

***In vivo* therapeutic evaluation of phage cocktail and probiotic in reducing *Salmonella* infection in Broilers**

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ABSTRACT

Antimicrobial resistance and ban on the usage of most of the antibiotics in food producing animals especially in poultry and pigs propelled research towards antibiotic alternatives. Food borne and multidrug-resistant (MDR) pathogens like *Salmonella* can enter food chain via consumption of the contaminated meat. Bacteriophage (phage) therapy could be used during rearing or pre-harvest stages of poultry production to overcome these emerging problems. The present study was conducted to determine the therapeutic effectiveness of bacteriophage cocktail and probiotics against *Salmonella gallinarum* in experimentally infected broiler chicks based on mortality, clinical manifestations, and faecal shedding. *In vitro* evaluation revealed that optical density (OD) of *Salmonella gallinarum* at MOIs of 10^2 was significantly reduced by individual phages as well as phage cocktail, with comparably less decrease in optical density in the culture treated with individual phage than the phage cocktail. Bacteriophage cocktail with concentration 10^{11} PFU/ml was able to protect 100% birds infected with *Salmonella gallinarum*. Faecal shedding rate was significantly low in the birds treated with bacteriophage cocktail and probiotics (1.2%) than untreated group. Significant improvement in body weight was seen in the bacteriophage treated groups as compared to untreated infected group. Based on the findings of the current study, administering high titre bacteriophages alone or in combination with probiotics for the effective management of *Salmonella* infection in broiler chicks may be suggested as an alternative to antibiotics as well as a useful strategy to control food borne pathogens in the food chain.

Keywords: Bacteriophages, Cocktail, Phage, Poultry, Probiotic, *Salmonella*, Therapeutic

Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented and/or treated by the administration of bacteriophages. As an alternative to antibiotic therapy, bacteriophage therapy is potentially a powerful approach for the treatment of bacterial infections, especially in antibiotic resistance era. Bacteriophages (phages) are natural predators of bacteria and are ubiquitous in the environment (Rohwer and Edwards 2002). Phages have unique advantages compared with antibiotics as they can replicate only on the targeted subset of bacteria and as long as the targeted bacterium is present, so are naturally self-limiting (Connerton and Connerton 2005). Intelligent combinations of different phages to make phage cocktail could contribute to the success of phage bio-control by reaching a broad host range and also keep phage resistance under control (Fischer *et al.* 2013). The use of phage cocktail was evaluated *in vitro* against *Escherichia coli* and *Salmonella enterica* serovar

Typhimurium and it was concluded that application of phages in the form of a cocktail can be used presumptively (Costa *et al.* 2019).

Probiotics are being considered as another alternative to antibiotics which when administered through the digestive route, are favourable to the host's health (Kabir 2009, FAO/WHO 2001). The use of probiotics in animals is well controlled and is regulated by Regulation (EC) no. 1831/2003 (Salminen *et al.* 2010).

In the light of India's position in the international scenario, the poultry and pig must be free from the infectious diseases having zoonotic importance. Contaminated meat with *Salmonella* spp. considered to be leading cause of human gastrointestinal infections as well as spread of multidrug-resistant (MDR) strains within the food chain (Thanki *et al.* 2023).

World Health Organization in 2017 issued a list of global priority pathogens based on their resistance pattern and therapeutic options, where phage therapy should be explored (Tacconelli *et al.* 2018).

The present study was designed to evaluate bio-control strategies including specific and well characterized bacteriophages (PSP 1, PSP 4, PSP 5, PSP 6, PSP 7) and probiotics to control *Salmonella* infection in broiler birds.

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Phages used to form cocktail were described previously and were collected from sewage puddles, where both poultry and swine waste accumulates (Kumar *et al.* 2017). These phages were previously examined and were found to be heat and pH stable and were able to lyse most of the *Salmonella* strains tested (Kumar *et al.* 2017).

