Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.302

RESEARCH ARTICLE

Seroprevalence, Molecular Confirmation and Application of Bacteriophage as A Potential Prophylactic Therapy Against Fowl Typhoid Disease in Commercial Poultry Birds

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ARTICLE HISTORY (24-445)

Received: July 25, 2024
Revised: October 28, 2024
Accepted: November 7, 2024
Published online: December 23, 2024

Key words:
Antibiotics
Bacteriophages
Fowl typhoid
Growth performance
Immunoprophylaxis

ABSTRACT

Fowl Typhoid (FT) is a septicemic disease caused by *Salmonella* Gallinaurm that affects poultry birds at any age. This study was aimed to determine the prevalence of FT and the use of bacteriophages as an effective prophylactic intervention. For this purpose, commercial layer farms located in district Layyah were visited and a total of 520 cloacal swabs and tissue samples (liver, spleen, and air sac) were collected from clinically FT-suspected birds followed by SG isolation and confirmation through conventional and molecular approach. Bacteriophages and antibiotics in diseased birds were administered and found that treated groups gained more weight, had less morbidity, and mortality than control groups. Among brown layers, 21.53% samples were found positive for *Salmonella* as compared to 12.69% among commercial white layers. Winter had the highest *Salmonella* distribution (18.46%) among white layers, followed by autumn (13.84%), spring (10.76%), and summer (7.69%). The disease was reduced by the bacteriophage supplementation, as it enhanced overall growth performance and reduced disease burden by elevating immunity levels. Phages improved development and immunological response, indicating their potential as an alternative to antibiotics for avoiding fowl typhoid in layer chickens.

To Cite This Article: Shakir MZ, Rizvi F, Masoud F, Rafique A, Usmani MW, Naseem MN and Usman M, 2024. Seroprevalence, molecular confirmation and application of bacteriophage as a potential prophylactic therapy against fowl typhoid disease in commercial poultry birds. Pak Vet J, 44(4): 1185-1192. http://dx.doi.org/10.29261/pakvetj/2024.302

INTRODUCTION

The poultry industry is a well flourishing business in Pakistan, but infectious and non-infectious diseases pose severe economic losses to farmers (Asghar *et al.*, 2022). Fowl typhoid (FT), caused by *Salmonella* Gallinarum (SG), is a bacterial disease affecting poultry, which is first described in 1888 by Daniel Elmer Salmon, a US Department of Agriculture veterinarian. The disease was initially thought to be a distinct species, later identified as *Salmonella* Gallinarum. In pre-antibiotic era the disease caused significant losses in poultry industry while in 1940s-1950s the antibiotics became widely used, reducing disease incidence. However, during 1960s-1970s the vaccine development and improved sanitation practices further controlled the disease.

FT is a severe bacterial disease in chickens caused by *Salmonella* Gallinarum, a Gram-negative rod. It affects chickens of all ages, causing septicemia, enlarged liver and spleen, and anemia. The disease can lead to high mortality in adult and growing birds (Mahmood *et al.*, 2022). A major economic impact includes reduced production in layers and breeders, which is worsened by mortality among the flock (Shakir *et al.*, 2021).

Antibiotics have been widely used in poultry as growth promotor, prevention, and treatment of bacterial diseases. However, their irrational, particularly in sub-therapeutic doses as feed additives, has led to antibiotic residues in meat and eggs (Shakir *et al.*, 2021). This misuse contributes to the development of antibiotic-resistant bacteria in consumers, posing a significant public health concern (Ishaq *et al.*, 2022). Therapeutic use of antibiotics

eliminates susceptible bacteria but is ineffective against resistant strains. Moreover, resistant microbes can transfer their genes to other bacteria, exacerbating the issue (Amjad *et al.*, 2022). To address this, reducing antibiotic use and exploring alternative therapies are crucial steps forward.

Bacteriophages are viruses that contain a DNA genome encased in a complex-tailed capsid and are known to selectively target bacteria. They were widely employed before antibiotics because of their ability to selectively destroy harmful germs while preserving normal microflora. Phages infect bacteria via the lytic cycle, resulting in bacterial lysis (Stanton, 2013).

It has been reported that bacteriophages can effectively combat lethal and resistant bacterial strains in laboratory settings (Stanton, 2013; Asghar *et al.*, 2022) . However, their application *in-vivo* requires adaptation due to differences in bacterial physiology between laboratory and live animals. Phages showed promising results as alternatives to antibiotics (Gadde *et al.*, 2017). Therefore, this study aims to investigate the molecular prevalence of FT in brown and white-layer birds and the application of bacteriophage as a potential immunoprophylactic therapy against fowl typhoid in poultry birds.

MATERIALS AND METHODS

Flock screening and sample collection: From September 2018 to August 2021, a total of 520 cloacal swabs and tissue samples (liver, spleen, and air-sac) were collected from randomly selected layer birds suspected of fowl typhoid. These collected samples were stored in sterile containers, labeled properly, and shifted to the Diagnostic Laboratory, Department of Pathology, University of Agriculture, Faisalabad Pakistan. The sampling was based on the type (brown & white layers), age (1-5 weeks, 6-10 weeks, 11-15 weeks, & >15 weeks) of layer birds, and seasons (winter, spring, summer and autumn) (Table 1).

