



In Vivo Effect of Novel Lytic Phage Cocktail against *Salmonella Pullorum*

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ABSTRACT

Despite advances, avian salmonellosis pose a major challenge to the poultry health, associated with high production losses. The study aimed to investigate the effect of oro-nasal phage delivery on bacterial population. As prophylaxis, oral phage delivery via drinking water and feed resulted in a significant ($P \leq 0.05$) reduction in bacterial numbers with a mean \log_{10} 1.43, \log_{10} 1.17, \log_{10} 1.92 and \log_{10} 1.625 on 3rd and 6th dpc, respectively. The least reduction was seen in the birds that received cocktail intranasally showing \log_{10} 0.85 on the 3rd day. As a delayed treatment, oral delivery significantly ($P \leq 0.05$) reduced count with a mean \log_{10} 1.2 and \log_{10} 1.55 on 3rd dpc, with efficiency decline on 6th dpc. No reduction was recorded for nasal delivery test subjects in delayed treatment group. As an immediate intervention, the oral delivery reduced bacterial count to \log_{10} 0.86 and \log_{10} 1.02 on the 3rd dpc. Phage-cocktail is promising pre-harvest biocontrol approach to curtail host-adaptive antibiotic resistant *Salmonella*.

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Authors' Contribution

AAS and MR presented the concept. RHD and AAS planned methodology. RHD and MAN performed formal analysis. MRK and TM did clinical evaluation. MU performed statistical analysis. AAS provided resources. RHD and SA curated data. RHD, AAS and SA wrote the manuscript while AZD, MU and MAN reviewed it.

Key words

Avian salmonellosis, Prophylaxis, Phage delivery, Phage cocktail, *Salmonella*

INTRODUCTION

With the increase in the global population (7.9 billion), poultry has become the fastest-growing livestock sector to meet the expanding nutritional needs and provide a livelihood to rural communities. The poultry industry is anticipated to contribute \$322.5 billion to the market, with Asia being the largest egg-producer with 64% of the output (FAO). The universal success of poultry products is owed to their inexpensive availability, consumer convenience, nutritional properties, and absence of any social and religious taboos (Petracci *et al.*, 2019). Despite advancements, avian salmonellosis continues to cause significant production and economic losses, especially in developing countries (Berhanu and Fulasa, 2020).

Pullorum disease (PD) is a septicemic disease of avian species caused by *Salmonella enterica* subsp. *enterica*

serovar Gallinarum biovar Pullorum that often manifests in young birds (2–4 weeks). Whereas atypical signs (asymptomatic carriers) are manifested in mature stock with loss of productivity leading to reduced hatchability and egg production. Horizontal and vertical spread are equally important with reference to the epidemiological standpoint. Game birds, backyard and free-range poultry serve as reservoirs of infection, while rodents and wild migratory birds can be potential vectors (OIE, 2020).

Developed countries have eliminated and controlled PD in their commercial sector through test and cull policies as treatment is never recommended because of asymptomatic carriers (MERCK, 2021). In contrast, the disease is common in low and middle-income countries with inefficient husbandry practices and higher ambient temperatures (Barrow *et al.*, 2012; Berchieri Jr *et al.*, 2001). Currently, there is great concern about animal welfare, hygiene and disease control resulting from great

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Abbreviations

CFU, Colony Forming Units; DPC, Days Post-Challenge; FAO, Food and Agriculture Organization; GLM, Generalized Linear Model; NRLPD, National Reference Laboratory for Poultry Diseases; OIE, Office International des Épidémiologies; PCR, Polymerase Chain Reaction; PD, Pullorum Disease; PI, Post-infection; PFU, Plaque Forming Units; QAC, Quaternary Ammonium Compound; SP, *Salmonella Pullorum*; SPF, Specific Pathogen Free.

genetic pressure to boost performance and productivity, which adversely affects animals (Hafez and Attia, 2020). Antibiotic prophylaxis is a common veterinary practice in the poultry and calf-rearing industry in many under-developed countries as a surrogate for inadequate management practices, but at the cost of escalating antibiotic resistance and drug residues (active metabolites) in the food chain at slaughter, if no withdrawal requirements are followed (Mohsin *et al.*, 2019).

The decreased efficacy of available antibiotics has led to renewed interest in lytic bacteriophages and derived products. There are numerous studies on the use of single phage or mixture of phages against pathogens of veterinary importance cited in literature includes *S. Enteritidis*, *S. Typhimurium*, *C. jejuni*, *C. coli*, *S. aureus*, *P. aeruginosa*, *E. coli* K1⁺, *E. coli* O2, enteropathogenic *E. coli*, *E. coli* O157: H7 and *A. pyogenes* in chickens, cows, dogs, calves, sheep, and steers (Morris Jr *et al.*, 2001). Keeping this in view, the present study was designed to test the *in vivo* efficacy of a four phage cocktail against *Salmonella* Pullorum to investigate the influence of delivery routes and schedule among experimental birds. For this purpose, log₁₀-reduction of bacterial count in intestinal tissue was used as an indicative parameter.

