Immune interference of bacteriophage efficacy when treating colibacillosis in poultry

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ABSTRACT A study was conducted to determine if prior exposure with bacteriophage would limit the ability of the same bacteriophage to treat colibacillosis. There were 5 treatments with 3 replicate pens of 20 birds per pen. The treatments consisted of 1) control, 2) birds treated with bacteriophage at 10 and 17 d of age, 3) birds challenged with Escherichia coli at 17 d of age, 4) birds challenged with E. coli and treated with bacteriophage at 17 d of age, and 5) birds treated with bacteriophage at 10 d of age and challenged with E. coli and treated with bacteriophage at 17 d of age. Colibacillosis was induced by injecting 0.1 mL of E. coli into the thoracic airsac containing 1 × 10^6 cfu, The bacteriophage was administered by i.m. injection of 0.1 mL into the thigh, providing a dose of 6.8 × 10^8 pfu. The study was concluded 14 d after E. coli challenge. Mortality in the birds challenged with E. coli and not treated with bacteriophage was 55% (treatment 3), and bacteriophage therapy significantly (P ≤ 0.05) reduced mortality to 8% (treatment 4), which was not significantly (P ≤ 0.05) different from the 2 non-E. coli-challenged controls (3%, treatment 1, and 2%, treatment 2). However, mortality in the birds administered bacteriophage before challenge with E. coli and treated with bacteriophage (treatment 5) was 33%, which was not significantly different (P ≤ 0.05) from the birds that were challenged with E. coli and untreated (55%, treatment 3). A kinetic in vitro assay of bacteriophage activity found that serum from birds pretreated with bacteriophage (treatment 5) inhibited bacteriophage activity. The IgG levels to the bacteriophage in serum from birds pretreated with bacteriophage (treatment 2) were significantly higher at all dilutions compared with control serum (treatment 1). These data demonstrate that prior exposure to bacteriophage will limit bacteriophage therapeutic efficacy and suggests that the reduced efficacy is due to an immune response to bacteriophage.

Key words: bacteriophage therapy, Escherichia coli, chicken, colibacillosis

INTRODUCTION

Bacteriophages are viruses that kill bacteria and have no known activity to animal and plant cells, which makes them an attractive natural agent for the control of bacterial diseases. Since they were co-discovered at the turn of the 20th century by Twort (1915) and d'Herelle (1917), bacteriophages have been shown to have efficacy to prevent and treat bacterial infections and to reduce foodborne pathogens. Using a colibacillosis model, we have been able to show that bacteriophages can both prevent and treat this disease, which suggests that bacteriophages could provide an alternative to antibiotics with possible applications to the control of animal and human bacterial infections (Huff et al., 2002a,b, 2003a,b). However, there is a concern that the efficacy of bacteriophages to treat bacterial disease would diminish if the same bacteriophage was used repeatedly in the same individual to treat a chronic bacterial infection, due in part to acquired immunity against the bacteriophage. The objectives of this study were to determine if the efficacy of bacteriophage therapy to colibacillosis would be altered after the birds were pretreated with bacteriophage, to determine the degree of the hypothesized decrease in efficacy, and to determine if acquired immunity to bacteriophage contributes to the hypothesized decrease in therapeutic efficacy of bacteriophages.

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MATERIALS AND METHODS

Bacteriophage Isolation and Amplification

A bacteriophage designated SPR02 was used in this study and was originally isolated from a municipal sewer treatment plant as described previously by Huff et al. (2002b). The bacteriophage was amplified and enumerated by procedures previously detailed (Huff et al., 2002b). Briefly, a 2.5-h culture of *Escherichia coli* was challenged with bacteriophage SPR02, incubated at 37°C under constant shaking, centrifuged to remove bacterial debris, and filter-sterilized. This bacteriophage lysate was enumerated using a soft agar overlay procedure described previously (Huff et al., 2002b) and was used to pretreat and treat the birds.

Bacteriophages used in an IgG ELISA assay were further purified by slowly (1 h) adding NaCl to the bacteriophage lysate with constant stirring at 4°C to a final concentration of 0.5 M. Polyethylene glycol (PEG 8000, EMD Chemicals Inc., Gibbstown, NJ) was then slowly added with constant stirring at 4°C to a final concentration of 10% (wt/vol). The mixture was kept at 4°C for 2 d to allow the bacteriophage to precipitate. The mixture was then centrifuged for 1 h at 11,000 × g and the supernatant was removed. The bacteriophage pellet was suspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 10 mM MgSO4. Polyethylene glycol was removed by mixing the bacteriophage preparation with chloroform 10% (vol/vol), which was centrifuged for 15 min at 1,000 × g. The upper bacteriophage layer was removed and stored at 4°C.

