https://doi.org/10.15218/zjms.2024.042

# Application of salmonella phage to reduce in-vitro and in-vivo colonization of *salmonella enteritidis* and *salmonella typhi*

Received: 08/09/2022 Accepted: 26/01/2023

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## Abstract

**Background and objective:** *Salmonella typhi* is the causative agent of enteric fever while *Salmonella enteritidis* causes gastroenteritis. Lytic bacteriophages can be applied as biocontrol agents to prevent Salmonella infections. The purpose of this study was to prepare a proper Salmonella phage therapy candidate to be used against pathogenic *Salmonella enteritidis* and *Salmonella typhi*.

**Methods:** We used clinical isolates of *Salmonella typhi* and *Salmonella enteritidis* as host bacteria, to isolate *Salmonella*-specific phages from raw sewage water in four locations (Bahrka, Farmnbaran neighborhoods, Korey and Shaqlawa towns) in Erbil district. We assessed the efficacy of this phage as a biocontrol agent against *Salmonella enteritidis* in-vivo using four groups of 8 pathogen-free duck chicks. A group was kept as uninfected control while the other three groups were artificially infected with a clinical strain of *Salmonella enteritidis*. Two of the infected groups were treated by oral administration of phage suspension using two different doses of *Salmonella enteritidis* phage (7 and 12 Log10 Plague Forming Unit respectively). To compare the bacterial growth dynamics among the infected groups, one group was kept untreated.

**Results:** We found that the four sewage samples contained bacteriophages for the two bacterial isolates with different plaque diameters and morphology. *Salmonella enteritidis* phage isolates collected from the Farmanbaran neighborhood showed the best lysing efficacy in-vitro hence it was selected to be tested in-vivo experiment. Phage-treatment significantly reduced the colonization burden of *Salmonella enteritidis* in feces and cecum contents of the experimentally infected chicks.

**Conclusion:** These results suggest that using Salmonella phage could be a good agent to control Salmonella.

**Keywords:** Salmonella-Phage; *Salmonella enteritidis*; Typhoid Fever; Gastroenteritis; Foodborne-Pathogen.

## Introduction

The bacterial genus Salmonella includes Gram-negative facultative anaerobic bacillus. The natural habitat of Salmonella bacteria includes the lower part of the digestive tracts in animals and humans. Therefore these bacteria are normally shed through feces and infections in human most often happens through the use of contaminated food and water with fecal materials.[1] Globally, infections due to Salmonella species are a major cause of mortality and morbidity. Typhoidal Salmonella such as *Salmonella typhi* is responsible for typhoid fever in humans. Typhoid fever which is a life-threatening infection especially to children, elders and immunosuppressed patients and it has been found to cause a high burden on public health.[2] Infection with non-typhoidal

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https://doi.org/10.15218/zjms.2024.042

Salmonella such as *Salmonella enteritidis* leads to gastroenteritis.[3] It has been found that *Salmonella enteritidis* represents 60% of salmonellosis in European countries.[4] Severe gastroenteritis and diarrheal diseases represent one of the leading causes of death in low-income countries with an estimation of 1.5 million deaths recorded in 2019.[5]

There are over 2600 different serotypes related to *Salmonella enterica* which explains that Salmonella has the capability to adapt to the human hostit infects. It is a zoonotic pathogen can be transmitted to humans by almost all the food chains including vegetables, fruits, beef, and poultry meats, and is implicated in the outbreaks of bacterial-associated foodborne disease in many countries.[6, 7]

Control of Salmonella in beef and poultry farms is challenging and it currently depends on the use of antibiotics causing a considerable financial burden for the farmers and consumers. Additionally, the continuous emergence of multi-drug-resistant Salmonella strains has been documented in poultry fields.[8] This indicates that the pathogen will be a significant future threat to global health. For example, there is evidence that some Salmonella isolates including *Salmonella enteritidis* and *Salmonella typhimurium* have developed resistance to multiple antibiotics including ampicillin, streptomycin, tetracycline, nalidixic acid, sulfisoxazole.[9] Therefore, there is a need for new cost-effective treatment strategies to replace conventional antibiotics for controlling such zoonotic bacteria.