MATERIALS AND METHODS

Shed preparation

The shed was disinfected with Medisept®, which is a quaternary ammonium compound (QAC) and fumigated with 40% of formalin per cubic meter of space. Following fumigation, the shed was sealed tight for the next 48 h (FAO Guidelines for Cleaning and Disinfection of Poultry Farms).

Experimental birds and their maintenance

Day-old SPF (*Salmonella*) Hyline w-36 chicks ($n=360$) were procured from a commercial (avg. 40–45 g/bird). Rapid slide agglutination test was performed to ensure that the birds were seronegative.

The conventional floor space was provided at 100 – 200 cm²/bird. For the first three days, the temperature was maintained at 33–35°C. The temperature was re-adjusted every three days as per the managemental guidelines (FAO, 2021a). The light intensity was kept uniform at 30–50 lux with 60% relative humidity. The air quality was maintained at 4 m³/kg/hour from the west to east direction (Hy-Line w-36 Managemental Guide).

Ready-made starter feed was offered to the birds during the trial. The feed and water intake were measured as 13–16 g/bird/day and 20 mL/bird/day, respectively. The feed and water were tested prior to being *Salmonella* negative via PCR. Chickens were cared for using protocols

approved by the Ethical Review Committee for Animal Handling of the University of Veterinary and Animal Sciences, Lahore.

Experimental design

The birds in the positive control group were given an oral challenge with *S. Pullorum* (10⁵ CFU/mL per 60 g of body weight). The birds in the negative control group remained unchallenged and untreated. Five experimental birds from the negative group were euthanized at 0, 7, 14 and 21 days to ensure sterility of the experiment (via PCR). The birds in the third control group were given phage cocktail via all three delivery routes to monitor shedding (i.e., internalization) and the occurrence of adverse reactions (if any). The birds in the fourth group were given phage cocktail as prophylaxis to monitor the effectiveness of the cocktail at preventing systemic infection or reducing the clinical severity of disease (upon challenge). The birds in the fifth group received challenge infection prior receiving phage cocktail 6 h post-infection (PI) to evaluate the impact of the cocktail as immediate therapy. The sixth group also received challenge infection prior to phage treatment, but with a 36h delay assessing the effect of the phage cocktail during active infection (Table I). The number of birds in all experimental subsets was 30 and were placed in different sheds with similar housing requirements. 05 birds from each treatment group on the 3rd, 6th and 9th day PI, were euthanized via cervical dislocation, and intestinal tissue (pooled sample ileum/cecum) was collected aseptically for estimation of log₁₀-reduction in *Salmonella* count. The quantitative enumeration of bacteria was carried out following method of Cappuccino and Sherman (2014).

Host bacterial strain and bacteriophage cocktail

The challenge bacterial strain, *Salmonella* Pullorum (SP-1628), was confirmed by conventional and molecular techniques at the national reference laboratory for poultry diseases (NRLPD). The phage cocktail was concoction of four lytic phages, isolated from sewage/sludge samples procured from commercial poultry units. The cocktail demonstrated optimal activity against motile serovars, such as *Salmonella* Enteritidis and *Salmonella* Typhimurium, tolerance to moderately high temperatures above 62°C with a log₁₀ of 8.73 PFU/mL, and activity in acidic and alkaline conditions. At 150 min, the cocktail reduced the bacterial count by log₁₀2.93 with 100 MOI. The growth kinetics of all four monophage suspensions (SalØ_ABF37, SalØ_RCMPF12, SalØ_MCOH26 and SalØ_DNLS42) showed an average latent time between 33 to 46.7 min with burst sizes given 73, 97, 75 and 132 PFU CFU, respectively. All isolates were revealed to possess dsDNA during nucleic acid characterization.

Table I. Experimental design with inoculation days and dose units.

Delivery schedule	Inoculation days	Dose units	Delivery route	
			Oral	Nasal
Positive Control (δ)	10 th day of age (Challenge \dagger)	10 ⁵ CFU/mL \dagger	✓	-
Negative Control (β)	Unchallenged and Untreated	--	-	-
Cocktail Control (γ)	7 th , 8 th , 9 th days of age (bacteriophage cocktail only)	10 ⁶ PFU/mL 10 ⁸ PFU/kg	✓	✓
Prophylaxis (ϵ)	7 th , 8 th , 9 th days of age Challenge \dagger on 10 th day	10 ⁶ PFU/mL 10 ⁸ PFU/kg 10 ⁵ CFU/mL \dagger	✓	✓
Immediate Therapy (λ)	Challenge \dagger on 10 th day of age Treatment 6 HPC*	10 ⁵ CFU/mL \dagger 10 ⁶ PFU/mL	✓	✓
Delayed Therapy (ρ)	Challenge \dagger on 10 th day of age Treatment 48 HPC*	10 ⁵ CFU/mL \dagger 10 ⁶ PFU/mL 10 ⁸ PFU/kg	✓	✓

\dagger represents oral gavage by using sterile syringe without needle; HPC refers to h post challenge.

