Therapeutic Efficacy of Bacteriophage and Baytril (Enrofloxacin) Individually and in Combination to Treat Colibacillosis in Broilers[1]

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ABSTRACT A study was conducted to evaluate the therapeutic efficacy of bacteriophage and the antibiotic enrofloxacin individually and in combination to treat colibacillosis. The experimental design was a $2 \times 2 \times 2$ factorial with 8 treatments and 4 replicate pens of 10 birds. The treatments were 1) control, 2) unchallenged birds treated with bacteriophage, 3) enrofloxacin, or 4) the combination; 5) birds challenged with *Escherichia coli*, and birds challenged with *E. coli* and treated with 6) bacteriophage, 7) enrofloxacin, or 8) the combination of bacteriophage and enrofloxacin. Birds in the *E. coli* challenged treatments were challenged at 7 d of age by injecting 10^4 cfu of *E. coli* into the thoracic air sac. The antibiotic treatment was initiated immediately after the birds were challenged and consisted of 50 ppm enrofloxacin in the drinking water for 7 consecutive days. The bacteriophage treatment consisted of a single intramuscular injection of 2 different bacteriophage (10^9 pfu) administered immediately after the *E. coli* challenge. Mortality in the birds challenged with *E. coli* and untreated was 68%, and the bacteriophage and enrofloxacin treatments significantly decreased mortality to 15 and 3%, respectively. There was total protection in birds that received both the bacteriophage and enrofloxacin representing a significant synergy. The decrease in mortality with enrofloxacin (3%) was significantly better than the decrease in mortality with bacteriophage (15%). Airsacculitis lesion scores and lesion incidence in surviving birds were significantly less in the enrofloxacin treatment compared with the bacteriophage treatment. Both bacteriophage and enrofloxacin provided effective treatments of colibacillosis, and the synergy between these 2 treatments suggests that bacteriophage combined with antibiotic treatment has significant value.

(*Key words*: bacteriophage, *Escherichia coli*, chicken)

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INTRODUCTION

The emergence of bacteria resistant to antibiotics poses a significant threat to animal and human health. There is growing concern that the use of antibiotics in animal production can result in the emergence of resistant bacteria that cause human infections that are difficult, if not impossible, to treat. The significance of the use of antibiotics in animal production vs. their use in human medicine to the emergence of resistance bacteria that pose a threat to human health has been debated for a long time and is equivocal. Recently, the approval of the antibiotic Baytril (enrofloxacin)[3] for use in poultry production has been withdrawn. Although enrofloxacin has been an extremely effective treatment of colibacillosis in poultry, it is a fluoroquinolone antibiotic, and fluoroquinolone and these compounds are critically important in the treatment of human bacterial infections. With the continued concern over the use of antibiotics in poultry production, there is a real need to find safe and practical alternatives in poultry production to both prevent and treat poultry diseases.

Bacteriophages are viruses that infect and kill bacteria and were codiscovered in the early 1900s by Twort (1915) and d'Herelle (1917). Bacteriophages appear to have evolved with bacteria and are ubiquitous in nature. We have had considerable and consistent success in preventing and treating colibacillosis in poultry with single and multiple bacteriophages and believe that bacteriophages may provide an alternative to antibiotics. We have isolated bacteriophage to a nonmotile serotype O2 *E. coli*, pathogenic to poultry, from municipal sewer treatment plants and poultry processing plants (Huff et al., 2002b). We found that when bacteriophage is mixed with *E. coli* prior to challenging the birds, total protection from colibacillosis could be achieved (Huff et al., 2002b). We have also demonstrated that when bacteriophage is administered as an aerosol spray prior to challenging the bird with *E. coli*, colibacillosis could be prevented for up to 3 d (Huff et al., 2002a). In addition, severe colibacillosis can

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be treated with bacteriophage when administered as an intramuscular injection (Huff et al., 2003b), and multiple injections of bacteriophage provide greater therapeutic value than a single injection (Huff et al., 2003a). The objective of the current study was to evaluate the efficacy of bacteriophage and enrofloxacin individually and in combination to treat colibacillosis in broiler chickens.

MATERIALS AND METHODS

Bacteriophage Isolation and Amplification

Bacteriophage were isolated to an *E. coli* poultry isolate (serotype 02) using waste water from either municipal sewer treatment plants or a poultry processing plant as described by Huff et al. (2002b). Two bacteriophage isolates, designated SPR02 and DAF6, were selected for these studies based on size and clarity of plaques. They were amplified by inoculating a culture of *E. coli* grown for 2.5 h in typtose phosphate broth[4] with bacteriophage and incubating overnight at 37°C. The cultures were then centrifuged (2,500 × *g*) and passed through a 0.2-μm membrane filter. Bacteriophage enumeration was conducted using the soft agar overlay procedures described previously (Huff et al., 2002b)