Bacterial isolation and characterization: The tissue samples (liver, spleen, air sac) were weighed, homogenized, and prepared for *Salmonella* isolation. Briefly, the cloacal swabs and homogenized tissue samples were suspended separately in the sterile saline to prepare 10% suspension, followed by a dilution at a 1:10 ratio in the nutrient broth and incubated aerobically at 37°C for 24 hours to enrich *Salmonella* growth. Post incubation, each sample was streaked onto selective and differential media *i.e.*, xylose lysine deoxycholate (XLD) agar, MacConkey's agar, and Salmonella-Shigella (SS) agar using a sterile loop followed by incubation at 37°C for 24-48 hours (Mahmood *et al.*, 2022). Later on, Gram staining was performed on suspected *Salmonella* colonies to determine their Gram-negative characteristics at 100X (oil immersion lens) under a light microscope. The Gram-negative isolates were further analyzed for their biochemical properties through biochemical tests *i.e.*, sugar fermentation/triple sugar iron (TSI), indole production, citrate utilization, and urease (Sarker *et al.*, 2021).

Molecular characterization of *Salmonella* Gallinarum: After the biochemical analysis, the positive colonies were further processed for *S.* Gallinarum confirmation through PCR. Briefly, the DNA was extracted and purified using the spin column protocol, mentioned in the commercially available GeneJET Genomic DNA Purification Kit (catalog # K0722, Thermo Fisher Scientific, Baltics UAB, Lithuania) followed by DNA quantification through nano-drop (ND-1000; Thermo Fisher Scientific, Baltics UAB, Lithuania). The extracted DNA was amplified by targeting the *ratA* gene as previously described by (Batista *et al.*, 2016) with some modifications using a specific set of primers (F= GACGTCGCTGCCGTCGTACC, R= TACAGCGAACATGCGGGCGG) in a thermal cycle (Bio-Rad T100™ – Thermal cycler, USA) with the following protocol: initial denaturation at 94°C for 3 minutes, followed by 26 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 64°C for 45 seconds, and extension at 72°C for 1 minutes. A final extension step was performed at 72°C for 7 minutes. Subsequently, the PCR products were analyzed by electrophoresis at 4 V/cm for 60 minutes in a 1.5% (w/v) agarose gel stained with ethidium bromide at a concentration of 0.6 µg/mL of gel running buffer (Usmani *et al.*, 2022). The bacterial strains were confirmed by the sanger sequencing by a commercial company.

Experimental studies: An experimental trial was conducted to evaluate the *Salmonella*-specific bacteriophage (SSB-Intralytix, USA) as a potential immuno-prophylaxis in layer birds against FT infection. The experimental design consisted of a total 140 nine-week-old layer birds, which were purchased from a local layer farm in Faisalabad (Fig. 1). Before the bird's arrival, the experimental station at the University of Agriculture Faisalabad was prepared *i.e.*, fumigation with 10% formalin, maintenance of 75°F temperature, proper ventilation, spreading of litter material, etc. The feed and water were provided *ad-libitum*. On day 3rd the layer birds were equally divided into 4 study groups *i.e.*, group A (negative control), group B (positive control), group C (SS-bacteriophage-treated), and group D (antibiotic-treatment). On day 14th, the layer birds of groups C and D were challenged with *S.* Gallinarum (8.5×10^8 CFU/bird). On day 21st, the birds in group C were supplemented (1g/kg of feed) with Salmonella-specific bacteriophages (SSB) as a feed additive, and group D birds were treated with sulfadiazine and trimethoprim (an antibiotic combination; 1mL/1L of drinking water) for seven days. Post-infection, the birds in each group were monitored for growth performance, morbidity, and mortality.

On days 28, 35, and 42, seven birds from each group were slaughtered to observe the gross and pathological changes in visceral organs. On the same days, the blood (with EDTA) was also collected for RBCs, WBCs, and Hb Conc. & PCV determination (Youssef *et al.*, 2023). The liver, spleen, kidneys, and intestine showing any gross lesions were collected for histopathological studies (Myburgh *et al.*, 2023). For cell-mediated immunity, the lymphoproliferative response (cutaneous thickness) against phytohemagglutinin (PHA-P) (Saleemi *et al.*, 2023), and the mononuclear phagocytic ability via carbon clearance assay (CCA) (Fraser-Smith, Waters, and Matthews, 1982) were assessed in the seven birds of each study group on the 40th day of the experiment.