MATERIALS AND METHODS

The present study was approved by Institutional Animal Ethics Committee (IAEC) vide number IAEC/VMC/CVASC/12/. Five bacteriophages namely PSP 4, PSP 5 and PSP 7 of family Siphoviridae and PSP 1 and PSP 6 of family Podoviridae, initially isolated and characterized (Kumar *et al.* 2017) were selected for the *Salmonella* challenge trial in poultry.

Bacterial challenge test: Before performing *in vivo* therapeutic study, the phage replication *in vitro* bacterial challenge test (O'Flynn *et al.* 2004) was performed with *Salmonella gallinarum* strain with PSP4, PSP7 and phage cocktail. Trial was performed at 37°C maintaining a MOI of 10^{11} PFU/CFU. Optical densities (OD) at 600 nm were measured every hour for 8 h and visual observations were made for up to 24 h.

Determination of colony forming unit (CFU): The number of viable counts of overnight (12 to 14 h) broth culture of isolates used throughout the period of study was determined by serial dilution method in triplicate. Serial 10-fold dilutions of each culture of the isolate were prepared in NSS ranging from 10^{-1} to 10^{-10} . A 100 µl of each dilution beginning from 10^{-7} onwards was spread plated on nutrient agar plates in triplicate. After incubation at 37°C the colonies were counted using colony counter. A number of viable counts of each of the three cultures were determined and the average of these three was considered as the viable count of overnight incubated culture of the bacterial isolates.

Determination of plaque forming unit (PFU): Serial 10-fold dilution of the bacteriophage stocks were prepared in SMG medium and 100 µl of each dilution beginning from 10^{-7} onwards was mixed with 100 µl pure broth culture of host bacteria and was incubated for 6 h in separate sterilized tubes. The phage and bacterial mixture were mixed well and incubated at 37°C for 20 min. To each tube 3 ml of melted soft agar at 47°C was added. The overlays were allowed to harden for 30 min, and the plates inverted in a single layer were incubated at 35°C overnight. Plaques were observed and counted. The titer of the original phage preparation was determined by using the calculation:

Number of plaques $\times 10 \times$ reciprocal of counted dilution = PFU/ml

Most phage lysates contain between 10^6 and 10^{11} PFU/ml. Dilutions of 10^{-5} to 10^{-9} was considered to have phage concentrations leading to countable numbers of plaques.

Therapeutic phage cocktail composition: The phage cocktail used to study the therapeutic effect on *Salmonella gallinarum* in broiler birds was composed of equal volumes of all phages, i.e. PSP1, PSP4, PSP5, PSP6 and PSP7

having 10^{11} PFU/ml in Trypticase soya broth.

Probiotic: Commercially available probiotic containing *Lactobacillus acidophilus* + *Streptococcus faecium* + yeasts + enzymes (protease, cellulase, amilase) was selected and procured from the market and mixed with the ration at the dose rate of 1 kg/ton of feed and the feed was fed to the chicks *ad lib*.

Experimental therapeutic trial: To determine the efficacy of bacteriophage cocktail alone and in combination with probiotics therapeutic trial was conducted on broiler chicks infected with *Salmonella gallinarum*. One hundred twenty, day old broiler chicks were procured from a commercial hatchery and were reared in cages under strict hygienic conditions adopting biosecurity measures. Birds were fed commercial feed available in the market along with clean drinking water. Chicks were kept under close observation for one week to ensure their normal health. Feed and water were provided to the chicks *ad lib*. Thereafter, all the chicks were randomly grouped having 20 birds each as per the experimental design: Group I: Healthy control; Group II: Non infected chicks treated with bacteriophage cocktail (10^{11} PFU/ml) and probiotic; Group III: Chicks infected with *Salmonella gallinarum* (10^9 CFU/ml) and were not given any treatment (infected control); Group IV: Chicks infected with *Salmonella gallinarum* (10^9 CFU/ml) and treated with bacteriophage cocktail (10^{11} PFU/ml); Group V: Chicks infected with *Salmonella gallinarum* (10^9 CFU/ml) treated with bacteriophage cocktail (10^{11} PFU/ml) and probiotic; Group VI: Chicks infected with *Salmonella gallinarum* (10^9 CFU/ml) treated with probiotic.