Experimental Design

A study was conducted to determine the efficacy of treating colibacillosis with a cocktail of 2 bacteriophage (DAF6 and SPR02), the antibiotic enrofloxacin, or a combination of the bacteriophage cocktail and enrofloxacin. Male broiler chicks (Cobb 500) were obtained from a local hatchery and maintained in electrically heated batteries with feed and water available ad libitum to 3 wk of age when the studies were concluded. The study was a 2 × 2 × 2 factorial arrangement with 8 treatments and 4 replicate pens of 10 birds per pen. The control treatments consisted of an untreated unchallenged control, birds not challenged with *E. coli* and treated with either of the 2 bacteriophage (3.7×10^9 and 9.3×10^9 pfu per mL, DAF6 and SPR02, respectively) at 7 d of age injected into the left thigh, 50 ppm of enrofloxacin administered in the drinking water that was started when the birds were 7 d of age and continued for 7 consecutive days, or a combination of bacteriophage and enrofloxacin. The remaining treatments consisted of birds challenged with *E. coli* at 7 d of age by injecting 0.1 mL of a 2.5 h culture of *E. coli* (serotype 02, nonmotile, lactose negative) containing 6×10^5 cfu/mL into the left thoracic air sac and the birds treated as described above with either bacteriophage, enrofloxacin, or the combination.

The birds were weighed each week. Any bird that died was weighed, and the severity of airsacculitis was scored as follows: 0, no inflammation; 1, opacity and thickening

[4]Sigma Chemical Co., St. Louis, MO.
[5]Remel, Lenexa, KS.

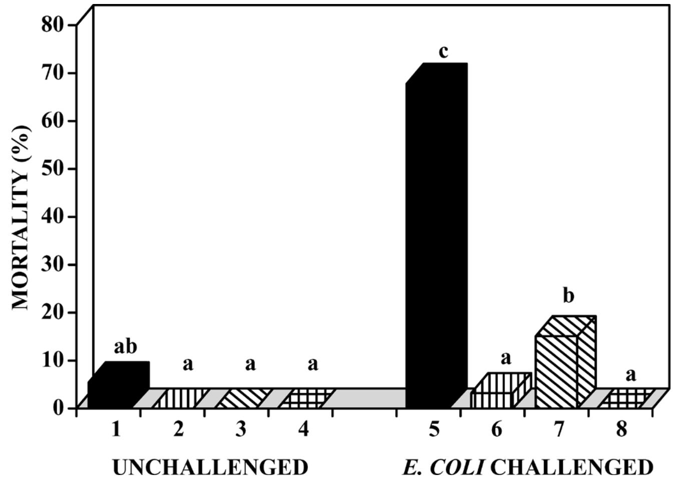

FIGURE 1. The individual and combined effects of enrofloxacin, bacteriophage, and the combination on mortality (%). Treatments: 1, control; 2, enrofloxacin; 3, bacteriophage; 4, enrofloxacin and bacteriophage; 5, challenged with *Escherichia coli*; 6, challenged with *E. coli* and treated with enrofloxacin; 7, challenged with *E. coli* and treated with bacteriophage; 8, challenged with *E. coli* and treated with enrofloxacin and bacteriophage. [a–c]Bars with different letters differ significantly ($P \leq 0.05$).

of the inoculated air sac; 2, mild airsacculitis and mild pericarditis; 3, moderate airsacculitis or pericarditis with spread to liver or abdominal cavity (perihepatitis or peritoitis); 4, severe fibrinous airsacculitis and severe pericarditis; 5, severe airsacculitis or pericarditis with spread to liver or abdominal cavity (Huff et al., 1998). The liver and air sac of each bird were cultured with sterile swabs and plated on MacConkey's agar.[5] The liver, heart, spleen, and bursa of Fabricius were excised and weighed. When the birds were 3 wk of age, they were humanely euthanized 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.

Statistical Analysis

These data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments by ANOVA (Snedecor and Cochran, 1967), using the GLM procedure of SAS software (SAS Institute, 1998). All data presented as percentages were transformed as the square root of the arc sine prior to statistical analysis. Pen means were the unit for statistical analysis. Significant differences between treatments were separated using least squares means procedures of SAS. All statements of significance are based on the probability level of 0.05.

RESULTS

The effect of treatments on mortality is shown in Figure 1. There was 68% mortality in the birds that were challenged with *E. coli* and not treated (treatment 5). Treatment of the birds with enrofloxacin in the drinking water at 50 ppm immediately after challenging the birds and

TABLE 1. The individual and combined effects of enrofloxacin and bacteriophage on airsacculitis lesion scores and the incidence of air sac lesions when birds were 3 wk of age

	Lesion score		Lesion incidence (%)	
Treatment	Unchallenged	Challenged	Unchallenged	Challenged
Control	0.000 ± 0.000[c]	1.308 ± 0.536[a]	0.0 ± 0.0[b]	35.0 ± 11.9[a]
Enrofloxacin	0.025 ± 0.025[c]	0.051 ± 0.051[c]	2.5 ± 2.5[b]	2.8 ± 2.8[b]
Bacteriophage	0.000 ± 0.000[c]	0.412 ± 0.212[b]	0.0 ± 0.0[b]	14.6 ± 8.5[ab]
Enrofloxacin + Bacteriophage	0.025 ± 0.025[c]	0.050 ± 0.035[c]	2.5 ± 2.5[b]	5.0 ± 2.8[b]

