Selection and Application of Bacteriophages for Treating Salmonella enteritidis Infections in Poultry

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Abstract: The objective of this study was to select appropriate bacteriophages that survive in the gastrointestinal tract of neonatal poultry and utilize those bacteriophages to reduce intestinal colonization of Salmonella enteritidis phage type 13A (SE) in challenged birds. Broiler chicks served as an in vivo biological filter to preferentially select bacteriophages from our bacteriophage library capable of surviving the gastrointestinal environment. A mixture of bacteriophage isolates designated PHL 1-71 was administered orally to three SE challenged chicks on three consecutive days. Each day, bacteriophages were recovered from the ileum, ileocecal junction and ceca for sequential administration the following day. The recovered bacteriophages were then administered to SE-infected turkey poults. In the first experiment, two-day old poults were challenged with 10⁶ cfu SE and treated 48 h later with 5mM Mg (OH)₂ followed by 2.5×10⁸ plaque forming units (pfu) of bacteriophages in 1mM Mg (OH)₂ solution. This treatment numerically reduced SE recovered from cecal contents at 12 and 24 h after treatment as compared to untreated controls. In a second experiment, two-day old poults were challenged with 1.6×10⁶ cfu SE and treated with 5mM Mg (OH)₂ followed by 7.5×10⁵ pfu phage in 1mM Mg (OH)₂ solution 48 h post-challenge. We recovered 79,728 cfu of SE per g of cecal contents in the control group and 11,224 cfu/g in the phage treated group 24 h post treatment. These data were not significantly different, but they suggest that bacteriophages can be preferentially selected in vivo to increase survival in the avian gastrointestinal tract. However, improved efficacy is required prior to useful application of the approach for reducing Salmonella infection.

Key words: Bacteriophage, Salmonella enteritidis, selection, poultry

Introduction
In the United States, it is estimated that 1.4 million humans contract salmonellosis and that the annual cost of this illness, including lost productivity, is $3 billion annually (WHO, 2006). In the year 2004, surveillance data indicated that the greatest number of foodborne illnesses was caused by Salmonella, comprising 42% of all laboratory diagnoses (FoodNet, 2005). Because many of these illnesses are associated with poultry and poultry products (Bean and Griffin, 1990; Persson and Jendteg, 1992), the reduction of microbial contamination during the production of poultry is important. Currently we are exploring the use of bacteriophages as a method to control Salmonella enteritidis (SE) infections in poultry. We previously isolated bacteriophages that lyse SE (Higgins et al., 2005) and here selected those that are most adapted to the gastrointestinal tract to potentially treat SE infected birds.

Poultry harboring Salmonella infections can be treated with antibiotics with some success (Goodnough and Johnson, 1991; Muirhead, 1994). However, Manning et al. (1992, 1994) reported increased Salmonella colonization when chickens are treated with selected antibiotics, possibly due to reduction of normal bacterial flora in the gastrointestinal tract that serve as a natural barrier to Salmonella infection. Additionally, Kobland et al. (1987) and Gast et al. (1988) have recovered antibiotic resistant Salmonella from experimentally challenged birds treated with antibiotics. Recently, the United States Food and Drug Administration (FDA) has banned the use of enrofloxacin in poultry production because of the increase in resistant Campylobacter infections in humans (FDA, 2005). Thus, it is important that effective and inexpensive methods or products to treat bacterial infections in sick birds be developed.

One possible alternative to antibiotic treatment, is use of bacteriophages to eliminate or control bacterial infections. Bacteriophages are viruses that infect and replicate in prokaryotic cells rather than eukaryotic cells (Cann, 1993; Voyles, 1993). They are ubiquitous in the environment and readily isolated from water sources, sewage and soil. Bacteriophages are also host specific and usually can infect only one serotype within a bacterial species (Ackermann et al., 1978).

Smith and Huggins (1983, 1987) have successfully used bacteriophages to eliminate Escherichia coli (E. coli) infections in calves, pigs and lambs. Bacteriophages have also been used with some success at eliminating poultry pathogens. Barrow et al. (1998) was able to prevent morbidity and mortality in chickens with bacteriophages lytic for E. coli. When chickens were challenged intramuscularly with E. coli and

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simultaneously treated with $10^6$-$10^8$ pfu of bacteriophage the mortality was reduced by 100%. Berchieri et al. (1991) also demonstrated efficacy at reducing Salmonella typhimurium in chickens when the challenge and treatment were administered simultaneously. This experiment also clearly demonstrated that the bacteriophage increased in numbers as long as the host was present. Recently, Huff et al. (2002, 2003 and 2005) demonstrated efficacy in the use of bacteriophages to treat airsacculitis caused by E. coli in chickens. Marked efficacy was achieved when administering the phages with the bacterial challenge inoculum by injection in the thoracic air sac. Despite progress toward effective use of bacteriophages in poultry, there are no established methods for selecting specific bacteriophages that can readily amplify in the gastrointestinal tract of poultry. Additionally, there is a need to select efficacious bacteriophages that can lyse the pathogen even when administered after infection.