Bacteriophage therapy is a promising way to replace antibiotics and it has recently gained considerable attention.[10] Bacteriophages are very abundant in the environment and they are powerful predators of specific bacteria. In comparison to antibiotics, bacteriophages have high host specificity granting them the major advantage as they target only specific bacteria without causing any harm to the beneficial normal flora in the gut. Unlike antibiotics, phages may cope with the bacterial defense mechanism, and with their smaller size and faster replication rate, they can evolve higher virulence when bacteria develop anti-phage resistance.[11] An additional advantage of bacteriophages includes their natural self-clearance straight after clearing targeted bacterium in the biotic environment where the phage-bacterium interaction occurs.[12]

Previous research has demonstrated that Salmonella infections pose a big challenge to public health in Iraqi Kurdistan, particularly in the growing urban.[2] Our aim in the current study was to isolate powerful lytic phages against Salmonella species. To do this, we carried out in-vitro and in-vivo trials to assess the antibacterial efficacy of eight Salmonella- phages isolated from sewage water sources collected in four different locations in Erbil city. We compared the phages' ability to control the growth of *Salmonella typhi* and *Salmonella enteritidis* in-vitro and then selected the best *Salmonella enteritidis* phage candidate to assess its lytic efficacy in reducing colonization of *Salmonella enteritidis* in-vivo.

## Methods

### Culturing of host bacteria

Bacterial samples including *Salmonella typhi and Salmonella enteritidis* were obtained from Medya Diagnostic Center- Erbil. To examine the effect of phages on bacterial load in-vivo, the duck fecal samples were analyzed using a standard microbiological approach.[13, 14] Briefly, one Gram of fecal sample was taken and directly transferred to tubes containing 9 ml selenite F broth then incubated at $37^0$C for 24 hours. After measuring the optical density for the tubes, 0.1ml from the broth mediums was used to streak on each Salmonella Shigella agar (SSA, Thermo Scientific, USA) and Xylose Lysine Deoxycholate (XLD, Thermo Scientific, USA) agar. Agar mediums were incubated for 48 hours at $37^0$C. After the

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colony counting procedure, and sub-culturing on nutrient agar plates, pure cultures were chosen for Gram-staining, motility testing, and oxidase testing.[14] Generally, colonies of approximately 3mm in diameter, Gram-negative rod-shaped were considered Salmonella species. Other biochemical tests used included catalase, oxidase, nitrate reduction test, glucose fermentation (and gas production), growth on citrate, production of $H_2S$ on triple sugar iron, and hydrolysis of urea and indole (Table 1).

*Salmonella enteritidis* was determined using Salmonella test kit- OxoidUK. This was carried out by mixing a drop of saline with 2 mm of suspected colonies from XLD agar on the kit reagent card to produce a thick suspension.[15] The suspension was gently mixed with the Latex Reagent using a clean stick for 30 seconds then it was gently shaken three times. The suspension was examined for agglutination/ clumping for two minutes. Agglutination within two minutes was considered a positive result.[15]

One day before the in-vivo assay 10mL of sterile Brain Heart Infusion Broth (BHI, Thermo Scientific, USA) inoculated with a loop full of Salmonella from XLD agar and incubated the bacteria at 37°C overnight. In the morning of the assay, 0.5 ml of the overnight culture was added to 10mL of fresh BHI broth. The newly incubated broth was incubated at 37°C in an incubator for approximately 2 hours until the bacterial culture was in log-phase (optical density = 600 nm) then the culture was kept at room temperature until the bacteria were added to the top BHI agar (0.6% agar) for plague assay.[16]