The fragment size from restriction digestion analysis showed the estimated sizes for genomes to be 35.9kB, 42.5kB, 35.5kB and 45.2kB for SalØ_ABF37, SalØ_RC-MPF12, SalØ_MCOH26 and SalØ_DNLS42, respectively (data not shown).

Statistical analysis

The data obtained from experimental trials was collected and organized into Microsoft Excel spread sheets (Office 2016). To check for significant differences in bacterial concentrations as a result of delivery routes and timing data was analyzed with generalized linear model (GLM) using Minitab 17.0.1. Differences were considered statistically significant at ($P \leq 0.05$). Post-hoc mean separation was used to test for pairwise differences among experimental groups.

RESULTS

Control group

The birds of positive control group developed non-specific signs like depression, weakness, somnolence, loss of appetite, drooping of wings, huddling, dehydration and ruffled feathers post 36 h of challenge infection. Gasping, white pasty diarrhea, and pasting of the vent feathers were also seen at 72 h PC. The bacterial count was enumerated to be \log_{10} 7.17 CFU in intestinal content (ileum/cecum).

5% mortality was observed in negative control group birds under normal circumstances (i.e., drowning in drinker). The birds remained free of infection for whole the duration of trial. 46.67% (drinking water) and 53.3% (feed additive) of birds shed bacteriophage in their feces. No bacteriophage shedding was observed in experimental

birds that received cocktail intranasally. All the birds remained clinically normal and maintained a healthy weight of between 60 – 73 g/bird.

Prophylactic group

In the prophylactic group, a significant ($P \leq 0.05$) \log_{10} reduction in *S. Pullorum* was seen in comparison to the untreated positive control group with a mean \log_{10} 7.17 CFU. The pairwise comparison was significant ($P \leq 0.05$) for all delivery routes, with higher \log_{10} -reduction seen in the oral groups than recipients with nasal delivery. On 3rd dpc, \log_{10} 1.43 (96.32%) and \log_{10} 1.92 (98.81%) CFU reduction was observed in birds that received cocktail through drinking water and as feed additive, while \log_{10} 0.85 CFU (86%) were recorded for nasal delivery subjects. Subsequently, on the 6th dpc, the counts were \log_{10} 1.17 (93.33%) and \log_{10} 1.629 (97.65%) CFU for oral delivery routes and no reduction in bacterial count was observed for nasal delivery route. The efficacy of phage cocktail as prophylaxis did not yield efficient \log_{10} -reduction on 9th dpc, by only reducing bacterial numbers by \log_{10} 0.334 (53.67%) and \log_{10} 0.44 (63.93%) CFU for oral delivery routes, i.e., by water and as a feed additive with no \log_{10} -reduction seen in experimental birds treated with phage cocktail via nasal delivery. The delivery of phage cocktail as pre-challenge choice resulted in reducing the clinical severity of disease with 15% mortality in immunocompromised chicks.

A significant ($P \leq 0.05$) \log_{10} reduction was seen in the second delivery schedule of phage therapy, i.e., immediate therapy in comparison to the untreated positive control group with a mean \log_{10} 7.17 CFU. Non-significant ($P \geq 0.05$) pairwise comparison was seen between delivery

routes. The phage cocktail delivery by oral routes, i.e., by water and as a feed additive, modulated *S. Pullorum* count by \log_{10} 0.85 (86%) and \log_{10} 1.02 (90.53%) CFU on the 3rd dpc. The efficacy of phage delivery as immediate therapy did not reduce bacterial count on 6th and 9th dpc, with 53.33% mortality in immunocompromised birds.

The group in which the treatment was delayed to 48 h generated similar results in terms of bacterial reduction with non-significant ($P \geq 0.05$) pairwise comparison recorded for the delivery routes. A reduction of \log_{10} 1.2 (93.78%) and \log_{10} 1.55 (97.21%) CFU was seen in experimental birds treated with a phage cocktail mixed in water and feed on the 3rd dpc. On the 6th dpc, the efficacy of phage cocktail mixed in water and feed was reduced to \log_{10} 0.77 (83.33%) and \log_{10} 0.66 (78.26%), respectively. On 9th dpc, the phage cocktail only reduced bacterial incidence to \log_{10} 0.319 (52.13%) and \log_{10} 0.228 (40.86%) CFU in oral delivery routes, i.e., by water and as a feed additive, respectively. 93.3% experimental birds that received cocktail through nasal route died with no significant reduction in bacterial numbers suggesting that nasal delivery did not modulate bacterial incidence in presence of active infection.