We hypothesized that effective bacteriophages against SE in turkey poult could be achieved by choosing bacteriophages that were better adapted to survive the gastrointestinal environment. As a selection tool, we used broiler chicks as a biological filter to select bacteriophages capable of surviving passage through the gastrointestinal tract. The selected bacteriophages were then evaluated in vivo in SE-challenged turkey poult to determine their effectiveness.

**Materials and Methods**

**Bacteriophage isolation:** Wastewater samples were obtained from a local municipal wastewater treatment plant and filtered through a 0.2 µm syringe filter. A combination of 100 µL of $10^7$ cfu/mL SE and 1 mL of the wastewater sample filtrate was added to 1.5 mL of Tryptic Soy Agar (TSA) and poured over a warm TSA petri plate. Plates were incubated overnight at 37°C and those with confluent lysis of SE were flooded with 15 mL of sterile 0.9% NaCl (saline). The fluid was then poured off the plate and filtered through a 0.2 µm filter. Serial ten-fold dilutions were made in saline. Thin agar overlay plates were poured as described above containing 1 mL of each bacteriophage dilution. Individual plaques resulting from this plating were differentiated on the basis of plaque morphology and selected by removing an agar plug. Each distinct plaque morphology was then sequentially passed on TSA plates three times to purify the isolate.

This technique yielded 71 bacteriophage isolates from four independent wastewater samples. Bacteriophage isolates were arbitrarily designated PHL 1-PHL 71 for identification purposes. While several different plaque morphologies were noted and isolates were obtained from four different wastewater samples, no attempts to differentiate bacteriophages based on molecular or physiological characteristics have been made. Therefore, the 71 wild-type bacteriophage isolates utilized in these experiments may contain an unknown amount of redundancy.

**Bacteriophage preparation:** All bacteriophages were maintained on TSA soft agar plates as described above. Bacteriophages were resuspended in solution for use in experiments by obtaining a single agar plug of the desired isolate and mixing it in sterile saline. Briefly, a sterile Pasteur pipette was pushed into the center of a bacteriophage plaque resulting from lysis of SE. This bacteriophage plug was then resuspended in 1 mL of sterile 0.9% saline and filtered through a 0.2 µm filter.

**Amplification of bacteriophages:** All 71 bacteriophage isolates were obtained from each plate in an agar plug as above and amplified in Tryptic Soy Broth (TSB) containing the mutagen ethidium bromide (0.125 µg/mL). Amplification of bacteriophages was accomplished by mixing the cell free bacteriophage suspension, a turbid SE culture (12h at 37°C) and fresh TSB combined at a ratio of 1:3:5 respectively and then incubated at 37°C for 1.5 h. This amplification procedure was repeated three times in vitro. The final titer of the bacteriophage mixture was approximately $10^{15}$ pfu/mL as determined by soft overlay plating.

**In vivo biological filter:** Ten, day-of-hatch Cobb 500 broiler chicks were orally challenged with $10^4$ cfu of SE in a 0.25-mL volume of TSB. Chicks used in all experiments were cared for using procedures approved by the University of Arkansas Institutional Animal Care and Use Committee. The chicks were maintained on clean pine shavings in a floor pen at age-appropriate temperature with feed and water ad libitum. Immediately prior to bacteriophage administration (0.25 mL containing $~2.5\times10^5$ pfu), a feed passage marker, ferric oxide (100 mg) was administered per os. When red feces were detected from an individual chick (1-3 h), that chick was humanely killed by CO₂ inhalation and samples from the gastrointestinal tract were obtained. The lower 4 cm of the ileum and ileocelecal junction and half of both cecal pouches were removed aseptically from each chick. The combined tissues from each bird were minced with scissors and placed in a sterile tube containing 10 mL of TSB and incubated at 37°C for 1 h. Samples were stored on wet ice until samples were obtained from all three chicks and each sample had been incubated for 1 h.

Following recovery of surviving bacteriophages, these bacteriophages were re-amplified in vitro prior to re-passage through chicks. The samples were centrifuged for 10 min at 3000 rpm (1864×g) and a total of 20 mL of
supernatant was pooled from all tubes and sterile filtered through a 0.2 μm syringe filter. Pooled filtrate was incubated with 10 mL of fresh TSB in the presence of 3×10⁷ cfu of SE at 37°C for 15 h. Following incubation, 20 mL was filtered and passed with 20 mL of fresh TSB and 0.4 mL of 10⁴ cfu of SE and incubated for 5 h. The bacteriophages underwent two additional amplifications utilizing the 1:3:5 ratio for bacteriophage, SE and TSB respectively and incubation for 2 h at 37°C. The final bacteriophage titer after the first passage through chicks and subsequent amplification was 10¹⁰ pfu/mL. This procedure for passaging bacteriophages through chicks was repeated two additional times.