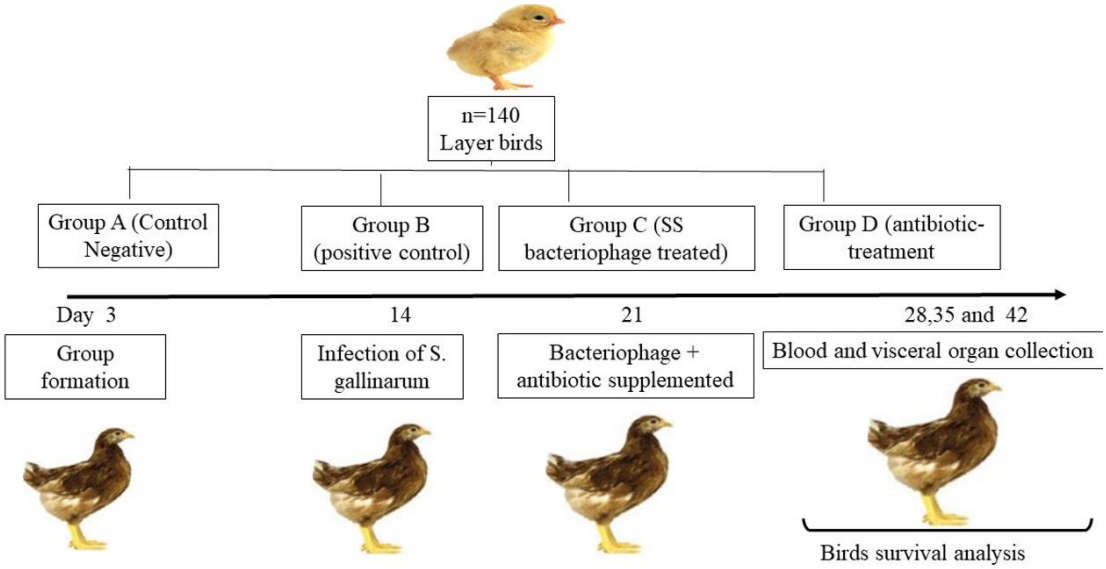

Fig. 1: Experimental layout of layer birds infected with *S.* Gallinarum supplemented with bacteriophage. Group A: Negative control Group B: positive control Group C: bacteriophages + *S.* Gallinarum Group D: antibiotics + *S.* Gallinarum

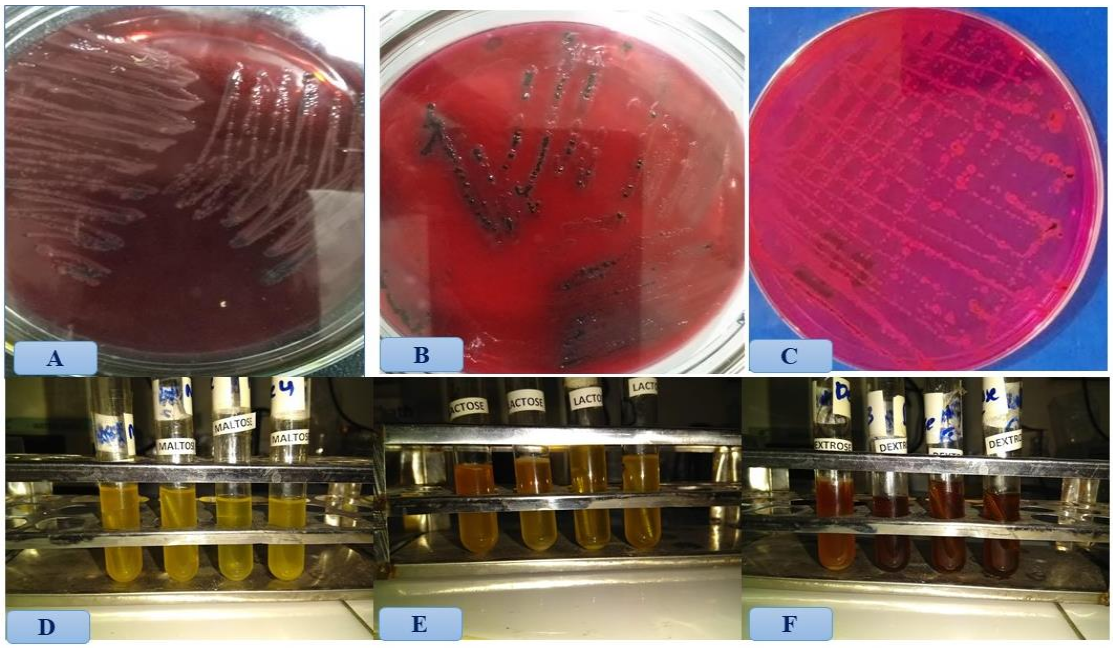

Fig. 2: Biochemical testing on different media. A: pink with a black center on XLD B: Black color colonies on SSA C: white on MacConkey agar were found D: maltose formation testing E: lactose fermentation testing with no production of gas in Durham's tube F: Dextrose formation testing with production of gas in tube.

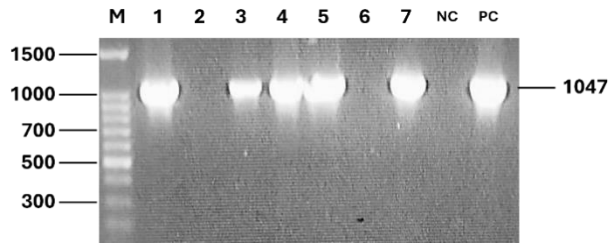

Fig. 3: PCR result of *S.* Gallinarum confirmation by amplifying rat*A* gene. Lane M= DNA gene labber (100bp Plus Thermo Scientific), lanes 1, 3, 4, 5, and 7 = positive (*S.* Gallinarum; 1047bp), lane 2 and 6= negative, NC = Negative Control, PC = Positive Control.

Statistical analysis: The data thus obtained about disease distribution was analyzed using Pearson's chi-square test by Statistix 8.1® software. The odds ratios were calculated by WinPepi software. The data obtained from experimental studies were analyzed statistically by one-way ANOVA, and group means were compared for significance using Duncan's Multiple Range (DMR) test through Minitab® software. The level of significance was ≥0.05.

RESULTS

Seroprevalence of Fowl typhoid: Analysis showed significant effects (P<0.05) of layer type, season, and age on disease occurrence (Table 2). Among brown layers, 21.53% tested positive for *Salmonella*, compared to 12.69% among commercial white layers. Winter had the highest *Salmonella* distribution (18.46%) among white layers, followed by autumn (13.84%), spring (10.76%), and summer (7.69%). In brown layers, winter also had the

highest distribution (36.92%), followed by autumn (21.53%), spring (15.38%) and summer (12.30%). Among white layers, 21.53% of Salmonella distribution was in birds aged less than 5 weeks, 18.46% in those older than 15 weeks, 6.15% in those aged 11-15 weeks, and 4.61% in the birds aged 6-10 weeks. In brown layers, 43.07% distribution was in birds less than 5 weeks old, 23.07% in those older than 15 weeks, 10.69% in the birds aged 11-15 weeks, and 9.23% in those aged 6-10 weeks.

