Efficacy of Bacteriophage Therapy on Horizontal Transmission of *Salmonella* Gallinarum on Commercial Layer Chickens

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SUMMARY. A *Salmonella* Gallinarum (SG)-specific bacteriophage isolated from sewage effluent was used to prevent horizontal transmission of SG in commercial layer chickens. Six-week-old chickens, each challenged with 5×10^8 colony-forming units of SG, cohabited with contact chickens treated with 10^6 plaque-forming units/kg of bacteriophage, prepared in feed additives, for 7 days before, and 21 days after challenge with SG. Mortality was observed for 3 wk after challenge and SG was periodically re-isolated from the liver, spleen, and cecum of chickens. SG re-isolation from organs was decreased and a significant ($P < 0.05$) reduction in mortality was observed in contact chickens treated with the bacteriophage, as compared to untreated contact chickens, indicating that bacteriophage administration in feed additives significantly prevented the horizontal transmission of SG. These results provide important insights into prevention and control strategies against SG infection and suggest that the use of bacteriophages may be a novel, safe, and effectively plausible alternative to antibiotics for the prevention of SG infection in poultry.

RESUMEN. Eficacia de la terapia de bacteriófagos en la transmisión horizontal de *Salmonella* Gallinarum en gallinas ponedoras comerciales.

Un bacteriófago específico para *Salmonella* Gallinarum aislado de aguas residuales se utilizó para prevenir la transmisión horizontal de *S.* Gallinarum en gallinas ponedoras comerciales. Pollos de seis semanas de edad que fueron desafiados con 5×10^8 unidades formadoras de colonias de *S.* Gallinarum, cohabitaron con pollos contactos que fueron tratados con 10^6 unidades formadoras de placa / kg del bacteriófago, preparado como aditivo para el alimento, durante 7 días antes y 21 días después del desafío con *S.* Gallinarum. Se observó la mortalidad durante 3 semanas después del desafío y *S.* Gallinarum fue periódicamente re-aislado del hígado, el bazo, y el ciego de pollos. El re-aislamiento de *S.* Gallinarum de los órganos se redujo y se observó una disminución significativa en la mortalidad ($P < 0.05$) en los pollos contacto tratados con el bacteriófago, en comparación con los pollos contacto no tratados, lo que indica que la administración de bacteriófagos como aditivo en el alimento previno de manera significativa la transmisión horizontal de *S.* Gallinarum. Estos resultados proporcionan información importante sobre las estrategias de prevención y control de la infección por *S.* Gallinarum y sugieren que el uso de bacteriófagos puede ser una alternativa novedosa, segura y eficaz en lugar del uso de antibióticos para la prevención de la infección por *S.* Gallinarum en la avicultura comercial.

Key words: *Salmonella* Gallinarum, bacteriophage, feed additives, horizontal transmission

Abbreviations: BGA = brilliant green agar; BP = bacteriophage; CFU = colony-forming units; dpc = day(s) postchallenge; FT = fowl typhoid; LB = Luria-Bertani broth; PFU = plaque-forming units; RSA = rapid serum agglutination; SG = *Salmonella* Gallinarum; SM = Super Optimal broth medium

Fowl typhoid (FT), which has been reported since 1992 in Korea, is an acute and septicemic disease of chickens caused by *Salmonella enterica* subsp. *enterica* serovar Gallinarum (SG) (11). FT is a disastrous disease in the poultry industry, not only because of its ability to cause enormous economic losses but also because it is extremely difficult to eradicate (2). Despite prevention and control strategies in commercial chicken flocks, which include increased biosecurity, vaccination, competitive exclusion, and antimicrobials, SG infection has posed a constant problem in the Korean poultry industry since 1992 (12). Moreover, the emergence of antibiotic-resistant SG strains against most currently available antimicrobials, and the restricted use of antibiotics, have increased the need for novel and effective SG control strategies (9).