Bacteriophage present in the tissue samples of the third in vivo passage were amplified by standard methods to generate a stock. These bacteriophages represent a heterogeneous subpopulation from the original wild-type pool that are tolerant of low pH and other enteric conditions as evidenced by survival in three consecutive passages through the proventriculus of chicks. Ten soft agar plates with undiluted bacteriophage were created to retain samples of these bacteriophages.

In vivo evaluation: In Experiment 1, One hundred twenty turkey poults were obtained on day-of-hatch and randomly divided into three groups of 40. Poults were placed in brooder rings on clean pine shavings, provided feed and water ad libitum and maintained at an age-appropriate temperature for the duration of the experiment as above. The control group was maintained in a BSL-3 capable isolation room separate from the two groups of poults receiving bacteriophage treatment. All poults received a challenge of 10⁴ cfu of SE by oral gavage (0.25 mL) 48 h after placement. Experimental treatment groups (groups 2 and 3) received bacteriophage and the control group (group 1) received sterile TSB by oral gavage 48 h after SE challenge. Group 2 was orally gavaged with 2.5×10⁹ pfu (0.25 mL) of the final bacteriophage mixture (passaged through chicks as above). Group 3 was first orally gavaged 30 min prior to bacteriophage treatment with 0.25 mL of 5mM Mg (OH)₂ in an attempt to buffer the acidity of the proventriculus. Then group 3 also received 2.5×10⁹ pfu bacteriophages in the presence of 1mM Mg (OH)₂ (0.25 mL).

Ten poults were randomly selected and humanely killed by CO₂ inhalation from each group 6 h, 12 h, 24 h, or 48 h post-bacteriophage treatment. At each time point, 0.2 g of cecal contents were aseptically collected and diluted in 1.8 mL of sterile saline in a sterile glass tube. Two serial ten-fold dilutions of each sample were made and 100μL of the three concentrations of cecal contents was spread plated on brilliant green agar plates containing novobiocin and nalidixic acid. The plates were incubated for 18 h at 37°C and the number of colony forming units of SE per gram of cecal contents was determined for each sample.

In Experiment 2, thirty day-of-hatch turkey poults were obtained and randomly divided into three groups of ten poults each. All poults were placed in brooder rings on litter, provided feed and water ad libitum and maintained at an age-appropriate temperature for the duration of the experiment. All poults received a challenge of 1.6×10⁷ cfu of SE by oral gavage 48 h after placement. The treatment groups received bacteriophage and the control group (group 1) received sterile TSB by oral gavage 48 h after SE challenge. Group 2 received 5mM Mg (OH)₂ by oral gavage 30 min prior to bacteriophage administration. Then, 7.5×10⁹ pfu of bacteriophages were administered by oral gavage with 1mM Mg (OH)₂ in a 0.25 mL volume. Group 3 received the same initial treatment as group 2, with subsequent oral administration of bacteriophages (7.5×10⁹ pfu) every six h for 24 h. Additionally, groups 2 and 3 received 0.1% MgSO₄ in the drinking water until termination of the experiment. At 24 h post-treatment, all poults were killed and 0.2 g of cecal contents were aseptically collected and diluted in 1.8 mL of saline. Two serial ten-fold dilutions were made and 100 μL of all three concentrations were spread plated on brilliant green agar plates containing novobiocin and nalidixic acid. The plates were incubated for 24 h at 37°C. SE colonies were counted and the number of cfu of SE per gram of cecal contents were calculated.

Statistical analysis: All data were analyzed within time points using a one-way analysis of variance. No means were found to be statistically significant.

