and after *Salmonella* and bacteriophage administration was examined. At 0 dpi, Firmicutes was the predominant phylum in the fecal bacterial flora (42.78, 25.08 and 28.06% in the PC, P and CC groups, respectively), and Bacteroidetes was the next most abundant phylum (22.02, 22.42 and 23.03% in the PC, PT and CC groups, respectively). At 2 dpi, Bacteroidetes was the predominant phylum in the fecal bacterial flora of all the groups (71.76, 76.01 and 51.16% for the PC, PT, and CC groups, respectively), and Firmicutes was the next most abundant phylum (26.72, 22.56 and 41.31% for the PC, PT, and CC groups, respectively; Fig. 2). At 8 dpi, however, Proteobacteria became the predominant phylum (44.37, 67.78 and 67.69%, for the PC, PT and CC groups, respectively), and the next most common phylum differed between the groups: Bacteroidetes, Actinobacteria and Firmicutes were the next most common phyla in the PC (30.09%), PT (24.94%) and CC (18.90%) groups (Fig. 2). Based on the results of the phylum levels, the fecal bacterial flora were identified at the family level (Table 6). Prevotellaceae, which belongs to the phylum Bacteroidetes, was the predominant family among the fecal bacterial flora found in all the groups at 2 dpi (64.495, 46.736 and 38.838% in the PC, PT, and CC groups, respectively; Table 6). At 8 dpi, Porphyromonadaceae (18.182%), which belongs to the phylum Bacteroidetes, and Xanthomonadaceae (12.885%) and Comamonadaceae (11.817%), which belong to the phylum Proteobacteria, were the predominant families in the fecal bacterial flora found in the PC group, whereas Xanthomonadaceae (18.070%) and Nocardiaceae (16.978%), which belong to the Actinobacteria phylum, and Comamonadaceae (15.010%) were the predominant families in the fecal bacterial flora found in the PT group (Table 6). However, Enterobacteriaceae (26.653%), which belongs to the phylum Proteobacteria and includes *Salmonella* species, was the predominant family in the fecal bacterial flora found in the CC group, and markedly lower percentages of Enterobacteriaceae were observed in the PC (2.292%) and PT (4.955%) groups at 8 dpi (Table 6).

DISCUSSION

The *Salmonella* serotypes London, Newport, Rissen, Schwanzengrund and Typhimurium have been isolated from human

Table 6. Composition of family level of fecal microbes in pigs with or without *Salmonella*-specific bacteriophage cocktail C

Phylum	Family	0 dpi			2 dpi			8 dpi		
		PC[a)]	PT	CC	PC	PT	CC	PC	PT	CC
Actinobacteria	Nocardiaceae	0.000[b)]	0.000	0.000	0.056	0.000	0.018	9.896	16.978	5.747
Bacteroidetes	Bacteroidaceae	17.069	17.487	12.656	1.568	2.899	2.922	9.416	0.187	0.152
	S24-7	0.000	0.000	0.000	2.819	0.590	7.358	0.201	0.101	0.350
	Porphyromonadaceae	0.085	0.021	0.074	3.211	22.413	4.792	18.182	0.244	1.095
	Prevotellaceae	4.320	4.394	9.807	64.495	46.736	38.838	0.635	2.269	1.657
	Rikenellaceae	0.544	0.514	0.494	1.512	0.052	0.089	1.301	0.158	0.137
Firmicutes	Acidaminococcaceae	9.959	4.555	6.870	0.075	0.052	0.214	0.046	0.000	0.000
	Erysipelotrichaceae	10.128	5.112	7.579	1.027	0.174	2.815	0.093	0.029	0.258
	Clostridiaceae	1.511	2.333	2.100	1.941	1.406	17.584	1.580	2.011	3.056
	Lachnospiraceae	5.717	2.551	0.829	9.334	8.472	2.298	1.301	0.172	0.958
	Lactobacillaceae	0.350	0.813	0.087	1.157	1.875	0.338	0.248	0.316	0.076
	Christensenellaceae	8.907	4.214	6.679	0.187	0.017	0.499	0.170	0.000	0.000
	Ruminococcaceae	6.013	5.458	3.846	6.384	6.667	4.810	0.883	0.158	1.140
	Peptostreptococcaceae	0.193	0.042	0.071	0.504	1.979	13.611	0.139	0.244	1.156
Proteobacteria	Comamonadaceae	0.000	0.000	0.000	0.000	0.017	0.018	11.817	15.010	6.599
	Enterobacteriaceae	0.048	1.165	1.107	0.000	0.000	0.018	2.292	4.955	26.653
	Pseudomonadaceae	0.000	0.000	0.000	0.000	0.017	0.053	6.257	8.475	8.925
	Xanthomonadaceae	0.000	0.000	0.000	0.000	0.069	0.000	12.885	18.070	7.450
Spirochaetes	Spirochaetaceae	19.966	9.865	7.733	0.019	0.000	0.285	0.015	0.000	0.000