Table 1: Number and type of samples collected from layer bird flocks in District Layyah, Pakistan from September 2018 to August 2021.

Type of Layer bird	Type of samples			
	Cloacal swabs	Tissue		
		Air sac	Liver	Spleen
Brown layer	120	32	44	44
White layer	140	36	52	52
Age (weeks) of layer bird				
1 – 5	42	18	23	23
6 – 10	76	18	24	24
11 – 15	86	18	24	24
> 15	56	18	23	23
Season (months)				
Autumn (Sept-Nov)	65	18	23	23
Winter (Dec-Feb)	65	18	24	24
Spring (March-May)	65	18	24	24
Summer (June-Aug)	65	18	23	23

Colony morphology and biochemical characteristics: *Salmonella*-specific traits *i.e.*, black centers with pink or transparent colonies on XLD agar, lactose-negative colonies on MacConkey agar, and black-centered colonies on Salmonella-Shigella agar were observed. Apart from lactose, sucrose, and dulcitol, all of the tested isolates demonstrated fermentation of dextrose and maltose with the production of acid and gas. The tested isolates were negative for indole and Voges-Proskauer (VP) tests, while positive for the methyl red test.

Growth performance: The layer birds of groups C (SS-bacteriophage) and D (antibiotic) showed a significant increase ($P<0.05$) in body weight gain as compared to the positive control (group B) layer birds (Fig. 4).

Morbidity and mortality rates of experimental trials: The morbidity and mortality rates were observed highest in group B (positive control), followed by group D (antibiotic-treated), group C (SS-bacteriophage-treated), and negative control (group A) layer birds. The morbidity rate in study groups was as follows; 91.4% in group B (positive control), 22.8% in group D (antibiotic-treated), and 17.1% in group C (SS-bacteriophage-treated), 0% in group A (negative control). The mortality rate in study groups was as follows; 71.4% in group B (positive control), 11.4% in group D (antibiotic-treated), and 8.6% in group C (SS-bacteriophage-treated). There was no morbidity or mortality observed in group A (negative control) as shown in Table 3.

Gross lesions: The necropsy examination of the layer birds of group B (positive control) showed an enlarged, friable liver with bronze discoloration and white necrotic foci. A mottled spleen and enlarged kidneys were also observed. The layer birds of groups C (SS-bacteriophage-treated) and D (antibiotic-treated) exhibited only mild gross lesions (Fig. 5).

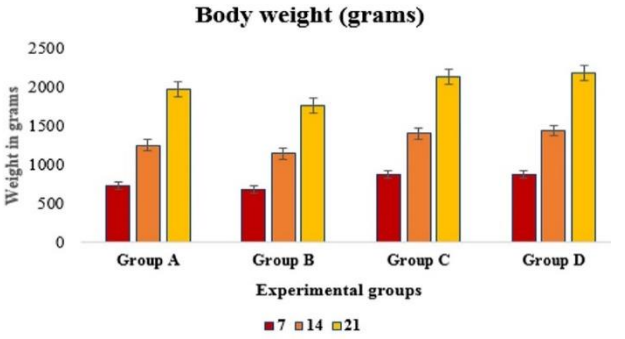

Fig. 4: Body weight (grams) gain of layer birds experimentally infected with *S. Gallinarum* treated with SS-bacteriophage and antibiotic. Group A: Negative control Group B: positive control Group C: bacteriophages + *S. Gallinarum* Group D: antibiotics + *S. Gallinarum*

Blood cell indices: The total erythrocyte (RBCs) count (10^6/µL), packed cell volume (PCV), and hemoglobin concentration (Hb. Conc.) were significantly higher in the layer birds of groups C (SS-bacteriophage-treated) and D (antibiotic-treated) as compared to group B (positive control) layer birds ($P<0.05$). Contrary to this, the total leukocyte (WBCs) count (10^3/µL) of the layer birds of group B (positive control) was significantly higher compared to that of the layer birds of groups A (negative control), C (SS-bacteriophage-treated) and D (antibiotic-treated) layer birds ($P<0.05$) (Table 4).

Microscopic examination of body tissues

Liver: The negative control birds had the normal hepatocyte arrangement and sinusoidal spaces in a radiating pattern around the central vein (Fig. 6A). In positive control birds, the hepatic cellular structure was disrupted, and well-dilated central vein, hepatic and sinusoidal cords were observed. There was severe vacuolation of the hepatocytes, coagulation necrosis of hepatocytes, and infiltration of mononuclear inflammatory cells in the interstitium of the liver (Fig. 6B). The liver of layer birds of groups C and D showed hydropic degeneration of hepatocytes, hemorrhages in the hepatic parenchyma as well as mild degeneration of the hepatic cord (Fig. 6C & 6D).

Kidneys: The negative control birds showed normal glomerular structure, surrounded by Bowman's capsule with normal renal tubules (Fig. 7A). In positive control birds, the cellular structure of renal tubules was badly disrupted, displaying clogged glomerular capillaries and interstitial blood vessels. Severe necrosis, desquamation of the renal epithelium (limiting glomerular filtration space), and tubular lumen obliteration were also observed. Some locations in the interstitial tissue of layer birds' kidneys showed lymphocyte infiltration (Fig. 7B). The layer birds of groups C and D showed mild swelling of renal epithelial cells at some focal points leading to the partial obliteration of the lumen (Fig. 7C & 7D).