Bacteriophages are ubiquitous viruses in nature that infect and kill bacteria (21). Although early experiences with bacteriophage therapy showed reduced success, and the rapid development of chemotherapy led to discontinuation of the research on phage therapy, recent interest in the therapeutic use of bacteriophages has been spurred by successes relating to their use in controlling infectious pathogens such as *Salmonella* (1), *Escherichia coli* (15), and *Clostridium* (17). A wide–host-range bacteriophage will have less impact to the host microflora than will antibiotics, but may still affect the overall population dynamics. However, host-specific bacteriophages are limited to attacking their targeted bacteria species, with very little affect on microflora populations; this ensures an excellent level of safety for therapeutic phage preparations and also enables targeted therapy of bacterial infections (3). However, previous studies demonstrated that cocktails of bacteriophage containing several lytic phages against the homologous bacterial strain do provide protective efficacy *in vivo* (4,20,22). Furthermore, the prophylactic and therapeutic effect of bacteriophages, although assumed in many instances, has also not been adequately evaluated in terms of clinical and practical significance in preventing the transmission of SG infection.

The present study evaluated whether treatment with a single strain of SG-specific bacteriophage delivered in feed additives could prevent or significantly reduce morbidity and mortality caused by the horizontal transmission of SG infections in commercial layer chickens. The result showed that bacteriophage therapy significantly reduced mortality caused by horizontal transmission of SG and provided suppression of organ invasion against SG.

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MATERIALS AND METHODS

Experimental animals. Six-week-old commercial layer chickens were obtained from a *Salmonella*-free chicken flock and were housed in a cage under strict biosecurity. The chickens were negative for antibodies against *Salmonella* Enteritidis and *Salmonella* Typhimurium by enzyme linked-immunosorbent assay (Biocheck, Foster City, CA) and against SG by a rapid serum agglutination (RSA) test. All experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the Konkuk University, Seoul, Korea.

Isolation and propagation of bacteriophage CJø01. Bacteriophage screening samples were collected from sewage effluent in Kunggi province by the CJ Research Institute of Biotechnology. Samples were centrifuged at $1550 \times g$ for 10 min and the supernatants were filtered through a 0.45-µm filter. Filtered samples were mixed with log-phase SG in 10× Luria-Bertani (LB) medium (tryptone 10 g; yeast extract 5 g; NaCl 10 g; in a final volume of 1 liter). The mixtures were incubated at 37 C for 18 hrs and then centrifuged at $1550 \times g$ for 10 min, after which the supernatants were filtered through a 0.2-µm filter. The filtered samples were mixed with fresh log-phase SG and 3 ml of 0.7% agar. The solutions were added to LB plates and incubated 37 C for 18 hrs. One distinct, clear plaque was selected and purified three times. The purified plaque was suspended in Super Optimal broth medium (SM) buffer (0.1 M NaCl, 1 mM $MgSO_4$, 0.2 M Tris [pH 7.5], 0.01% gelatin) and stored at 4 C until use. The selected bacteriophage CJø01 was cultured in large quantities using SG. The SG was cultured with shaking, and an aliquot of 1.5×10^{10} colony-forming units (CFU) was centrifuged at $1550 \times g$ for 10 min and the pellet was re-suspended in 4 ml of SM solution. The 7.5×10^7 plaque-forming units (PFU) of bacteriophage were inoculated (multiplicity of infection = 0.005) and left at 37 C for 20 min. This solution was then inoculated into 150 ml of an LB media in a flask and cultured at 37 C for 5 hrs. The solution was centrifuged at $1550 \times g$ for 10 min and filtered through a 0.2-µm filter. The bacteriophage was enumerated by making serial dilutions and by preparing soft agar overlay plates. This procedure produced bacteriophage preparations containing 10^{11} bacteriophage/ml. The bacteriophage was prepared as a powder, using a spray dryer, and the powder was diluted to obtain 10^6 PFU/kg as a final concentration for use in feed additives.