a) Phage control (PC), Phage treatment (PT), Challenge control (CC), b) % of mapped reads.

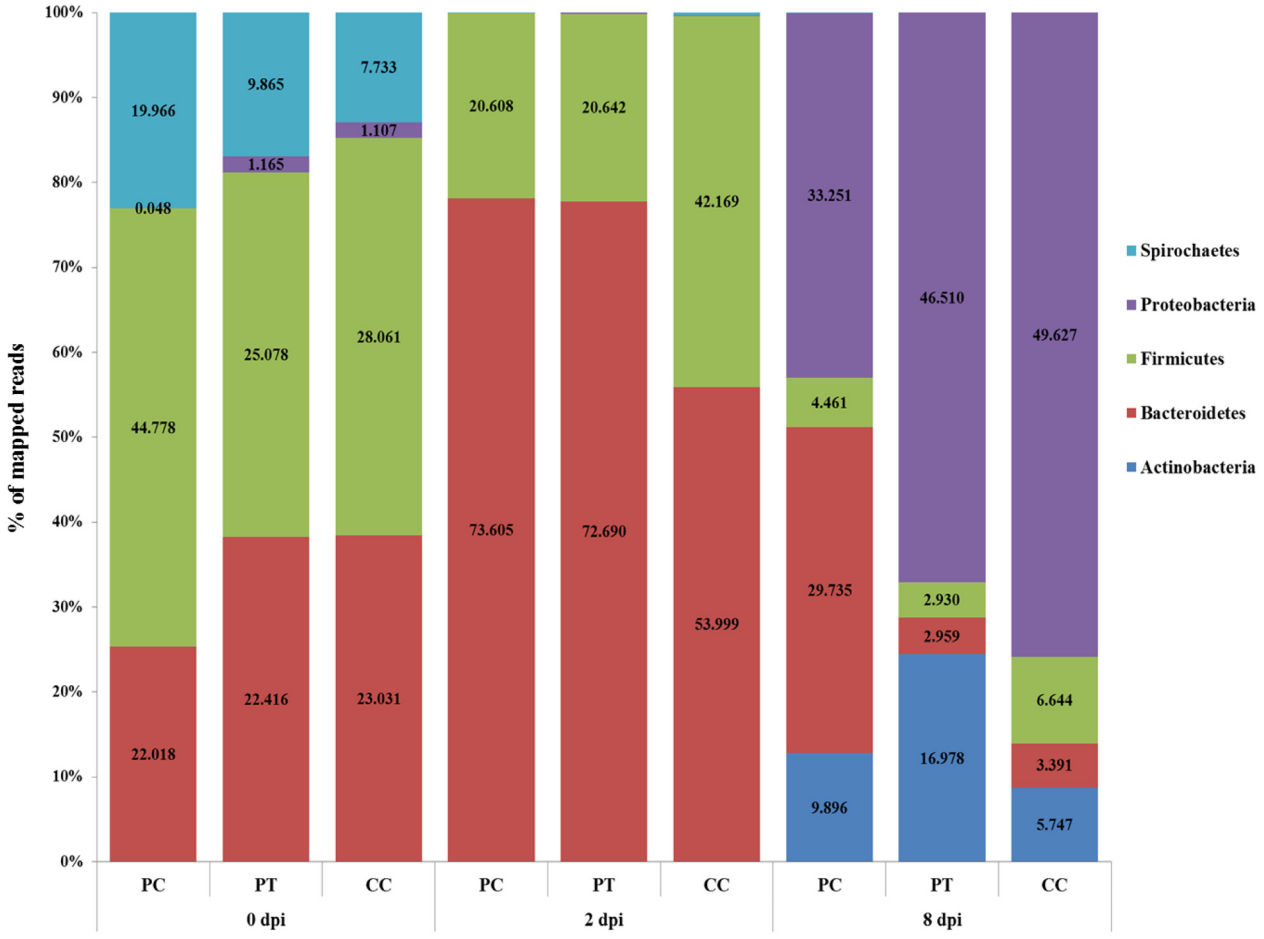

Fig. 2. Phylum-level metagenomic analyses of fecal bacteria in pigs treated or not treated with *Salmonella*-specific bacteriophages after challenge with *Salmonella* Typhimurium. Five fecal samples were pooled for each group. PC, Phage control; PT, Phage treatment; CC, Challenge control.

clinical samples in Korea. Among these, Typhimurium is the most common serotype that causes human salmonellosis [13]. In this study, *Salmonella* Typhimurium isolates exhibited markedly higher antibiotic resistance than other serotypes. Additionally, among 107 resistant isolates, 80 (74.8%) were MDR, and most of these were *Salmonella* Typhimurium (Table 4). These results are consistent with the results of *Salmonella* prevalence surveys conducted at swine farms in Korea [34]. *Salmonella* Typhimurium is an important public health concern because it is a zoonotic bacterium that is frequently isolated from swine production systems and exhibits high resistance to antibiotics [19, 20, 26]. Thus, a standard management protocol is required to avoid the introduction and transmission of *Salmonella* in swine farms and during processing. Previous studies have suggested that the direct feeding of bacteriophages to supplemented feeds or water, which results in decreased bacterial shedding and colonization, the spraying of bacteriophages in the environment to reduce bacterial contamination, or the rinsing of carcasses are practical and effective means for reducing transmission to humans [21, 25, 48]. Although *Salmonella* can be controlled by vaccines, bacteriophages are cost effective and as efficient as vaccines. Thus, bacteriophage treatments could be a good candidate for animal disease control [41].