Spleen: The negative control birds showed normal splenic parenchyma *i.e.*, white pulp and red pulp with the nodular artery (Fig. 8A). However, the positive control birds, severely infected spleen leading to the disorientation of white and red pulp with infiltration of lymphocytes. Depletion of the lymphoid tissue coupled with scattered or multifocal necrosis of lymphoid follicles (Fig. 8B). In

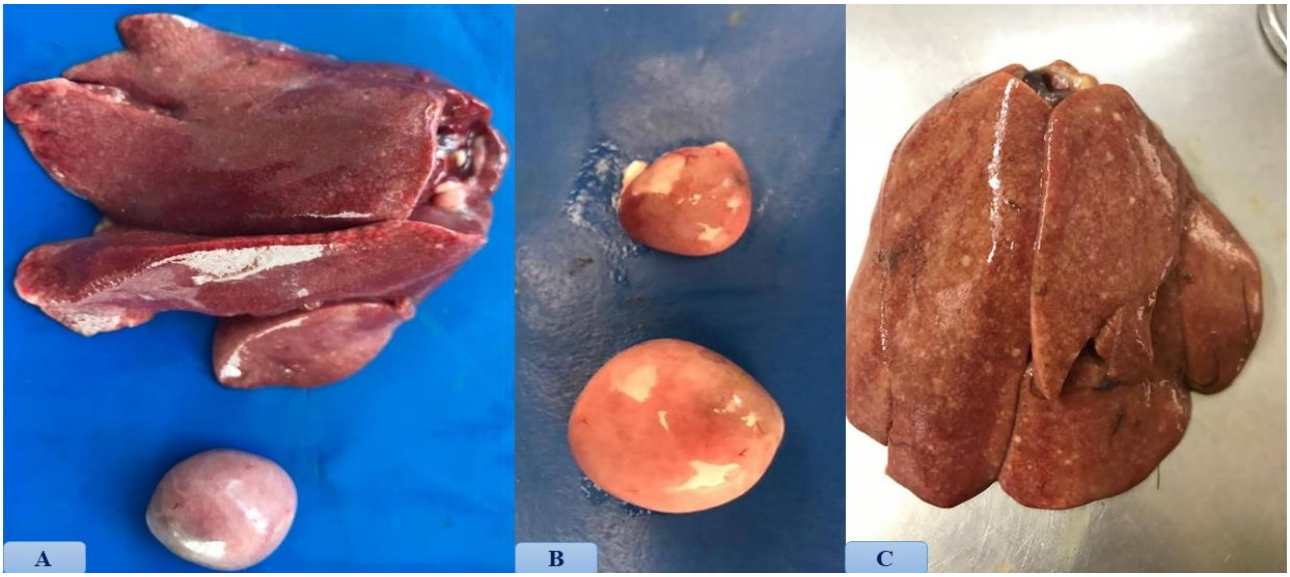

Fig. 5: Postmortem leison shown by positive conntrol birds. A: indicate the Enlarged liver, B: indicates the mottled spleen, and C: indicates the liver with white necrotic foci.

Table 2: Distribution of avian *Salmonella* based on type, season, and age of layer birds (white and brown) by rapid plate agglutination test.

Distribution based on Type of layer birds						
Type of layer birds	Negative	Positive	P-value	Odds ratio	95% CI	
					Lower limit	Upper limit
Brown layer	204	56 (21.53%)	0.005	1.89	16.98	26.93
White layer	227	33 (12.69%)	0.0074	-----	9.18	17.29
Chi-Square = 7.17, DF = 1, P-Value = 0.0074						
Distribution based on Season in White layer						
Winter (Dec-Feb)	53	12 (18.46%)	0.195	2.71	10.89	29.55
Autumn (Sept-Nov)	56	9 (13.84%)	0.764	1.92	7.46	24.27
Spring (March-May)	58	7 (10.76%)	1	1.44	5.32	20.6
Summer (June-august)	60	5(7.69%)	0.294	-----	3.33	16.77
Chi-Square = 3.71, DF = 3, P-Value = 0.2941						
Distribution based on Season in Brown layer						
Winter (Dec-Feb)	41	24 (36.92%)	0.003	4.17	26.23	49.07
Autumn (Sept-Nov)	51	14 (21.53%)	0.475	1.95	13.29	32.97
Spring (March-May)	55	10 (15.38%)	1	1.29	8.57	26.05
Summer (June-august)	57	8 (12.30%)	0.0031	-----	6.37	22.45
Distribution based on Age group in the White layer						
1 to 5 weeks	51	14 (21.53%)	0.009	5.67	13.29	32.97
>15 weeks	53	12 (18.46%)	0.033	4.67	10.89	29.55
11 to 15 weeks	61	4 (6.15%)	1	1.35	2.42	14.78
6 to 10 weeks	62	3 (4.61%)	0.005	-----	1.58	12.72
Chi-Square = 12.88, DF = 3, P-Value = 0.005						
Distribution based on Age group in the Brown layer						
1 to 5 weeks	37	28 (43.07%)	0.009	7.44	31.76	55.17
>15 weeks	50	15 (23.07%)	0.089	2.95	14.52	34.65
11 to 15 weeks	58	7 (10.69%)	1	1.18	5.32	20.61
6 to 10 weeks	59	6 (9.23%)	0.001	-----	4.31	18.71
Chi-Square = 28.22, DF = 3, P-Value = 0.0019						

groups C and D, mild lymphoid depletion and normal distribution of white and red pulp in the splenic parenchyma was observed (Fig. 8C & 8D).