The swabs of faecal samples were collected from each individual bird prior to the start of the experiment to ensure the absence of any *Salmonella* strain. The *Salmonella gallinarum* and bacteriophage cocktail was administered by oral gavage and probiotic was administered with feed (Fig. 1). At three days of age, the chicks in groups III, IV, V and VI were challenged with 1 ml of 10^9 CFU/ml suspension of *Salmonella gallinarum* in phosphate-buffered saline (PBS). At four days of age the chicks in groups II, IV and V were inoculated with 1 ml of 10^{11} PFU of bacteriophage cocktail in PBS containing 30% (wt/vol) calcium carbonate as an antacid. Efficacy of bacteriophage



Fig. 1. Administration of phage cocktail by oral gavaging.

cocktail and probiotics was assessed on the basis of mortality, clinical manifestations and faecal shedding. After infection mortality rate was recorded daily for six days. The performance of birds was also assessed by determining the body weight. For faecal shedding sterile cotton swab was inserted into the cloaca of each bird and rotated gently to collect the cloacal contents and inoculated in 10 ml of Trypticase soya broth. Incubation was done overnight at 37°C. Next day a loopful from broth was streaked on BGA to check the growth of *Salmonella* spp. The suspected colonies were identified morphologically and biochemically.

Statistical analysis: The statistical analysis of data of protection and faecal shedding was carried out using Chi-Square (Greenwood and Nikulin 1996) and the statistical analysis of average body weight was examined using analysis of variance (ANOVA) (Shott 1990).

RESULTS AND DISCUSSION

Bacterial challenge test: The replication dynamics of phage-host system *in vitro* showed that two phages namely PSP4 and PSP7 as well as phage cocktail containing all the isolated phages in equal concentration was able to significantly reduce the OD of *Salmonella gallinarum* at MOIs of 10^2 (Fig. 2). Visual observations of *Salmonella gallinarum* at 24 h confirmed that broth remained clear indicating bacteriophage cocktail as well as individual bacteriophages suppressed *Salmonella gallinarum* growth. There was steady increase in *Salmonella gallinarum* OD over time in bacteriophage free bacterial control. However, the decrease in OD was comparatively less to that of the

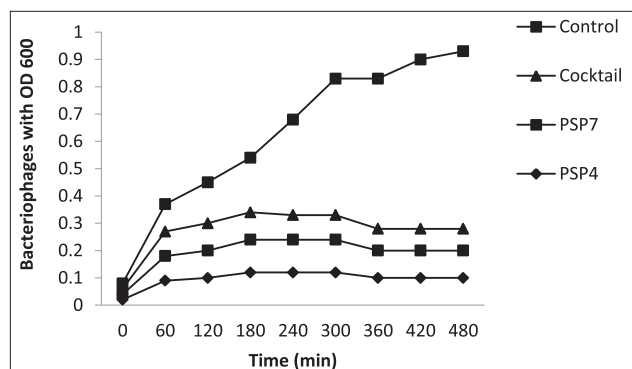


Fig. 2. Comparison of lytic ability of individual phage and phage cocktail applied to *Salmonella gallinarum*.

culture infected with phage cocktail. This made the base for selecting phage cocktail rather than single phage for *in vivo* therapeutic trial. Present study corroborates with the findings of O'Flynn *et al.* (2004) and Atterbury *et al.* (2007). Phage cocktails were more effective than individual phages at bacterial lysis and lowered the re-growth of *S. Typhimurium* compared to when individual phages are used, presumably because there was less development of phage resistance (Thanki *et al.* 2022). Turki *et al.* (2012) concluded that when mixture of *Salmonella* serotypes was challenged with phage cocktails at different MOIs a consistent retardation of the growth rate was observed.