Challenge bacterial strain. *Salmonella* Gallinarum 2293 (SG2293), purchased from Salmonella Genetic Stock Center (Calgary, AB, Canada), was used and was designated as SG. Inocula for challenge were prepared from 18–24-hr Luria-Bertani broth cultures maintained at 37 C. After overnight incubation, the broth was centrifuged at $2500 \times g$ for 10 min and the bacterial pellet was suspended and serially diluted in sterile phosphate-buffered saline (pH 7.2). Bacterial enumeration of the suspension was performed using LB agar. The SG challenge strain is virulent for chickens and the 50% lethal dose of SG challenge strain, in challenged and contact chickens, was determined to be 5×10^6 CFU/ml and 5×10^8 CFU/ml, respectively.

Experimental design. Six-week-old commercial layer chickens ($n = 175$) were divided into three experimental groups (Table 1). Group 1 contained 70 birds; 35 birds were each orally-challenged with 5×10^8

Table 1. Experimental design.

Group	Number of chickens	Treatment
1 Challenged	35	SG challenged[A] and BP treated[B]
Contact[C]	35	SG unchallenged and BP treated
2 Challenged	35	SG challenged and untreated
Contact	35	SG unchallenged and untreated
3 Negative control	35	SG unchallenged and untreated

[A]Six-week-old chickens were orally challenged with SG (KP-93 strain) at a concentration of $5 \times 10^{8.0}$ CFU/bird.

[B]BP = bacteriophage; chickens were treated with a bacteriophage as a feed additive at a concentration of $10^{6.0}$ PFU/kg.

[C]Chickens were housed in the same cage with challenged chickens.

CFUs of SG and 35 contact birds were treated with 10^6 PFU/kg of bacteriophage, contained in the feed additive, for 7 days before and 21 days after SG challenge. Group 2 contained 70 birds; 35 birds were orally challenged with SG and 35 contact birds did not receive treatment. Group 3 contained 35 birds that served as the unchallenged and untreated negative controls.

To avoid a high density of chickens in any cage, which could change the dynamics of the spread of *Salmonella*, 70 chickens of groups 1 and 2 were divided into 4 cages, respectively. Forty chickens were housed in two cages (Cage 1 = 10 challenged chickens + 10 contact chickens [$n = 20$]; Cage 2 = 10 challenged chickens + 10 contact chickens [$n = 20$]) to observe mortality caused by SG horizontal transmission. Thirty chickens were housed in two cages (Cage 3 = 7 challenged chickens + 8 contact chickens [$n = 15$]; Cage 4 : 8 challenged chickens + 7 contact chickens [$n = 15$]) to observe the re-isolation rate of SG.

The birds were monitored for mortality daily for 21 days after challenge. Sera samples were collected for SG antibody detection using RSA at 2 wk after challenge. At 7, 14, and 21 days postchallenge (dpc), the liver, spleen, and cecum were aseptically collected from 10 chickens per group (five challenged and five contact chickens) to re-isolate the SG challenge strain.

Bacteriologic analysis. An approximately 1 g tissue sample was macerated in 10 ml of buffered peptone water broth (Difco, Detroit, MI) and incubated overnight at 37 C. A 0.1-ml volume of culture was inoculated into Rappaport-Vassiliadis broth (Difco) and incubated at 37 C for 48 hr prior to plating on xylose-lysine desoxycholate (Difco) and brilliant green agar (Difco). Plates were incubated at 37 C for 24 hr and examined for the presence of SG. The identity of the challenge strain was confirmed using *Salmonella* antiserum (Difco).

Serologic tests. SG antibodies were detected by the RSA plate test used as an authorized method in Korea. The serum plate agglutination antigen was prepared with a homologous SG strain as described previously (5). For the reaction, 30 µl of antigen was mixed with an equal volume of serum on a clean, white tile marked into squares of about 3×3 cm². The mixture was observed for agglutination after 2 min of constant rotation. A positive reaction was indicated by easily visible clumping of the antigen within 2 min.

Statistical analysis. Mortality rate in chickens and SG re-isolation rate in organs were analyzed using a one-tailed Fisher's exact test. A P-value < 0.05 was considered to be statistically significant.

