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ORIGINAL ARTICLE

Efficacy of a bacteriophage cocktail in controlling *Salmonella* Enteritidis infection in broiler chickens

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ABSTRACT

Background and Objectives: *Salmonella* Enteritidis is widespread in the world and is known to be among the most common agents of zoonotic food-borne illnesses. This study evaluates the efficacy of a bacteriophage cocktail — comprising SEPL01 (Siphovirus) and SEPL13 and SEPL20 (Myoviruses) — in controlling *Salmonella* Enteritidis infection in poultry.

Materials and Methods: A total of 168 one-day-old desi chicks were procured and randomly divided into five different groups: negative control, positive control, prophylactic, 6 h and delayed post-challenge treatment group. Birds in the positive control and trial groups were orally infected with 10^5 CFU/ml *S.* Enteritidis on the fourth day. The bacteriophage was given in 10^7 PFU/ml through oral gavage, drinkers and aerosol spray. Bacterial enumeration was done using dilution plate counting on XLD agar and bacterial reduction was determined using log10 reduction.

Results: Among the groups, the prophylactic group showed the highest $\log_{10}$ bacterial reduction: 1.92 (Day 3), 1.79 (Day 7), and 1.23 (Day 14). Drinking water administration resulted in a $\log_{10}$ reduction of 1.62, 1.44, and 0.91, respectively, while aerosol spray was the least effective with a $\log_{10}$ reduction of 1.12, 0.85, and 0.52 across the same days. The treatment group receiving therapy 6 hours post-challenge exhibited a moderate level of reduction: 1.63, 1.37, and 1.22 via oral gavage; 1.43, 1.15, and 0.94 via drinkers; and 0.46, 0.15, and 0.12 via aerosol spray. The delayed post-challenge group showed smaller reductions: 1.56, 1.24, and 0.92 by oral gavage; 1.25, 0.99, and 0.79 by water; and 0.42, 0.13, and 0.10 by aerosol spray.

Conclusion: The bacteriophage therapy is highly effective in reducing *Salmonella* Enteritidis, demonstrating potential as an antibiotic alternative.

Keywords: Bacteriophage therapy; *Salmonella enterica* serovar enteritidis; Drug resistance; Microbials; Zoonoses

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INTRODUCTION

Salmonellosis, due to *Salmonella enterica* serovar enteritidis, continues to represent a major global public health threat and one of the main vehicles for transmission is poultry products (1). *Salmonella* Enteritidis has been shown to be a leading cause of foodborne gastroenteritis worldwide, resulting from contaminated eggs, meat and poultry products (2). Since the 1980s, the rapid dissemination of *Salmonella* Enteritidis across continents has been facilitated by the globalization of poultry supply chains, through centralized sourcing and international trade of breeding stocks (3).

Approximately 10% of the more than 2,500 known *Salmonella* serovars are associated with poultry, and *Salmonella* Enteritidis is responsible for approximately 75% of poultry-associated Salmonella outbreaks because it thrives in and persists within poultry systems (4). Prevalence rates also vary widely, ranging from 5% in Europe to 93.7% in Asia, highlighting the widespread threat posed by this pathogen. In addition, low- and middle-income countries often have inadequate biosecurity, and the use of antibiotics further increases the risk of antimicrobial resistance (AMR) in *Salmonella* Enteritidis strains (5). For example, poultry in Pakistan, India, and Nigeria has been reported to harbor multidrug-resistant *Salmonella* Enteritidis isolates, making treatment and control of these infections difficult (6, 7).

Traditional control strategies such as vaccination, improved farm hygiene and antibiotic therapy, have been only partially successful in eradicating *Salmonella* Enteritidis in poultry flocks (8, 9). Vaccines leave people only partially protected and antibiotics no longer work well because of a growing resistance. Chicken meat and its products with antibiotic residues present a food safety and public health issue and exacerbate the global antimicrobial resistance epidemic (10).