**Phage preparation and in-vitro experiment**

Four sewage samples of 5ml were collected during November 2021 from each of four geographical locations including Bahrka, Farmnbaran neighborhoods, Korey, and Shaqlawa towns in Erbil district. To each of the sample tubes, 0.1 ml Chloroform was added and kept in the fridge until processing. On processing day, tubes containing sewage samples were centrifuged at 10,000 rpm for 5 minutes and filtered using sterile Batman filter paper. This was followed by mixing 250µl of the phage sample with 250µl of a freshly incubated bacterial sample (described above), 4 ml of top BHI supplemented with 0.6% agar at 45 °C and 500µl of sterile $CaCl_2$ then gently shaken for 30 seconds. The mixture was poured on the bottom BHI supplemented with 1.5% agar and left at room temperature to solidify then incubated at 37°C overnight. The plates were examined for plaque under a manual colony counter (IUL - Spain). Phage samples coded from 1 to 4 depending on the average plague size they could produce on the plates of *Salmonella typhi* (STPH1, STPH2, STPH3, and STPH4) and *Salmonella enteritidis* (SEPH1, SEPH2,

**Table 1** Biochemical tests were carried out to confirm the identification of *Salmonella typhi*

| No | Biochemical tests | Results |
|---|---|---|
| 1 | oxidase | Negative |
| 2 | Catalase | Positive |
| 3 | Reduce nitrate to nitrite | Positive |
| 4 | Growth on sole carbon source using citrate | Positive |
| 5 | Glucose fermentation and production of acid and gas | Positive |
| 6 | $H_2S$ production triple sugar iron | Positive |
| 7 | Hydrolyzing indole and urea. | Positive |

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SEPH3, and SEPH4). Where the sample numbers represented plague size ascendingly from the smallest plague in diameter ranging from an average of 1mm to the largest 3 mm. Progeny of one strain of "SEPH4" phage was prepared by selecting a single plague from the Farmanbaran sample and propagating on its *Salmonella enteritidis* hosts before starting an in-vivo trial.[16]

### Experimental infection of duck chicks

After granting ethical approval by the Human Research Ethics Committee (HREC) at Salahaddin University-Erbil (SUE), thirty-two commercial duck chicks of 1-day old were obtained from a local market in Erbil and divided into 4 groups of 8 isolated cages. To examine whether the duck chicks were free of Salmonella, all the chick groups were housed for 2 days and their droppings were daily cultured to confirm that they are not contaminated with Salmonella. After confirming the absence of Salmonella from droppings, on day three, the chicks were randomly divided into four groups (A, B, C, and D) using a 1.5 m2 cage leaving a 1.5 m distance between the cages. Three groups (B, C, and D) were artificially infected with *Salmonella enteritidis* by orally inoculating 1ml of the bacterial sample with a bacterial dose of 1*105 Colony Forming Unit (CFU) while group A was kept as an uninfected control group in which the chicks were administered an equal volume of sterile water. After the infection procedure, on a subsequent day, groups "B" and "C" were treated by orally inoculating chicks with 1ml of SEPH4 bacteriophage samples using 7 log10 PFU for group "B" and a higher dose of 12 log10 PFU for group "C". Group "D" was kept as untreated infected control and the chicks were orally administered with 1ml of sterile water. Live weight, photographs of head and beak, and mortality were recorded for all the birds on daily basis. Clean disposable plastic table sheets were placed under the cages every night to be able to collect fresh fecal samples. Every day starting from day 2 to 8, the total number of Salmonella was assessed using fecal samples pooled for each group. Briefly, the total count of Salmonella was assessed by taking 1gm of pooled fecal sample from each of the four groups and transferring it to 9ml of selenite F broth. Homogenized suspension of fecal samples (1gm weight per 9ml volume) prepared and serially diluted to 1/105 using the same broth medium. An amount of 0.1ml from each dilution was spread plated onto SSA and incubated under the aerobic condition at 37°C for 24 hours. Salmonella colonies were counted using a manual colony counter (IUL Spain). On day 9, all the chicks were euthanized and cecum content samples were taken for the enumeration of the total number of the bacterial host per Gram of cecum content as described above.[16, 17]

### Statistical Analysis

An ordinary One-Way ANOVA test was used to examine the differences in Salmonella CFUs in fecal samples among the 3 experimental groups. An average of three replicas of pooled fecal samples were taken for every day counted from day 1 to 8 for each of the three of infected groups and considered as dependent variables while bird groups as independent factors. CFU values were transformed to natural logarithms (Ln) to achieve normal distribution. Differences in the bacterial CFUs (dependent variables) of cecum contents among the three infected groups (independent variables) were examined at the last day of the experiment using ordinary One-Way ANOVA. All the statistical tests were performed in Prism biograph-7. *P* value <0.05 was considered statistically significant.