In the present study, *Salmonella*-specific bacteriophage cocktails demonstrated broad-spectrum lytic capability against *Salmonella* reference strains and field isolates (Tables 1 and 2). Atterbury *et al.* [2] reported that among 232 *Salmonella* bacteriophages, three bacteriophages were effective against the broadest host range of the serotypes Enteritidis, Hadar and Typhimurium. Zhang *et al.* [52] reported that 10 bacteriophages were effective against *Salmonella* Typhimurium isolated from diseased pigs. Unexpectedly, among the 23 isolates of *Salmonella* Rissen, only 16 isolates (69.6%) were completely lysed with cocktails B and C, and *Salmonella* Essen (n=1), one of least frequently isolated serotypes in Korea, was not sensitive to any of the tested bacteriophage cocktails (Table 2). *S.* Rissen and *S.* Essen are generally detected in samples from pigs at a slaughterhouse rather than in clinical samples, as observed in previous studies. Therefore, the risk of clinical disease outbreaks caused by these particular isolates was considered very low [7, 8, 30]. In general, the bacteriophage cocktails B and C showed more efficient lytic capability against various *Salmonella* species than the other two cocktails. The bacteriophage cocktail B ($\geq 10^{11}$ pfu/m*l*), which had the same bacteriophage components as cocktail A ($\geq 10^{9}$ pfu/m*l*) except for the bacteriophage titer, was more efficient against *Salmonella* than cocktail A. Bacteriophage cocktail C, which had a different bacteriophage titer and components compared with cocktail B, showed similar levels of lytic performance against *Salmonella*. Thus, these results demonstrated that the bacteriophage titer and bacteriophage components are important factors determining the lytic capability of bacteriophages. This result is similar to the *in vivo* results obtained in previous studies, in which the use of a high bacteriophage titer significantly reduced *Salmonella* colonization in chicken [2, 4, 24].