An alternative or complementary approach to control SE infections in poultry is bacteriophage therapy. Bacteriophages, viruses that primarily target and lyse bacteria, possess various advantages due to their great specificity for bacterial hosts, their lack of impact on beneficial microbiota, and their inability to develop in response to resistant bacterial populations (11). Phage therapy has been shown in experimental studies to dramatically decrease *Salmonella* Enteritidis colonization in the gastrointestinal tract of poultry, resulting in reductions in bacterial loads by several orders of magnitude. The combination of multiple phage types into phage cocktails has demonstrated improved phage efficacy and reduced the likelihood of bacterial resistance (12).

The feasibility of phage therapy in commercial poultry settings has been improved by recent advances in phage isolation, characterization and delivery methods. Phages can be delivered through drinking water, feed additives or aerosol sprays which provides for flexible inclusion in current farm practices (13). Most importantly, phage therapy proved effective against multi-drug resistant *Salmonella* Enteritidis strains isolated from poultry farms in Pakistan and in other countries and provides a clue to dealing with the challenges posed by antibiotic resistance. The current research is planned to evaluate the efficacy of bacteriophage therapy in controlling *Salmonella* Enteritidis infections in poultry birds.

MATERIALS AND METHODS

Experimental site. The study was conducted at the Post Graduate Research Institute for Poultry Diseases, Rawalpindi.

Preparation of shed. The poultry shed was thoroughly cleaned and disinfected using a Quaternary ammonium compound, and fumigation was carried out with 40% formalin mixed with potassium permanganate. After fumigation, the shed was sealed for 48 hours. Overcrowding and cross-contamination were prevented by installing separate partitions. Temperature, humidity, and light intensity were maintained at 32-35°C, 60%, and 30-35 lux, respectively. For the first 10 days, paper lining was used to collect fecal buildup, after which the birds were moved to rice husk litter (14).

Experimental birds. A total of 168 one-day-old desi chicks (each weighing approximately 40-45 g) were sourced from *Salmonella*-free flocks. Vitamin and mineral supplementation was withheld from the birds, which were then reared under standard management conditions. Housing temperature and humidity were regulated within the recommendations of the Hy-line W-36 guidelines. Feed and water were tested and confirmed to be free of *Salmonella*. Slide

agglutination was used to confirm the birds were negative for *Salmonella* antibodies, and the birds were housed under biosafety-controlled conditions (15).

Bacterial strains. For in vivo assessment of phage lytic activity, confirmed *Salmonella* Enteritidis isolates were procured from stock cultures developed in a PARB-funded project (P-680) at the diagnostic laboratory of the Central Lab Complex, UVAS Ravi Campus, Pattoki. For challenge inoculation, bacterial suspensions were prepared in LB broth, and the optical density (OD625) was adjusted to 0.08–0.1 (~10^5 CFU/mL). To achieve appropriate bacterial concentrations, the suspension was serially diluted in phosphate-buffered saline (16).

Bacteriophages. The purified and confirmed bacteriophage cocktail—comprising SEPL01 (Siphovirus) and SEPL13 and SEPL20 (Myoviruses)—was collected from PARB Research Project 680 at the Central Lab Complex, UVAS Ravi Campus, Pattoki. The phage cocktail, consisting of three bacteriophages, was isolated and characterized from sewage samples collected from commercial poultry (17).

Experimental design. A controlled in vivo experimental study was conducted where broiler chickens were randomly distributed into five experimental groups: Negative Control Group, Positive Control Group, Prophylactic Group, Delayed Post-Challenge Group, and 6-Hours Post-Challenge Therapy Group. Table 1 has a detailed overview of the bird allocation, inoculation days, phage routes of administration and the day on which they were sampled per group and thus makes the methodology clear and repeatable. The bacteriophage cocktail was administered once daily via oral gavage at a concentration of 1×10^7 PFU/mL. The same dose was repeated for three consecutive days prior to challenge to ensure adequate gut colonization. Post-challenge, birds in phage-treated groups continued to receive the same daily dose for five days, thereby maintaining a consistent phage exposure throughout the critical infection window. No additional booster doses were used outside this scheduled regimen. During the study, morbidity and mortality were closely monitored daily in all birds (18).