## Results

Clinical isolates of *Salmonella enteritidis* and *Salmonella typhi* were used as indicator hosts for lytic bacteriophage selection from wastewater samples. Straight after the collection, phage-containing samples were treated with

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chloroform to get rid of unwanted bacteria and other microbial backgrounds. After the enrichment step, plaque assay was used to investigate the existence of a virulent phage. Using *Salmonella enteritidis*, bacteriophages with a range of lytic activities were isolated from the four wastewater samples. The phage isolates significantly differed in their plague size, One-Way ANOVA, F (3, 36) = 24.36, *P* <0.001 (table 2 and figure 1A).

Bonferroni's multiple comparisons test showed that plague size of Farmanbaran's sample with a mean size of 3.16 mm, SD = 0.81 was significantly larger than the mean of other three samples including Bahrka, (mean = 2.3, SD = 0.46), Korey (mean = 1.5, SD = 0.38) and Shaqlawa (mean = 1.2, SD = 0.50). Depending on its lytic activity, a phage sample from Farmanbaran (SEPH4) was selected for an in-vivo study.

All four wastewater sources also contained *Salmonella typhi* phage with a large difference in their plague size, One-Way ANOVA, F (3, 36) = 103.1, *P* <0.001 (Table 2 and Figure 1B). Similarly, Farmanbaran's sample formed the most virulent phage with significantly larger plaque size (mean = 5.12, SD = 0.67) than Bahrka (mean = 2.03, SD = 0.5) and Shaqlawa (mean = 1.82, SD = 0.38) with *P* <0.001. The difference in the mean plague size between Farmanbaran and Korey (mean = 4.63, SD = 0.55) was not significant (*P* >0.5).

**Table 2** Plague size, plague morphology, and the initial concentration for 8 bacteriophage isolates from four locations in Erbil City

| Phage isolate | Location in Erbil | Mean plaque diameter in mm (SD) | *P* value | Plaque morphology | PFU/ml |
|---|---|---|---|---|---|
| SEPH1 | Shaqlawa | 1.2 (0.50) | <0.001 | clear | $6 * 10^2$ |
| SEPH2 | Korey | 1.51 (0.37) | <0.001 | clear | $5 * 10^2$ |
| SEPH3 | Bahrka | 2.3 (0.46) | <0.001 | turbid | $6 * 10^2$ |
| SEPH4 | Farmanbaran | 3.16 (0.81) | <0.001 | clear | $8 * 10^2$ |
| STPH1 | Shaqlawa | 1.82 (0.38) | <0.001 | turbid | $7 * 10^2$ |
| STPH2 | Bahrka | 2.03 (0.49) | <0.001 | turbid | $9 * 10^2$ |
| STPH3 | Korey | 4.63 (0.55) | <0.001 | clear | $3 * 10^2$ |
| STPH4 | Farmanbaran | 5.12 (0.67) | <0.001 | clear | $2 * 10^2$ |