**E. coli Challenge Culture**

The *E. coli* strain used in these studies is serotype 02, nonmotile, and lactose-negative and was initially isolated from blood of chickens with colisepticemia (Bayyari et al., 1997, Huff et al., 1998). The *E. coli* culture was prepared by inoculation of tryptose phosphate broth (Sigma Chemical Co., St. Louis, MO) that was incubated in a shaking water bath for 2.5 h. The culture was removed from the water bath and held at 4°C. The culture was enumerated by making duplicate 10-fold serial dilutions of the culture and duplicate spread-plate the appropriate dilutions on tryptose phosphate agar plates, which were enumerated after overnight incubation at 37°C. The challenge cultures were made by diluting this *E. coli* stock culture and verified with serial dilutions of the challenge culture and enumeration by spread-plateing.

**Experimental Design**

Male broiler chicks (Cobb 500) were obtained at day of age from a local hatchery and maintained in floor pens on fresh wood shavings with a lighting regimen of 23 h light and with feed and water available for ad libitum consumption. There were 5 treatments and 3 replicate floor pens of 20 birds for each treatment. The treatments consisted of 1) control, 2) birds treated with bacteriophage at 10 and 17 d of age, 3) birds challenged with *E. coli* at 17 d of age, 4) birds challenged with *E. coli* and treated with bacteriophage at 17 d of age, and 5) birds treated with bacteriophage at 10 d of age and challenged with *E. coli* and treated with bacteriophage at 17 d of age. In addition, 5 extra birds per replicate pen were maintained in the control (treatment 1) and bacteriophage non-*E. coli*-challenged treatment (treatment 2) to 17 d of age. These extra birds were bled and killed at 17 d of age to collect serum for an IgG and bacteriophage kinetic activity assay. The experiment was concluded when the birds reached 31 d of age.

At 17 d of age, treatments 3, 4, and 5 were challenged with *E. coli* administered as a 0.1-mL injection into the left thoracic airsac containing 1 × 10⁶ cfu. In the treatments that received bacteriophage twice at 10 and 17 d of age (treatments 2 and 5), the bacteriophage was administered i.m. into the left thigh at 10 d of age and right thigh at 17 d of age. The bacteriophage was administered only once to the birds in treatment 4 into the right thigh. The bacteriophage lysate contained 6.8 × 10⁸ pfu in the 0.1-mL dose at both times (10 and 17 d of age) and at 17 d was administered immediately after *E. coli* challenge in treatments 4 and 5.

The birds were individually weighed each week and at the conclusion of the study at 31 d of age. Any bird that died was weighed, the severity of airsacculitis was scored (Huff et al., 1998), and the liver and airsac were cultured with sterile transport swabs and plated on MacConkey’s agar (Remel, Lenexa, KS). The liver, heart, spleen, and bursa of Fabricius were excised and weighed. When the birds were 31 d of age, they were humanely killed by cervical dislocation and necropsied as described for the mortalities. All procedures described in these studies were approved by the University of Arkansas Animal Care and Use Committee.

**Bacteriophage Kinetic Activity Assay**

Blood was collected when the birds were 17 d of age via cardiac puncture, allowed to clot, and serum was harvested from the 5 extra birds in the 3 replicate pens of the control treatment (treatment 1) and the birds administered bacteriophage at 10 and 17 d of age (treatment 2). The serum was maintained at −20°C until used to develop a bacteriophage kinetic activity assay and an IgG ELISA assay. The serum from both treatments was thawed and diluted 1:2 (vol/vol) with PBS, and 10 μL was placed into each well of a microtiter plate, followed by 10 μL of bacteriophage lysate at a concentration of 3.5 × 10⁸ pfu per mL, followed by 40 μL of tryptose phosphate broth, followed by 100 μL of a stock *E. coli* grown in tryptose phosphate broth at a concentration of 1.8 × 10⁹ cfu per mL. The microtiter plate was incubated in a microtiter plate spectrophotometer (Spectra MAX 250, Molecular Devices, Sunny-
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**IgG ELISA**