Sample collection and bacterial enumeration. Three birds in each group were slaughtered at 3-, 7- and 14-days following infection. The ileum and

Table 1. Experimental Design

Group	No. of birds	Treatment route	Bacterial challenge	Bacteriophage treatment	Sampling days
Negative control	18	Not applicable	No bacterial challenge	No bacteriophage treatment	Days 3, 7, and 14
Positive control	15	Oral gavage	Day 4 of age; 10^5 CFU/mL	No bacteriophage treatment	Days 3, 7, and 14
Prophylactic group	45 (15 per route)	Aerosol spray; drinking water; oral gavage	Day 7 of age; 10^5 CFU/mL	Days 4, 5, and 6 of age; 10^7 PFU/mL	Days 3, 7, and 14
Delayed post-challenge group	45 (15 per route)	Aerosol spray; drinking water; oral gavage	Day 4 of age; 10^5 CFU/mL	Day 6 of age; 10^7 PFU/mL	Days 3, 7, and 14
6-hour post-challenge therapy group	45 (15 per route)	Aerosol sprayy; drinking water; oral gavage	Day 4 of age; 10^5 CFU/mL	6 hours post-challenge; 10^7 PFU/mL	Days 3, 7, and 14

cecum were harvested in a sterile state, ground into a homogenate and serially diluted in phosphate-buffered saline. Samples were cultured on Xylose Lysine Deoxycholate (XLD) agar and placed in an incubator at 37°C for 24 hours. Bacterial load reduction was assessed by counting the salmonella colonies on the petri dishes (19).

Statistical analysis. The results of all experiments were subjected to statistical analysis through Repeated Measure ANOVA by SPSS (SPSS V.20, IBM, Armonk, NY, USA). The significant difference among treatments was considered at p-value < 0.05 (20).

Ethical approval statement. All animal procedures were conducted in accordance with institutional guidelines and were approved by the Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore.

RESULTS

Throughout the experiment, no mortality was observed in any group. The mean intestinal counts of *Salmonella* Enteritidis (CFU/mL (log10)) in the positive control group were 4.88, 5.11, and 4.87 on the 3rd, 7th, and 14th days post-challenge, respectively. In the prophylactic group, a log10 reduction in *Salmonella* Enteritidis was observed for all delivery routes compared with the positive control group. On the 3rd day post-challenge, log10 reductions of 1.62 and 1.92 were observed in birds that received the bacteriophage cocktail through drinking water and oral gavage, respectively, whereas the aerosol spray route showed a log10 reduction of 1.12. On the 7th day post-challenge, the reductions were 1.44, 1.79, and 0.85 for drinking water, oral gavage, and aerosol spray, respectively. On the 14th day post-challenge, the reductions were 0.91, 1.23, and 0.52 for drinking water, oral gavage, and aerosol spray, respectively, as shown in Fig. 1.

In the delayed post-challenge group, the treatment by a cocktail of bacteriophages of 10^7 PFU/ml was given after 48 hours of the *Salmonella* Enteritidis challenge. The efficacy of phage cocktail in bacterial reduction by drinking water, oral gavage, and aerosol spray was 1.25, 1.56, and 0.42 Log10 respectively on the 3rd days post challenge. Subsequently, on 7th day bacterial reduction was 0.99, 1.24 and 0.13 Log10 for all three routes. The efficacy of bacterial reduction was lowered to 0.79, 0.92 and 0.10 Log10 on 14th days post challenge for drinking water, oral gavage and aerosol spray routes respectively as shown in Fig. 2.

In 6 hours post-challenge therapy phages were administered 6 hours after the challenge of *Salmonella* Enteritidis. In this group drinking water and oral gavage routes reduced bacteria to 1.43 and 1.63 Log10 while aerosol spray reduced to 0.46 on the 3rd days post challenge. A reduction of 1.15, 1.37, and 0.15 was seen in drinking water, oral gavage, and aerosol spray routes on 7th days post-challenge. The phage cocktail efficacy given by drinking water, oral gavage, and nasal spray routes was reduced to Log10 0.94, Log10 1.22, and Log10 0.12 respectively on the 14th days post challenge as shown in Fig. 3. As the p-value is more than 0.05, the results are not significant and therefore do not indicate a meaningful difference between the groups. In this experiment, the most effective group in bacterial reduction was the prophylactic group, and in different routes oral gavage route was most effective, and aerosol spray was least effective in control of infection.