The distribution and duration of bacteriophages cocktail C in various organs, blood and feces of mice after oral inoculation with bacteriophages were investigated until 35 dpi. As a result, bacteriophages were detected in all the organs, blood and feces until 21 dpi and in the digestive organs (small and large intestine) of the mice until 28 dpi (Table 5). The detection of bacteriophages in all the tested organs might be due to bacteriophage delivery through the blood circulation. Therefore, further research is needed to evaluate the possibility of the induction of antibodies against bacteriophages during long-term bacteriophage treatment. In addition, even though the bacteriophages are quite specific for *Salmonella* spp., they might be able to replicate in other bacteria of the Enterobacteriaceae family at low levels because the orally injected bacteriophages were detected in intestines for a relatively long duration.

To evaluate the protective effects of bacteriophage cocktail C against *Salmonella* infection, we measured the levels of *Salmonella* shedding in feces until 8 dpi. The highest levels of *Salmonella* shedding were observed in the PT group at 2 dpi, *Salmonella* shedding gradually decreased from 3 dpi, and no *Salmonella* shedding was detected at 7 dpi. However, the levels of *Salmonella* shedding continued to increase from 6 until 8 dpi, which indicated that *Salmonella* had been successfully colonized and started replicating in the pig intestines (Fig. 1). Thus, this result demonstrated that bacteriophage cocktail C can efficiently resolve *Salmonella* infection in pigs. Callaway *et al.* [10] reported that the groups treated with a bacteriophage cocktail for 48 hr presented a decreased *Salmonella* population in the cecal and rectal intestinal contents of pigs compared with the controls. Jamalludeen *et al.* [28] reported that six bacteriophages were effective in enterotoxigenic *Escherichia coli* (ETEC)-challenged pigs, and six days after the inoculation, the bacteriophage-treated group showed a decreased average number of *E. coli*.

One of the most important changes in the pig intestinal microbial flora due to the administration of *Salmonella*-specific bacteriophages is the decline in the abundance of phyla and families containing *Salmonella* in the PT groups until 8 dpi. The PC and PT groups showed increases in the phylum-level percentages of mapped reads of Proteobacteria due to increases in Comamonadaceae and Xanthomonadaceae until 8 dpi. Enterobacteriaceae, which include *Salmonella* spp., was observed at a low percentage of mapped reads in the PT group, probably due to the effect of bacteriophage cocktail C. However, the CC group showed an increase in the phylum-level percentage of mapped reads of Proteobacteria due to an increase in Enterobacteriaceae until 8 dpi. As previously reported, dietary treatment of pigs with bacteriophages resulted in decreases in the *Salmonella* and *E. coli* concentrations in the fecal microflora compared with the concentrations obtained with a normal diet [50]. Thus, these metagenomic analyses revealed that treatment with *Salmonella* bacteriophage cocktails could reduce *Salmonella* colonization in pig intestines without causing any significant negative impacts on the normal intestinal flora (Table 6 and Fig. 2).

In conclusion, *Salmonella*-specific bacteriophage cocktails might be a safe and efficient tool for controlling *Salmonella* infection in pigs.

ACKNOWLEDGMENT. This study was supported by a grant from the Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries (No. 1121314).

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