In vivo study: *Salmonella gallinarum* culture with 10^9 CFU/ml concentrations was obtained and used to infect the broiler chicks. Titre of bacteriophage cocktail was standardised to 10^{11} PFU/ml. Probiotic with dose of 1 kg/ton was used in the trial. The results of induced infection of broiler chicks during present investigation indicated that the chicks of all the infected groups (III, IV, V and VI) started showing clinical manifestations like depression, anorexia, less intake of water after 24 h of infection. No mortality was observed till 24 h of infection. Treatment with bacteriophage cocktail and probiotics was started after 24 h of infection in different groups. The severity of signs was more in the birds that were only infected (group III) than those of treated (group IV, V, and VI). Mortality rate (Table 1) was high in group III (45%) followed by group VI (30%). No mortality was recorded in other groups. Idea of considering phages as powerful alternatives to antimicrobials is shared by several researchers who reported successful trials with phages conferring high protection levels against infections (Huff *et al.* 2005, Wagenaar *et al.* 2005). The results of present study revealed that phages were highly effective when administered 24 h after the experimental infection with *Salmonella gallinarum* in chicks. The protection rate was calculated as per the formula:

Protection rate was 100% in birds of groups treated with

$$\text{Protection rate} = \frac{\text{Survived test} - \text{Survived control positive}}{\text{Dead control positive}} \%$$

phage cocktail and probiotics (V) as well in the groups receiving only bacteriophages (IV), whereas, protection rate was zero in untreated infected group (III) and 33.3% in birds infected and treated with probiotic only (VI). Theoretically, if a bacteriophage reaches the site

Table 1. Protection rate of bacteriophage cocktail and the probiotic treatment against *Salmonella gallinarum* infection in broiler chicks

Group	Total no. of birds	No. of dead birds/day post challenge						Total no. of dead birds	Mortality rate	Total no. of survived birds	Protection %
		1 st	2 nd	3 rd	4 th	5 th	6 th				
I	20	0	0	0	0	0	0	0 ^a	0 ^a	20 ^a	100 ^a
II	20	0	0	0	0	0	0	0 ^a	0 ^a	20 ^a	100 ^a
III	20	0	3	3	2	1	0	9 ^b	45.0 ^b	11 ^b	0 ^b
IV	20	0	0	0	0	0	0	0 ^a	0 ^a	20 ^a	100 ^a
V	20	0	0	0	0	0	0	0 ^a	0 ^a	20 ^a	100 ^a
VI	20	0	2	2	2	0	0	6 ^c	30.0 ^c	14 ^c	33.3 ^c

^{a,b,c} Letters within the same column having different superscripts differ significantly ($P \leq 0.05$).

Table 2. Faecal shedding rate of *Salmonella gallinarum* in broiler chicks after treatment with bacteriophage cocktail and probiotic

Group	Days post treatment								+ve/Total	%
	1 st day		2 nd day		3 rd day		4 th day			
	+ve/ Total	%	+ve/ Total	%	+ve/ Total	%	+ve/ Total	%		
I	0/20	0	0/20	0	0/20	0	0/20	0	0	0 ^a
II	0/20	0	0/20	0	0/20	0	0/20	0	0	0 ^a
III	14/17	82.3	14/14	100	12/12	100	11/11	100	51/54	94.4 ^a
IV	3/20	15.0	2/20	10	2/20	10	2/20	10	9/80	11.2 ^b
V	1/20	5.0	0/20	0	0/20	0	0/20	0	1/80	1.2 ^c
VI	10/18	55.5	10/16	62.5	9/14	64.3	9/14	64.3	38/62	61.3 ^d

^{a,b,c,d} Letters within the same column having different superscripts differ significantly ($P \leq 0.05$).

of a bacterial infection, it is supposed to be effective in eliminating the disease. The lower mortality rate in broilers recorded during present study after treatment with probiotics corroborate with the findings of many researchers (Soomro *et al.* 2002, Timmerman *et al.* 2006, Wafaa *et al.* 2006).