RESULTS

In a serologic examination, all the chickens were determined to be *Salmonella* seronegative prior to challenge and were demonstrated to be efficiently seroconverted to SG at 2 wk after bacterial challenge, suggesting that horizontal transmission of SG had occurred (Table 2). Mortality of challenged chickens was first observed at 7 dpc (Fig. 1). Mortality rates of challenged and contact chickens that did not received the bacteriophage were 55% and 35%, but challenged and contact chickens treated with the bacteriophage were 50% and 5%, respectively (Table 2, Fig. 1). The mortality rate of the contact chickens treated with the bacteriophage was significantly decreased ($P < 0.05$) when compared with that of the untreated contact chickens.

In the re-isolation study, untreated contact chickens displayed a 40% re-isolation rate of the SG challenge strain in the liver and spleen, while the challenge strain was not isolated in contact chickens treated with bacteriophage at 2 wk postchallenge (Table 3). In addition, the untreated contact group showed a 40–60% re-isolation rate of the challenge strain in the liver and spleen, while the re-isolation rate in bacteriophage-treated contact chickens was 20–40% at 3 wk postchallenge. Although there were no significant differences in the re-isolation rate of the challenge strain between bacteriophage-treated and untreated contact chickens, bacteriophage treatment reduced the number of chickens colonized with the pathogen after challenge.

Table 2. Effect of BP on mortality caused by horizontal transmission of SG in commercial layers.

Group	No. of chickens	Antibodies against SG[A]		Mortality[B] (%)
		Prechallenge	Postchallenge	
Phage-treated				
Challenged	20	0/15	15/15	10/20 (50)
Contact	20	0/15	15/15	1/20 (5)*
Untreated				
Challenged	20	0/15	15/15	11/20 (55)
Contact	20	0/15	15/15	7/20 (35)
Negative control	20	0/15	0/15	0/20 (0)

[A]RSA tests were performed for SG antibody detection at 2 wk after challenge; number of chickens positive/number of chickens tested.
[B]Mortality was observed for 3 wk after SG challenge; number of dead chickens/number of chickens tested.
*$P < 0.05$ by Fisher's exact test; as compared to untreated contact chickens.

DISCUSSION

SG is characterized by severe morbidity with moderate to very high mortality (10–50%) (18). Thus, prevention of SG infection is important for the profitable expansion of the poultry industry in Korea and elsewhere. In general, septicemia, caused by the ability of SG to survive and multiply in internal organs, particularly liver and spleen, causes a high incidence of mortality (19). However, in this study, bacteriophage therapy decreased the incidence of organ invasion and produced a significant ($P < 0.05$) reduction in mortality in the contact chickens when compared to the untreated contact chickens (Table 1). Considering the fact that the horizontal transmission of *Salmonella* species usually occurs following ingestion of feces of clinically infected chickens or carriers (10), these results suggest that the presence of the bacteriophage in the intestinal tract of contact chickens might inhibit the SG growth that causes septicemia, as well as provide protection from the horizontal spread of SG due to reduced bacterial shedding and environmental contamination. The single strain of bacteriophage presently used (CJø01) significantly decreased the incidence of horizontal transmission of SG, indicating that bacteriophage CJø01 possesses high bactericidal activity. Thus, the use of bacteriophage CJø01 might be more practical in poultry flocks because the production of a single strain of bacteriophage is much more economical than cocktails that contain several types of bacteriophage. Furthermore, the use of two or more bacteriophages reduces the possibility of selection for resistance against a specific bacteriophage. For this reason, the
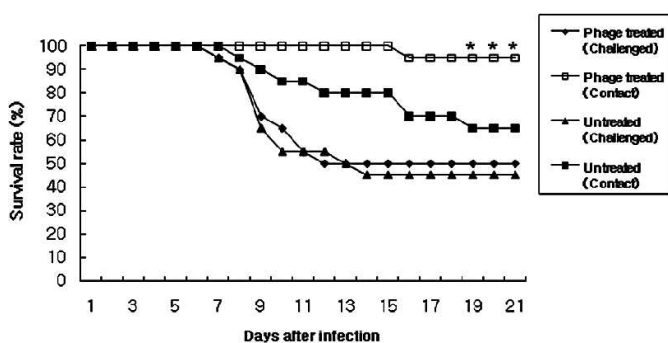

Fig. 1. The efficacy of a bacteriophage on survival rate of SG-challenged and contact chickens. Six-week-old chickens, each challenged with 5×10^8 CFUs of SG, cohabited with contact chickens treated with 10^6 PFU/kg of bacteriophage prepared in feed additives for 7 days before, and 21 days after challenge with SG. Mortality was observed for 3 wk after challenge. Asterisk (*) indicates significant difference ($P < 0.05$) between bacteriophage-treated and untreated contact chickens.