Results
In experiment 1, poults were challenged with SE and then treated with bacteriophages alone or in combination with Mg (OH)₂. At 6, 12 or 24 h post-treatment, SE recovery was numerically less in bacteriophage-treated birds when compared to non-treated controls (Table 1). However, these differences were not statistically significant at any of the time points evaluated. Additionally, by 48 h post-treatment the cfu’s of SE in the control group had declined from 21,329 cfu of SE to 5,189 cfu of SE and the group that received Mg (OH)₂ in combination with bacteriophages had increased from 4,064 cfu to 227,527 cfu at 48 h. In the group receiving bacteriophages in the absence of Mg (OH)₂, no numerical difference in SE was demonstrated at any time point evaluated. From this group we also recovered SE in numbers exceeding the untreated control at 48 h post-treatment. Although the bacteriophages administered were selected for the ability to survive the gastrointestinal tract, there was no suggestion of efficacy noted in the absence of Mg (OH)₂ (Table 1).
In experiment 2, we hypothesized that repeated administration of bacteriophages could reduce or possibly eliminate SE and potentially overcome the development of bacteriophage-resistant SE. In this experiment we compared a non-treated control group with pouls treated once or every 6 h for 24 h with bacteriophages and Mg (OH)₂. Additionally, we added 0.1% MgSO₄ to the water of pouls in the treatment groups to provide a continuous source of Mg²⁺. Again, at 24 h post-treatment, we recovered numerically less SE from the group that received bacteriophage and Mg(OH)₂, but the data was not significantly different than untreated controls (Table 2). Similarly, repeated administration of Mg(OH)₂ and bacteriophage treatment every 6 h for 24 h did not reduce the recoverable SE as compared to the control.

**Discussion**

In these experiments we found that the selected bacteriophages were ineffective with co-administration of Mg(OH)₂ at reducing SE in pouls. Recently Koo et al. (2001) described increased survival of bacteriophages against *Vibrio cholerae* in a simulated human gastrointestinal tract with antacid (aluminum hydroxide hydrate and magnesium hydroxide) present. Because of the buffering capabilities of the Mg(OH)₂, perhaps more bacteriophages are intact after passing through the duodenum without the aid of an antacid. If so, lack of efficacy would then be a matter of too few bacteriophages to overcome the increasing SE population. Additionally, the properties of the Mg²⁺ ions may render the bacteriophages better able to infect the *Salmonella*. It is common practice to use Mg²⁺ ions such as MgSO₄ in bacteriophage media to better enable adsorption of the bacteriophage to the host cell (Eisenstark, 1967). This interaction in the avian gastrointestinal tract could enhance the ability of the bacteriophages to adhere to host cells and thus proliferate.

We also observed in Experiment 1 increased SE recovery at 48 h in the treatment groups. This could be due to mutations in the SE that rendered it resistant to the bacteriophages administered. Although the numbers of SE in this treatment group dropped dramatically compared to controls, the bacteriophage treatment did not eliminate SE. Further, due to the populations of bacteriophage administered as treatments and their subsequent amplification in susceptible host SE, it is highly unlikely that the SE remaining in the ceca of treated pouls did not encounter bacteriophages. Data from our laboratory indicates that resistant host bacteria occur in most *in vitro* cultures containing bacteriophages (unpublished data). This phenomena has also been reported by other investigators (Smith and Huggins, 1983). However, we did not evaluate the recovered SE to determine if it was still susceptible to the bacteriophages utilized as treatment in this study.

An alternative explanation for the observation of increased SE in the treated groups is lack of host specificity. Recently our laboratory has observed that our original wild-type bacteriophage library is not stringently host specific (unpublished data). Some of the bacteriophage isolates PHL 1-71 have demonstrated the ability to lyse non-pathogenic bacteria found as natural microflora of the ceca. This observation led us to consider the possibility that the pool of bacteriophages can potentially destroy normal microflora in addition to SE, giving bacteriophage-resistant SE the advantage of less competition. Although it is speculation, this could allow resistant SE to proliferate to high numbers as demonstrated at 48 h in the treatment groups.

In Experiment 2, re-administration of the bacteriophages
did not prove efficacious. Repeated handling of the poults every 6 h could have contributed to stress and possibly immunosuppression in that group. In fact, the group that received repeated administration of bacteriophages showed qualitative behavioral changes, including increased cannibalism which was not observed in the other two groups.

Little research on bacteriophage treatment of poultry diseases has focused on oral administration of bacteriophages. Bechieri et al. (1991) showed efficacy with oral administration of bacteriophages specific for Salmonella typhimurium, however the bacteriophages were only effective if administered simultaneously with the challenge. Recently Huff et al. (2002, 2003 and 2005) showed remarkable efficacy at treating airsacculitis caused by E. coli with bacteriophages administered intramuscularly. When the same bacteriophages were administered orally in the drinking water, they were not effective.

Possibly, oral administration of effective bacteriophages is difficult because of the harsh conditions encountered within the gastrointestinal tract. Enzymes and variable pH conditions are likely detrimental to the structure of the bacteriophages. Further research is needed to increase the efficacy of bacteriophage therapy for treating Salmonella infections.

References
Higgins et al.: Selection and Application of Bacteriophages


Abbreviation Key: SE = *Salmonella enteritidis* phage type 13A, TSA = tryptic soy agar, TSB = tryptic soy broth, BGA = brilliant green agar, NO = novobiocin, NA = naladixic acid
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