Summing up, the logarithmic reduction was concurrent with the GLM which affirmed that delivery schedules are significantly different in reducing bacterial incidence. However, the oral delivery routes, on the other hand, are non-significant i.e., they produced similar \log_{10} -reduction in bacterial count, with nasal delivery being least effective out of all delivery routes, as seen in Figure 1.

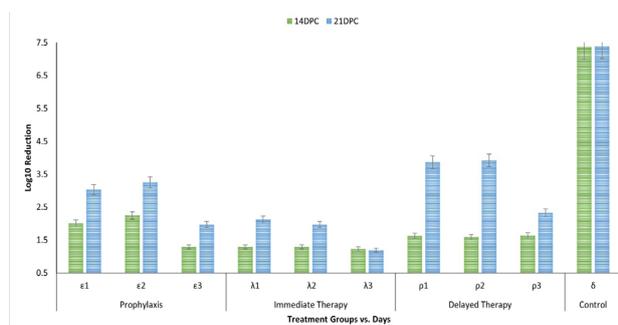


Fig. 1. Comparative efficacy of delivery schedules and routes (x-axis) on modulation (\log_{10} -reduction) of *S. Pullorum* on y-axis incidence in intestinal tissue

DISCUSSION

Salmonellosis and poultry have been linked epidemiologically and economically since the commercialization of poultry industry. Although PD or white bacillary diarrhea, caused by *S. Pullorum* is largely eliminated from developed countries, but PD remains

an under-reported problem in many under-developed countries with free-range backyard and intensively reared commercial poultry. The experiment carried out in the present study resulted in significant reduction in bacterial incidence for oral and nasal phage delivery. The findings of the present study are supported by literature where several routes of administration have been shown to influence the impact of therapy (Andreatti-Filho *et al.*, 2007; Borie *et al.*, 2009; Carlos *et al.*, 2009; Hong *et al.*, 2013; Huff *et al.*, 2006; Rozema *et al.*, 2009).

The number of days also had a significant effect on bacterial reduction. The timing of delivery although yielded significant effects in all experimental groups, but phage delivery 72 h before and 48 h after challenge showed significant results, whereas the group that received immediate treatment of cocktail produced the lowest reduction in bacterial numbers. The present study is in accord with several studies cited in literature that suggest delivery of phages too early may result in low threshold of multiplication but can significantly reduce the clinical severity and delay attachment or colonization (Hong *et al.*, 2013; Lim *et al.*, 2011; Wagenaar *et al.*, 2005).

The positive control group of study had an incidence that reached 100% where 46% birds exhibited acute mortality within 36 h of challenge, while the rest expired within 72–96 h post-challenge. The results are in accord with the study of Carlos *et al.* (2009) who suggested a 100% incidence of infection in positive control group with their 3 phage cocktail lowered *S. Enteritidis* incidence to 80% alone and 75.5% in presence of a competitive exclusion product broilact (Carlos *et al.*, 2009).

The feces from cocktail control group also showed that the candidate isolates passed down the gastrointestinal system upon re-isolation of lytic bacteriophages with 3 different plaque morphologies i.e., 3 out of 4 strains survived in the digestive tract while one lost its viability. This is a significant finding in regard to the *in vivo* application of lytic phages, as the particles were able to survive and replicate in host as cited by (Colom *et al.*, 2015; Toro *et al.*, 2005).

The use of quadcocktail in the present study was to reduce the possibility of selection of resistance against a particular candidate bacteriophage. For this purpose, cocktails having two or more bacteriophage isolates are chosen as combating tool cited in literature. Additionally, using cocktails is efficient to reduce the risks associated with the multiple dose and continuous administration of bacteriophages and to make this methodology more cost-effective and practical under commercial prospects (Bielke *et al.*, 2007; Borie *et al.*, 2008).

The results obtained in this preliminary study can lay foundation for cost-effective futuristic approach

towards biocontrol of salmonellosis with further work can be established in terms of stability and formulation parameters for efficacious delivery and greater retention.

CONCLUSION

The results of this study are a preliminary effort to measure the efficacy of bacteriophages in controlling bacterial infections in chickens. As reported above, lytic bacteriophages significantly reduced the concentrations of target bacteria in the experimentally infected birds. As such, the application of phages in such a manner could provide clinicians and animal producers with additional means to treat bacterial infections.

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Data availability

The data of the study would be available on fair request to corresponding author.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board and institutional biosafety committee of University of Veterinary and Animal Sciences, Pakistan. The official letter (no. DR/47) would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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