Intestine: The negative control birds showed normal intestinal tissue consisting of villi lined by ciliated columnar epithelium and submucosal glands in the mucosa. The muscular mucosa was composed of a thin layer of myocytes (Fig. 9A). Layer birds of the positive control group showed shrunken and sloughed villi, disrupted mucosal epithelium and submucosal gland along with infiltration of inflammatory cells (Fig. 9B). However, the layer birds of groups C and D showed mild sloughing of intestinal villi and epithelial cells with lack of cellular infiltration in the mucosa and submucosa of the intestine (Fig. 9C & 9D).

Table 3: Morbidity and mortality rates of experimental trial birds.

Experimental groups	Mortality	
	Morbidity %	Mortality %
A		
B	32 (91.4%)	25 (71.4%)
C	8 (22.8%)	3 (8.5%)
D	6 (17.14%)	4 (11.4%)

Group A: Negative control Group B: positive control Group C: bacteriophages + *S.* Gallinarum Group D: antibiotics + *S.* Gallinarum.

Lymphoproliferative response to phytohemagglutinin (PHA-P): Post 24 and 48 hours of PHA-P inoculation, the lymphoproliferative response (cutaneous thickness) was significantly higher ($P<0.05$) in the layer birds of group C (SS-bacteriophage treated) as compared to positive control (group B) layer birds (Table 5).

Mononuclear phagocytic activity: Post 3 and 15 minutes, the phagocytic activity was significantly higher ($P<0.05$) in the layer birds of group C (SS-bacteriophage treated) as compared to positive control (group B) layer birds (Table 6).

DISCUSSION

Infectious diseases cause significant economic losses in poultry due to morbidity and mortality. The effective control of these diseases in poultry is achieved through the implementation of strict biosecurity measures (Islam *et al.*, 2024). Controlling bacterial diseases such as *S. enterica subspp. enterica* serovar Gallinarum biovars Gallinarum and Pullorum require early screening of the flock and

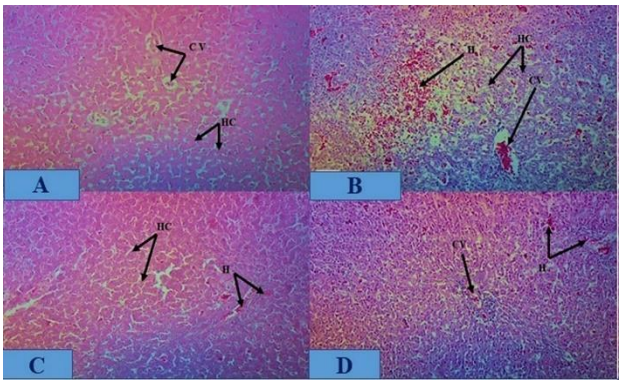

Fig. 6: Photomicrograph of liver of layer birds experimentally infected with *S.* Gallinarum treated with SS-bacteriophage and antibiotic. In the negative control group (6A), the hepatic tissue displayed normal orientation of hepatocytic cords (HC) around the central vein (CV). In contrast, the positive control group (6B) exhibited HC degeneration, an edematous CV, and extensive hemorrhaging (H). In groups treated with bacteriophages + *S.* Gallinarum (6C) and antibiotics + *S.* Gallinarum (6D), the hepatic tissue showed restored cellular architecture with reduced hemorrhaging (H) and HC degeneration. (Hematoxylin and eosin staining, 1000X magnification).

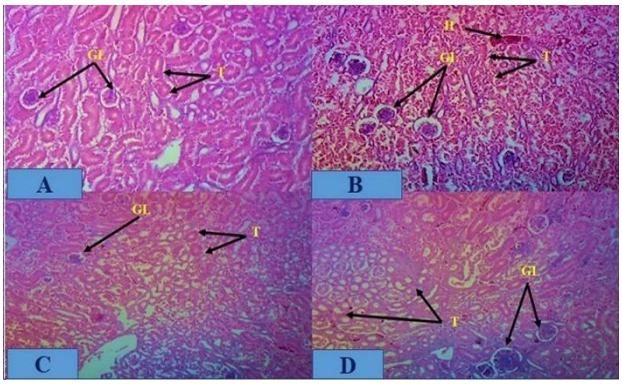

Fig. 7: Photomicrograph of kidneys of layer birds experimentally infected with *S.* Gallinarum treated with SS-bacteriophage and antibiotic. The renal tissue of the negative control group (7A) exhibited normal glomerular (GL) and tubular (T) structures. In the positive control group infected with *S.* Gallinarum (7B), the glomeruli were found to be shrunken and there was severe degeneration of tubules (T), accompanied by hemorrhages (H). Groups 7C (bacteriophages + infected with *S.* Gallinarum) and 7D (antibiotic + infected with *S.* Gallinarum) showed recovery of glomerular (GL) and tubular (T) structures towards normal, indicating the protective effect of the vaccine. (H & E staining, 1000X magnification).