DISCUSSION

The research was carried out using a controlled in vivo experimental design based on established phage-therapy models reported in poultry research. The birds were randomly allocated to treatment groups according to standard allocation procedures to eliminate selection bias. The challenge model, timing, and dosing were based on studies of phage-bacterium interactions published previously, which demonstrated increased therapeutic efficiency in broiler chickens. This design enabled reproducible comparisons across all treatment arms and ensured scientific rigor comparable to that of existing methods. The research suggests that bacteriophage therapy greatly minimizes the presence of *Salmonella* Enteritidis in poultry by controlling when and how it is applied. Phage therapy given to the prophylactic group ahead of infection with bacteria saw the greatest reduction in *Salmonella* Enteritidis of up to 1.92 Log10 CFU/mL on day 3 after challenge, as shown in previous studies. Phages are best at stopping bacterial infections when they are introduced before the pathogens can take hold in the gut (21).

Administering the bacteriophages via oral gavage gave better outcomes than insertion into drinking

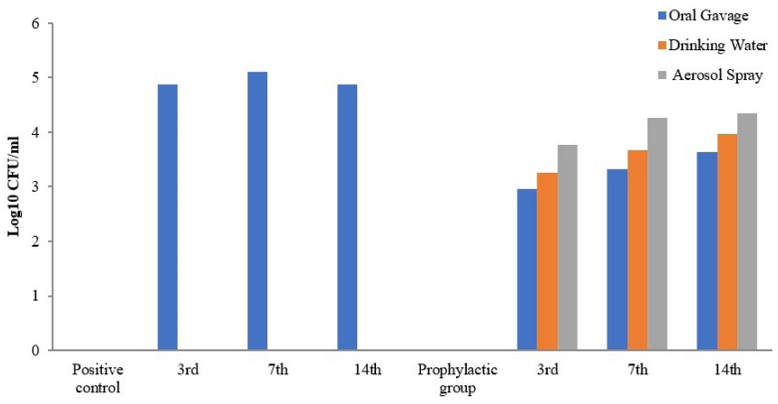

Fig. 1. Comparison of CFU Log10 values in between Prophylactic and Positive control groups.

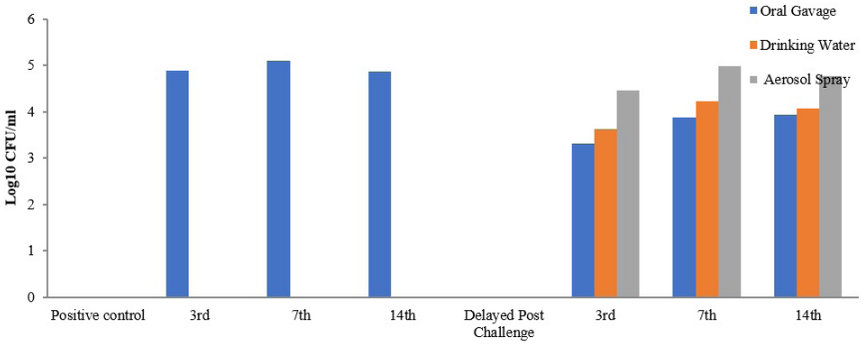

Fig. 2. Comparison of CFU Log10 values in between Delayed post challenge and Positive control groups.