[a–c]Values represent the mean ± SEM of 4 replicate pens of 10 birds per pen. Values with different superscripts within the parameters lesion score and lesion incidence differ significantly ($P \leq 0.05$).

continued for 7 consecutive days significantly decreased mortality to 3% (treatment 6). The bacteriophage treatment administered as a single injection immediately after the birds were challenged significantly decreased mortality to 15% (treatment 7). There was a significant difference between the antibiotic and bacteriophage treatments (15 vs. 3%). No mortality was observed in the combination treatment, and the interaction between enrofloxacin and bacteriophage was significant ($P < 0.0012$).

Airsacculitis lesion scores and the incidence of lesions in the birds necropsied at 3 wk of age are presented in Table 1. In the surviving birds the highest lesion scores were observed for those challenged with *E. coli* and not treated. The birds that survived and were treated with bacteriophage had lesion scores that were significantly less than the challenged untreated birds and significantly higher than the birds treated with enrofloxacin. Thirty-eight percent of the birds challenged with *E. coli* had lesions present at 3 wk of age compared with 3, 15, and 5% in the enrofloxacin, bacteriophage, and combination treatments, respectively.

Body weights were generally higher in the antibiotic treatments but not significantly different from the controls (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 negative when cultured (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.

DISCUSSION

Based on mortality, enrofloxacin and bacteriophage were effective in treating severe airsacculitis. The enrofloxacin was administered in the drinking water at 50 ppm for 7 d, compared with a single injection of bacteriophage. Based on mortality this treatment was more effective than the bacteriophage treatment. There were less severe and fewer lesions in the surviving birds treated with enrofloxacin compared with the bacteriophage treatment, which supports the conclusion that the enrofloxacin treatment was a more effective treatment. There was no mortality in the enrofloxacin and bacteriophage combination treatment; this interaction was significant, supporting the concept that a synergy occurs when antibiotics and bacteriophage are used in combination that improves the efficacy of these treatments. This synergy between enrofloxacin and bacteriophage suggests that there may be a real advantage to combine antibiotic treatments with bacteriophage treatments. It may be possible to reduce the levels of antibiotics used in treating bacterial diseases if they are used in combination with bacteriophage, which could increase the effective life of antibiotics and decrease the development of resistance to antibiotics. This concept of improving the effective life of antibiotics by combining antibiotic with bacteriophage therapy was proposed by Carlton (1999), and these data support this concept.

Bacteriophages kill bacteria, and it should be possible to develop them as effective prophylactic and therapeutic agents. Our work indicates that bacteriophage can be used to effectively prevent and treat colibacillosis in broiler chickens (Huff et al., 2002a,b, 2003a,b). In addition, the efficacy of bacteriophage has been demonstrated by others using a variety of models. The use of bacteriophage to control *E. coli* induced diarrhea in calves, piglets, and lambs has been previously reported (Smith and Huggins, 1983; Smith et al., 1987). The ability of bacteriophage to treat *E. coli* infections in mice has also been demonstrated (Smith and Huggins, 1982). Barrow et al. (1998) demonstrated the ability of bacteriophage to protect chickens from an intramuscular challenge with *E. coli* when bacteriophage is simultaneously injected at different sites. Soothill (1992) was able to use bacteriophage to protect mice from infection with *Acinetobacter baumanii* and *Pseudomonas aeruginosa*. There is significant research and considerable clinical experience with the use of bacteriophage in human medicine conducted in Russia as reviewed by Alisky et al. (1998) and in Poland (Ślopek et al., 1981, 1984, 1985, 1987; Weber-Dabrowska et al., 1987). More recent work has demonstrated that bacteriophage is able to rescue mice from a lethal challenge with a vancomycin-resistant *Enterococcus faecium* (Biswas et al., 2002). There are also a number of research programs looking at the efficacy of bacteriophage to decrease foodborne pathogens on agricultural products. Research demonstrating the efficacy of bacteriophage to reduce *Listeria monocytogenes* on fresh cut produce has been demonstrated by

Leverentz et al. (2003). Lytic bacteriophage has been shown to decrease *Salmonella* and *Campylobacter* contamination on chicken skin (Goode et al. 2003).

Bacteriophage used in the current study was an effective treatment of severe colibacillosis with enrofloxacin being slightly but significantly more effective. Smith and Huggins (1982) found that bacteriophage is superior to the antibiotics tetracycline, ampicillin, chloramphenicol, and trimethoprim in treating *E. coli* infections in mice. The synergy between antibiotics and bacteriophage observed in this study suggests that combining antibiotic and bacteriophage to treat infections has considerable value and could decrease the effective therapeutic level of antibiotics, which would help prolong the effective life of a given antibiotic. Bacteriophage do provide an effective alternative to antibiotics; the challenge is to develop practical ways to use bacteriophage in poultry and animal production systems.

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