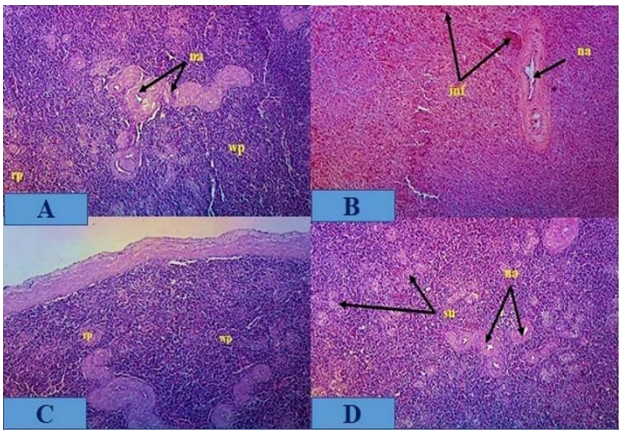

Fig. 8: Photomicrograph of spleen of layer birds experimentally infected with S. Gallinarum treated with SS-bacteriophage and antibiotic. In the negative control group (8A), the splenic structure appeared normal with distinct splenic nodules and red pulp. In group 9B, infection with S. Gallinarum led to severe disruption of splenic parenchyma and lymphocyte infiltration. However, splenic sections from the group treated with bacteriophages and infected with S. Gallinarum (9C) showed nearly normal parenchymal structure. Sections from group 9D (treated with antibiotics and infected with S. Gallinarum) exhibited mild lymphoid depletion in the white pulp. Abbreviations used: splenic artery (na), trabecular artery (tr), red pulp (rp), white pulp (wp), lymphocytic infiltration (INF). (Hematoxylin and eosin staining, 1000X magnification).

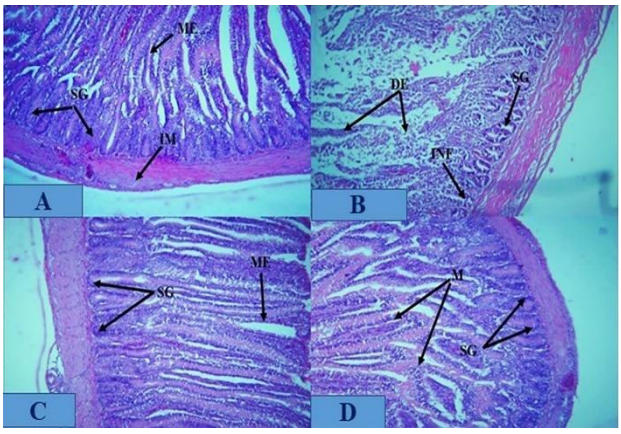

Fig. 9: Photomicrograph of intestine of layer birds experimentally infected with *S.* Gallinarum treated with SS-bacteriophage and antibiotic. In group G1, the intestine exhibited a normal mucosal epithelium (ME) lined with intestinal villi, and the submucosal glands (SG) in the submucosa appeared mucous as in the control group (9A). In positive control group (9B) the normal cellular structure of the intestine was disrupted, characterized by damaged ME and shrunken villi. The SG showed necrotic epithelium and atrophied intestinal smooth muscle, accompanied by inflammatory cell infiltration. In groups C and D, the intestinal sections displayed significant restoration of ME and SG without infiltration of inflammatory cells (INF) as shown in 9C and 9D, respectively (Hematoxylin and eosin staining, 1000X magnification).

culling of morbid birds from the house (Abreu *et al*., 2023). Serological tests are routinely used to detect *Salmonella* in infected and non-infected flocks and should be confirmed through various available biochemical tests due to non-precise reactions. Therefore, early screening of the flock and differentiation between the two biovars Gallinarum and Pullorum are more precise ways to control the disease (Farhat *et al*., 2024). The two biovars are similar antigenically, but they cause different diseases in poultry. The main objective of the present study was to isolate *Salmonella* from local field samples through different serological, biochemical, and molecular testing.

In the current study, the distribution of *Salmonella* in layer birds from collected samples was found to be 19.21, with 16.9% in commercial white layers and 21.53% in commercial brown layers. The results of our findings were higher than those of previous studies (Majid and Siddique, 2000). *Salmonella* prevalence of 13.53% was reported from Pakistan. Likewise, *Salmonella* prevalence of 11.93 and 7.14% were reported by Li *et al.* (2013) and Rhon *et al.* (2012), respectively. However, our results were lower than those Rahman *et al.* (1970) who reported 36.5% and Andoh *et al.* (2016) reported 38% prevalence, respectively from Pakistan. *Salmonella enterica* prevalence was 41% (41.7% in broiler and 40% in layer) reported by Sarker *et al.* (2021). Shahzad *et al.* (2012) observed the prevalence of *Salmonella* in eggs (29.36%), egg shells (38.88%) egg contents (28.78%), and egg storing trays (43.93%) from

Table 4: Hematological parameters of chickens experimentally infected with fowl typhoid infection followed by bacteriophage and antibiotic treatments.