Purified bacteriophage was serially diluted in 0.2 M Na carbonate-bicarbonate buffer (pH 9.4) and 100 µL containing 10⁸ pfu/mL was added to the wells of an ELISA plate and incubated overnight on a rocking platform. The plate was washed with Tris-buffered saline (TBS, pH 7.2) and 200 µL of a solution of 1% fish gelatin in TBS (vol/vol) was added to wells as a blocking agent, and the plate was mixed for 2 h. The plate was washed as described above and 50 µL of serum diluted in TBS was added to each well up to a dilution of 1:320 and the plate was mixed for 3 h. The plate was then washed in TBS containing 0.05% Tween 20 and 100 µL of a 1:5,000 dilution of rabbit anti-chicken IgG alkaline phosphatase conjugate in TBS was added to the plate, which was mixed for 1 h. The plate was washed and 100 µL of p-nitrophenyl phosphatase substrate (1 mg/mL of alkaline phosphatase buffer, 100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, pH 9.5) was added and mixed for 30 min. The reaction was stopped by the addition of 100 µL of 1 N sodium hydroxide, and the plate was read at 405 nm in a microtiter plate spectrophotometer (Spectra MAX 250, Molecular Devices). The negative controls contained only TBS in place of the serum dilutions.

**Statistical Analysis**

These data were analyzed by ANOVA (Snedecor and Cochran, 1967), using the GLM procedures of SAS software (SAS Institute, 1998). All data presented as percentages were transformed as the square root of the arcsine before statistical analysis. Pen means was the unit for statistical analysis. Significant differences between treatments were separated using Duncan’s multiple range test (Duncan, 1955). All statements of significance are based on the probability level of ≤0.05.

**RESULTS**

The effects of the treatments on mortality are presented in Figure 1. Mortality was 55% in the birds that were challenged with *E. coli* and not treated with bacteriophage (treatment 3). Treating the birds with bacteriophage immediately after they were challenged with *E. coli* (treatment 4) significantly reduced mortality compared with the birds challenged with *E. coli* and untreated and mortality was not significantly different from the 2 control treatments (treatments 1 and 2). However, the mortality observed in the birds that were pretreated with bacteriophage at 10 d of age and then treated immediately after challenge with *E. coli* at 17 d of age (treatment 5) was not significantly lower than the mortality observed in the birds challenged with *E. coli* and untreated (treatment 3).

Graphs of the effects of the kinetics of bacteriophage activity in control serum and serum from birds administered bacteriophage and bled 7 d post bacteriophage administration (17 d of age) are shown in Figure 2. In this assay, as the bacteriophage lyse the bacteria, the optical density decreases. As can be seen in Figure 2, the serum from birds administered bacteriophage affected the kinetics of bacteriophage activity. The onset of bacteriophage activity was delayed by approximately 10 min (45 vs. 35 min), and after 2.5 h (150 min), there was a difference in the optical density, suggesting in-
Figure 2. The effect of control serum (treatment 1) and serum from birds administered bacteriophage at 10 d of age (treatment 2) on a bacteriophage kinetic assay. The serum samples were collected when the birds were 17 d of age. As the bacteriophage lyises the bacteria, the *Escherichia coli* optical density is decreased. Optical density was measured at 600 nm every 5 min for 2.5 h.

The concentrations of IgG to bacteriophage in both the control (treatment 1) and birds bled 7 d post bacteriophage administration (17 d of age, treatment 2) are presented in Figure 3. The serum was diluted out to 1:320 and there was significantly more IgG antibody to the purified bacteriophage in the bacteriophage serum at every dilution compared with the control serum.

Body weights of the birds only given bacteriophage (treatment 2) were not significantly different from the control birds (treatment 1), and all of the birds challenged with *E. coli* had significantly reduced BW from both controls (treatment 1 and 2). Body weights of the birds challenged with *E. coli* and not treated with bacteriophage (treatment 3) were significantly lower compared with all other treatments (data not shown). Necropsy results of birds that died were consistent with colibacillosis lesions characterized by airsacculitis and pericarditis; an increase in the relative weights of the liver, spleen, and heart; and a decrease in the relative weight of the bursa of Fabricius (data not shown). Our challenge strain of *E. coli* was isolated from swabs of the air sac and liver in affected birds with over 90% of the cultures being pure, and swabs taken from our control birds were culture-negative (data not shown). The challenge culture of *E. coli* is a lactose-negative, nonmotile serotype 02 that is easily identified on MacConkey agar on the basis of fermentation of lactose and colony morphology.