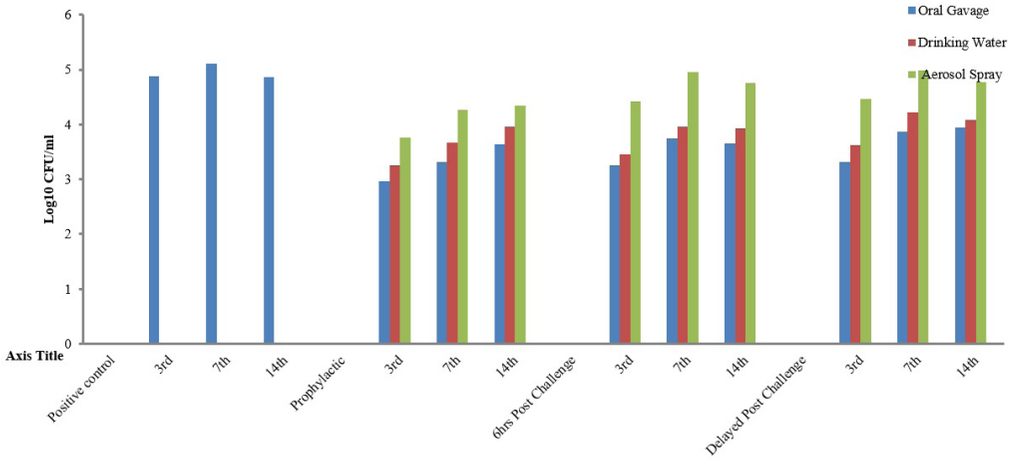

Fig. 3. Comparison of *Salmonella* Enteritidis counts (CFU Log10) in between Prophylactic, 6 hrs post challenge and Delayed post-challenge groups.

water or aerosol spray for all groups. Oral gavage is likely more effective because it sends the phages right into the intestinal tract, where *Salmonella* Enteritidis usually begins colony formation (22). Similarly, when *Salmonella* Enteritidis was tested in quail models, intragastric usage of phages was a better approach for *Salmonella* Enteritidis reduction than delivering the phages by vent lip routes (21). However, using aerosol spray to deliver these phages did not show great results, proving the importance of direct-

ing phages to the area of infection (23).

Significantly, phage's effectiveness dropped after day 14 following the challenge. The reduction in numbers could be because the phages were cleared from the digestive system, as seen in mouse and poultry experiments that often require regular treatment with phages (24). Administration of repeated phages in chickens decreased *Salmonella* Enteritidis counts by two to four $\log_{10}$ within twelve days which suggests that frequent doses can help phages last longer in real-world use.

The noticeable differences in the treatment groups show that the success of phage therapy depends greatly on timing. The significant advantage of early intervention for the prophylactic group means that fast action can stop *Salmonella* Enteritidis from settling and growing in the intestines (25). The result is in line with the concept that once bacteria form a biofilm, become highly populated or are recognized by the immune system, phage therapy becomes more difficult. It is also evident from the delayed treatment studies that phage therapy alone may not be enough against well-established infections, so using it with other biosecurity actions is recommended (26).

The decreases in *Salmonella* Enteritidis in the current study, especially in the Prophylactic Group, are consistent with a previous study, which reported a 2-3 log10 CFU/g reduction in bacterial colonization when phages were administered before exposure to the pathogen (21). In the same manner, another study proved that early phage intervention blocks initial colonization of the gut significantly, which is similar to the greater reduction that we measured in the post-challenge phage group, when compared to post-challenge therapies. There were moderate, albeit significant, changes in our Delayed Post-Challenge and 6-Hours Post-Challenge Therapy groups, which are consistent with the outcomes of other research, which have shown that there are limited but significant therapeutic effects when phages are delivered once the infection is established. The present findings support the already known trend where phage efficacy is the greatest when used prophylactically or extremely within the infection window, compared to therapeutic dosing after colonization which only partially controls but not altogether suppresses bacterial persistence, which has been repeatedly observed in past experimental phage work in poultry. Notably, the study finds that phage therapy is safe, as no negative effects or deaths happened, proving it's appropriate for use in commercial poultry farms. In general, the research supports that bacteriophage therapy is useful in preventing and managing Salmonella infections in poultry, but demonstrates that planning its application is key for best results (27).

CONCLUSION

The findings indicate that bacteriophage therapy works effectively in stopping *Salmonella* Enteritidis in poultry, especially when applied prophylactically using oral methods. More research is needed to streamline phage delivery methods, fine-tune the timings for phage administration and check phage cocktails against multiple strains and serovars of *Salmonella enteritidis.* Overcoming these challenges, phage therapy may prove to be an ongoing replacement for antibiotics and become good for both the poultry industry and the global food supply.

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