SG-specific bacteriophage developed and evaluated in the present study could be of considerable value as a better tool to combat bacteria when combined in cocktails with other bacteriophages that have been developed by different groups (6,8).

In general, the viability of an orally administered bacteriophage may be rapidly reduced under the acidic conditions of the stomach and in the presence of enzymes and other digestive compounds such as bile (14). Thus, a bacteriophage might not survive during gastric passage. However, in the present study, sufficient bacteriophage was identified in feed during the experiment, and was isolated from organs and feces of chickens that received bacteriophage in the feed additive (data not shown), to indicate that the bacteriophages are stable in feed and did pass through the digestive tract, reach the infection site, and kill the SG. This scenario is plausible because the stomach pH is likely to be much higher after feeding due to the buffering effect of the ingested food (23). The ingested feed constituents may protect a bacteriophage against extreme pH values, and a bacteriophage could still be effective against SG in the intestine as a result of survival after passage through the stomach. Therefore, application of bacteriophages as feed additives would allow prolonged efficacy against SG infection due to their stability. Different routes of bacteriophage administration, such as in drinking water, coarse spray, or intramuscularly has been shown to influence the success of therapy (7,8). Further studies involving bacteriophage administration by different routes could provide more information about the most effective delivery of bacteriophages in the poultry environment.

In many poultry industries, live and inactivated killed SG vaccines have been applied to prevent and control the incidence of the disease. Although SG vaccines can reduce clinical signs, they do not provide complete protection against bacterial shedding in SG-infected chickens (13). Therefore, the sole use of SG vaccine in poultry farms may allow chickens to shed bacteria although remaining symptomatically subclinical, which could encourage horizontal transmission and complicate SG eradication. The use of bacteriophage therapy in combination with vaccines or competitive exclusion has proven very successful in limiting *Salmonella* infections in chickens (16,22). Therefore, based on our results, SG-specific bacteriophage-containing feed additives in combination with SG vaccine could be helpful in controlling SG in the poultry industry.

In conclusion, the results from this study demonstrate that bacteriophage therapy could markedly curtailed the mortality and organ invasion in chickens exposed to virulent strains of SG via horizontal transmission. These results provide important insight into preventive and control strategies against SG infection and suggest that use of bacteriophage may constitute a novel, safe, and effectively plausible alternative to antibiotics for the prevention of SG infection in poultry.

Table 3. Effect of BP on organ invasion caused by horizontal transmission of SG in commercial layers.

| | | No. of chickens with SG re-isolation from organs (%)[A] | | | | | | | | |
| | | 7 dpc[B] | | | 14 dpc | | | 21 dpc | | |
Group	No. of chickens	Liver	Spleen	Cecum	Liver	Spleen	Cecum	Liver	Spleen	Cecum
Phage-treated										
Challenged	15	5/5 (100)	2/5 (40)	0/5 (0)	3/5 (60)	4/5 (80)	0/5 (0)	0/5 (0)	3/5 (60)	0/5 (0)
Contact	15	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	2/5 (40)	1/5 (20)	0/5 (0)
Untreated										
Challenged	15	4/5 (80)	4/5 (80)	0/5 (0)	4/5 (80)	3/5 (60)	1/5 (20)	0/5 (0)	1/5 (20)	0/5 (0)
Contact	15	0/5 (0)	0/5 (0)	0/5 (0)	2/5 (40)	2/5 (40)	0/5 (0)	3/5 (60)	2/5 (40)	0/5 (0)
Negative control	15	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

[A]Number of chickens positive/number of chickens tested.
[B]Day postchallenge.

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