Dead birds were subjected to post-mortem examination for specific lesions of *Salmonella gallinarum* as well as for isolation of *Salmonella* from tissue samples. The positive samples were confirmed by colony characteristics as well as by biochemical testing. Most prominent lesions observed were bronze discoloration and congestion of liver.

The results of the faecal shedding rate of *Salmonella gallinarum* in broiler chicks after treatment with bacteriophage cocktail and probiotic is illustrated in Table 2. The results revealed a significant ($P \leq 0.05$) difference between the treated groups and the untreated infected group. The faecal shedding rates in the treated groups IV, V and VI were 11.2%, 1.2%, and 61.3%, respectively which was significantly ($P \leq 0.05$) lower than untreated infected birds (94.4%). So, the intestinal carrier status of *Salmonella* contamination during transportation and processing of broilers can be dealt with the use of bacteriophages and probiotics although combination of specific bacteriophage cocktail and probiotic proved to show better results. Similar reports were documented by others too (Atterbury *et al.* 2007, Dorea *et al.* 2010) where cecal colonization of *Salmonella enterica* serotypes *Enteritidis* and *Typhimurium* was studied.

Birds performance of each group was evaluated with measured parameter as average body weight (BW) on daily basis for 9 days. Birds after treatment with phage cocktail

and probiotics were compared with untreated infected birds (Table 3). There is significant ($P \leq 0.05$) improvement in the treated groups (II, IV, V and VI) as compared to untreated infected group (III). The improvement in the performance parameters caused by bacteriophage and probiotic administration may be due to synergistic antibacterial effect of oral probiotic applied together with specific bacteriophage cocktail. The positive effect was expected because of stimulation of the host's appetite, improving feed conversion ratio (Ayed *et al.* 2004), producing digestive enzymes (Saarela *et al.* 2000), synthesizing of vitamins, stimulating lactic acid production and beneficial effect on the health of the host (Soomro *et al.* 2002). Thanki *et al.* (2023) found that phage treatment at three different doses, i.e. 0.1 $\times$, 1 $\times$, and 10 $\times$ had a positive impact on growth performance in challenged birds with increased weight gains in comparison to challenged birds with no phage diet. They showed that delivering phages via feed was effective at reducing *Salmonella* colonization in poultry.

In present study it is revealed that phage cocktail did not cause any adverse effect that could be attributed to the rapid lysis of bacteria. Bacteriophages present in the administered cocktail were isolated from the natural sources mainly of poultry or pigs waste, so their use to control *Salmonella* infection in live birds would not pose any threat of introduction of the new biological entity into the food chain. The data presented in the study showed that probiotics containing *Lactobacillus acidophilus*, *Streptococcus faecium*, yeasts and enzymes can be used to control *Salmonella* infection in broiler chicks. Although different issues like development of bacteriophage resistance and different methods of administration of

Table 3. Average body weight of broiler chicks before and after infection in different groups

Group	Average body weight (g)								
	Age in days								
	Before infection			After infection					
	1	2	3	4	5	6	7	8	9
I	40	52	62	85	95	110	135	156	175
II	42	51	61	84	94	105	132	155	175
III	41	51	61	83	90	93	105	120	137.5
IV	42	52	62	84	94	108	130	157	175
V	40	51	61	83	95	112	140.5	168	187.5
VI	42	52	62	84	93	110	125	145	175

phages like in feed or spray need to be studied in detail so that bacteriophage therapy can be easily and successfully be used under real commercial poultry farming.

Leaving with some issues like safety, long term effectiveness, host immune response etc. unresolved, the results of present study indicated the important role that bacteriophages and bacteriophage cocktails can play either alone or in combination with probiotic preparations for effective management of *Salmonella* infection in broiler chicks paving the way for an effective alternate to antibiotics in food producing animals.

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