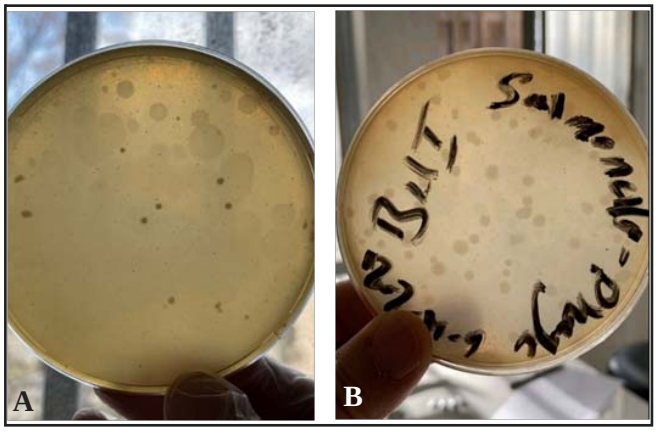


**Figure 1** Showing plaque formation by *Salmonella enteritidis* phage (A) and plague formation by *Salmonella typhi* phage (B) on BHI agar plates

https://doi.org/10.15218/zjms.2024.042

In the in-vivo experiment, pooled fecal samples from each of the four groups included A: uninfected control, B: infected and treated with a low dose of *Salmonella enteritidis* phage (7 log10 PFU), C: infected and treated with a high dose of *Salmonella enteritidis* phage (12 log10 PFU) and D: infected and kept as untreated control until the end of the experiment. No colonies of *Salmonella enteritidis* were detectable in fecal samples of Group A (uninfected control) and hence data of this group was excluded from all the subsequent analyses. There was a significant difference in the average burden of live Salmonella in the pooled fecal samples collected over 8 days of the three infected groups (B, C and D), One-Way ANOVA test ($P < 0.001$) (Figure 2). Bonferroni's multiple comparisons test showed that the untreated group D had significantly higher bacterial counts (total CFUs) in their pooled fecal samples than Group B which received 7 log10 PFU phage ($P = 0.001$) and Group C which received 12 log10 PFU ($P = 0.002$). Difference in the bacterial counts between the two treated Groups (B and C) was not significant ($P > 0.05$).

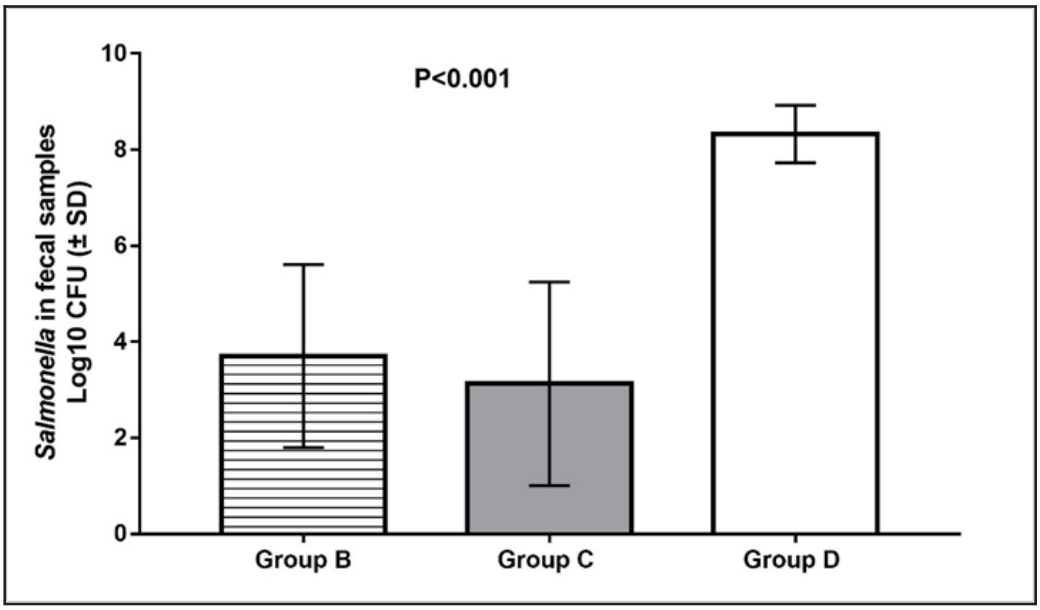


**Figure 2** Effect of phage administration on reducing *Salmonella enteritidis* counts in chick feces (total number of *Salmonella* per 1gm feces, mean of three duplicates combined± SD). Group B (lined bar) was treated with 7 log10 PFU and Group C (shaded) was treated with12 log10 PFU of *Salmonella enteritidis* phage against untreated control group D (plane bar).