Figure 3. The concentration of IgG in control serum (treatment 1) and serum from birds administered bacteriophage at 10 d of age (treatment 2). The serum samples were collected when the birds were 17 d of age. The serum was diluted 1:10 (vol/vol) and then 1:2 out to 1:320. The values represent the mean ± SEM of 8 samples. The asterisk (*) designates statistical difference (*P* ≤ 0.05) between the 2 treatments at a given serum dilution.
Bacteriophages kill bacteria, making them an attractive alternative to antibiotics for the prevention and treatment of bacterial diseases. Bacteriophages represent a natural and safe alternative to antibiotics, having no known activity for animal or plant cells. Bacteriophages are generally very specific and can be used to target pathogenic bacteria without harming commensal bacteria, unlike antibiotics that generally have broad activity. Bacteriophages are plentiful in nature so the emergence of resistance of bacteria to a single bacteriophage can be anticipated and overcome with isolation and selection of bacteriophages with activity to these resistant bacteria. The emergence of bacteria resistant to antibiotics is much harder to overcome, requiring the discovery of new antibiotics or the modification of the structure of existing antibiotics to recover treatment efficacy to pathogenic bacteria. In addition, commercial production of bacteriophage-based therapeutic products can be accomplished at a fraction of the cost of the production of antibiotics. Although bacteriophage-based therapeutic products would have some advantages over antibiotic-based products, there are some disadvantages inherent in bacteriophage therapy compared with antibiotics. Antibiotics given orally can be readily absorbed to obtain therapeutic levels in circulation, whereas oral administration of bacteriophage has little or no therapeutic efficacy. We have demonstrated that colibacillosis can be effectively treated in poultry with i.m. administration of bacteriophage having efficacy similar to the antibiotic enrofloxacin (Huff et al., 2004) but have little therapeutic efficacy when administered orally or as an aerosol spray (Huff et al., 2003a). We have also demonstrated that bacteriophage can be very effectively used to prevent colibacillosis in poultry with an aerosol spray (Huff et al., 2002a).

Data reported in this manuscript demonstrate another challenge of bacteriophage therapy over the use of antibiotics. Based on mortality and an in vitro bacteriophage activity kinetic assay, prior exposure to the same bacteriophage used to treat the birds decreases the therapeutic effectiveness of this specific bacteriophage. This effect was anticipated and represented a 40% decrease in therapeutic efficacy. It was expected that there would be an acquired immune response to the systemic administration of bacteriophage that could limit the efficacy of using the same bacteriophage to repeatedly treat a systemic bacterial infection. The significant increase in IgG titers in the birds administered bacteriophage supports this hypothesis. In addition, it would be expected that antibody would bind to the bacteriophage, essentially reducing the effective bacteriophage titer. This would result in an increase in the time and rate of lysis, which is what was observed in our bacteriophage kinetic activity assay. Therefore, it may be possible, but maybe not practical, to overcome immune interference of bacteriophage therapeutic efficacy by overwhelming the immune response by increasing bacteriophage titers. Another approach to reducing the effect of immunity on bacteriophage therapeutic efficacy would be to isolate bacteriophages that are antigenically distinct.

There is a lot of interest in developing bacteriophage-based products to prevent and treat bacterial diseases and reduce foodborne pathogens. We have shown that bacteriophage can be used to both prevent and treat colibacillosis in poultry (Huff et al., 2002a,b, 2003a,b). Research has shown efficacy of bacteriophage against E. coli infections in calves, piglets, lambs, and mice (Smith and Huggins 1982, 1983; Smith et al., 1987; Barrow et al., 1998; Jamalludeen et al., 2009). Bacteriophages have been found to protect mice from infections with Acinetobacter baumannii and Pseudomonas aeruginosa (Soothill, 1992), rescue mice from lethal challenges with vancomycin-resistant Enterococcus faecium (Biswas et al., 2002), protect fish from Pseudomonas plecoglossicida (Park et al., 2000), protect mice from a lethal injection of Staphylococcus aureus (Matsuzaki et al., 2003), and treat Vibrio vulnificus infections in mice (Cerveny et al., 2002). A review of bacteriophages to prevent and treat infections in cattle, poultry, and pigs was published by Johnson et al. (2008). Bacteriophages have also been demonstrated to have some efficacy to reduce foodborne pathogens on several products (Goode et al., 2003; Leverentz et al., 2003; Higgins et al., 2005; Borie et al., 2008). However, as data presented in this manuscript demonstrate, bacteriophage-based prophylactic and therapeutic agents do have limitations and should not be viewed as a replacement of antibiotics but as an alternative to antibiotics. There are clinical conditions and bacterial diseases that can be more effectively prevented and treated with bacteriophage-based approaches rather than antibiotic-based approaches, but a great deal more research is needed to be able to effectively target bacteriophage applications to disease prevention and treatment, make these applications practical, and find ways to overcome the limitations of bacteriophage-based prophylaxis and therapy.

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REFERENCES