	DPI	Group A	Group B	Group C	Group D
Total erythrocyte count (10^6/µl)	7	3.12±0.01^a	2.50±0.09^c	2.91±0.04^b	2.97±0.115^b
	14	3.08±0.01^a	2.33±0.04^c	2.94±0.019^b	3.08±0.08^a
	21	3.0±0.01^a	2.32±0.04^c	2.95±0.014^b	3.13±0.07^a
Total leukocyte count (10^3/µl)	7	24.54±0.07^b	32.85±0.13^a	27.45±0.98^b	25.07±0.73^b
	14	25.47±0.15^b	29.17±0.11^a	26.18±0.40^b	25.27±0.59^b
	21	23.81±0.10^b	28.20±0.14^a	24.43±0.32^b	23.58±0.42^b
Pack cell volume (%)	7	35.98±0.14^a	26.78±0.49^b	33.67±0.36^a	34.32±0.47^a
	14	32.63±0.15^a	23.75±0.13^b	34.39±0.34^a	35.26±0.45^a
	21	30.54±0.10^b	22.97±0.09^c	35.30±0.28^a	35.60±0.35^a
Hemoglobin concentration (g/dl)	7	11.53±0.08^a	8.97±0.11^b	11.62±0.21^a	11.90±0.14^a
	14	12.15±0.09^a	7.98±0.11^b	11.92±0.27^a	12.27±0.21^a
	21	11.65±0.10^a	7.12±0.16^b	12.23±0.27^a	12.57±0.30^a

Values in each column followed by different letters are significantly different (P<0.05). DPI: Days post infection, Group A: Negative control Group B: positive control Group C: bacteriophages + *S.* Gallinarum Group D: antibiotics + *S.* Gallinarum.

Table 5: Lymphoproliferative response to Phytohemagglutinin (PHA-P)

Response time	A	B	C	D
response at 24 hours	0.45±0.01^a	0.18±1.0^c	0.36±0.01^b	0.34±0.01^b
Response at 48 hours	0.33±0.01^b	0.56±0.42^a	0.27±0.01^c	0.24±1.0^c

Values in each column followed by different letters are significantly different (P<0.05). Group A: Negative control Group B: positive control Group C: bacteriophages + *S.* Gallinarum Group D: antibiotics + *S.* Gallinarum

Table 6: Mononuclear Phagocytic system function assay (Carbon clearance assay)

Response time	A	B	C	D
3 minutes	288.47±1.45^d	480.31±0.77^a	311.15±1.007^c	352.87±1.54^b
15 minutes	41.52±1.030^d	146.55±1.040^a	68.55±0.98^c	76.14±1.03^b

Values in each column followed by different letters are significantly different (P<0.05). Values were determined as k= (logn OD1- logn OD2)/T2-T1), where OD1 and OD2 are optical densities (640nm) at times T1 and T2 respectively. Absorbance from a 0 min sample was considered as zero value for each sample. Group A: Negative control Group B: positive control Group C: bacteriophages + *S.* Gallinarum Group D: antibiotics + *S.* Gallinarum

different farms and poultry markets of Faisalabad. The difference in results could be due to different housing and environmental conditions at the farm. Also, the various other risk factors (density of flock, type of housing, age of flock, and vaccination schedule) play a vital role in disease occurrence.

In the current experiment, birds from control group A and treated group (C and D) were alert and had shiny skin with normal feed intake. The degree of apparent clinical signs and organs gross lesions was markedly reduced due to the antibacterial activity of bacteriophages. Our findings were in line with Hosny *et al.* (2023), who reported that bacteriophage reduced the severity of clinical signs in the positive control group of *Salmonella*. The body weight of positive control group B was lower significantly, due to stress caused by *Salmonella* infection, the birds were unable to take feed. Our current findings were alike the results of Wang *et al.* (2013) who reported that *Salmonella* infection causes a decrease in body weight due to poor feed intake. However, the body weight of treated groups was found significantly higher due to the growth-promoting potential of bacteriophages and antibiotics, similar findings were reported by Upadhaya *et al.* (2021), who found that bacteriophage supplementation enhances the daily weight compared to the control group. The morbidity and mortality percentage was reduced in the treated group due to the antibacterial activity of bacteriophage, similar activity was reported by Hong *et al.* (2013) in broiler birds against *S.* Gallinarum infection.

In the current study, the hematological parameters (Red blood cells, Hb concentration, and Hematocrit) were found significantly lower in layer birds of the positive control group, which is due to the destruction of erythrocytes leading to anemia (Elaroussi *et al.*, 2006). The bacteriophage decreases the destruction caused by bacteria describing higher hematological parameters in treated groups, similar results were documented by Li *et al.* (2013). Bacteriophage and antibiotic supplementation improved the concentration of RBCs and WBCs in broiler birds.

The bird's immune system is made up of several soluble components and cells that provide immunity (Schilling *et al.*, 2019). Elevated levels of cellular and humoral immune response were reported by indirect ELISA when the birds were supplemented with bacteriophages (Huff *et al.*, 2010). The production of B and T-cells plays a significant role in cell-mediated immunity of birds. Administration of PHA-P must stimulate the T-cells which results in cytokines/chemokines and ultimately elevate the skin thickness (Sattar *et al.*, 2016). The lymphoproliferative response was significantly lower in the positive control group after 24 h of injection as compared to the negative control group A. A similar immune response was reported by Elaroussi *et al.* (2006), who described immune suppression in broiler birds given different doses of toxin. In our study, the bacteriophage improved the protection against *Salmonella* in layer birds.

Conclusions: The current study shows that *Salmonella* was more prevalent in brown layers. The age, season, and type of poultry have a significant effect on disease production. The disease can be minimized through bacteriophage supplementation, as it enhances overall growth performance and reduces disease burden by elevating immunity levels. The exact mechanism of bacteriophages needs further study. The study showed that bacteriophages may be used as a replacement for antibiotics to overcome drug resistance, which is a major concern for public health and the poultry industry. Therefore, it is necessary for the poultry industry to use bacteriophages to control fowl typhoid, which is a serious threat to layer birds.

Contribution of authors: MZS and FR: Conceptualization of idea, original draft writing AR: Methodology and supervision FM: Formal analysis and Validation, MWU, NN and MU: visualization, investigation and data collection.

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