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There was a large difference in total counts of Salmonella in the cecum contents between the treated and untreated groups, One-Way ANOVA test, (*P* <0.001) (figure 3). Bonferroni's multiple comparisons test showed no significant difference (*P* >0.05) in the bacterial counts of cecum contents between group B (treated with a lower dose of phage) and group C (treated with a higher dose of phage).

## Discussion

Salmonella is considered to be one of the most common foodborne pathogens affecting millions of people worldwide.[18] It has been estimated that at least 535 000 disease cases due to *S.enterica* occurred during 2017 which has led to over 77 500 deaths and the fatality cases are likely to increase with the continuously evolving antimicrobial resistance risks.[19] WHO 2015 report suggested that diarrhea caused mostly by non-typhoid Salmonella represents 70% of all foodborne diseases.[20] In this study, eight strains of Salmonella phages were isolated from four geographically separate locations, prepared and tested them against two subspecies of Salmonella including S. *typhi* in-vitro and the non-typhoidal S.*enteritidis* in-vitro and in-vivo. It was found that phage isolates from the Farmanbaran neighborhoods had the highest lytic efficacy against both subspecies of Salmonella. The most virulent phage strain was chosen and propagated in the lab before using it in an in-vivo trial using duck chicks as an animal model. Examination of fecal samples and cecum contents showed that phage therapy could significantly reduce Salmonella colonization in chicks in comparison to the untreated group of experimentally infected birds which consistently harbored a high burden of Salmonella.

Isolation and selection of the appropriate bacteriophage strain are critical to successfully treating bacterial infections.[21] In the present study, four separate locations were found to contain Salmonella phages, but the biological characteristics of the phage isolates greatly varied both within and between the locations. Phages with the largest plague diameter were more abundant in the wastewater sample collected from the Farmanbaran neighborhood. Generally, the existence of bacteriophage in an environment might

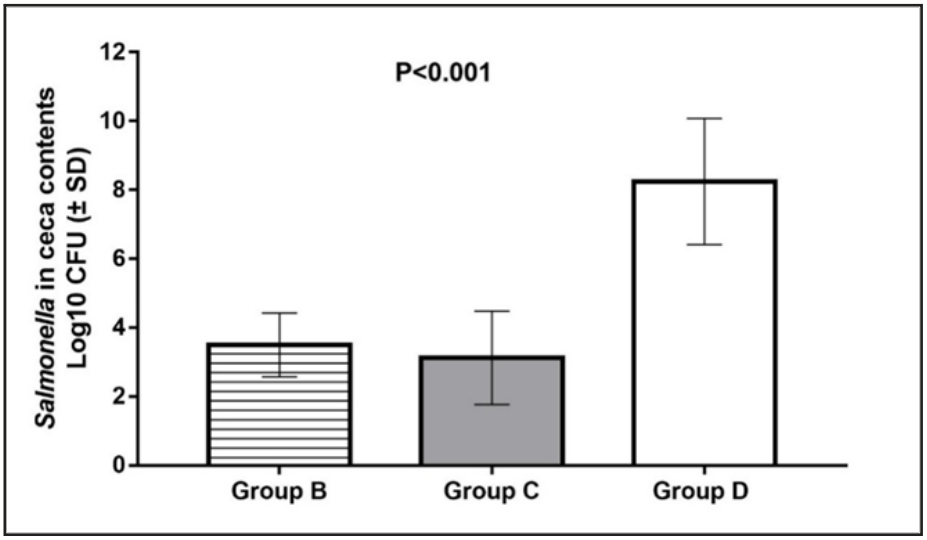


**Figure 3** *Salmonella enteritidis* counts in ceca contents (mean of the total number of Salmonella per 1gm cecum for 8replicas/group± SD). Group B (lined bar) was treated with 7 log10 PFU and Group C was treated with 12 log10 PFU (shaded bar) of *Salmonella enteritidis* phage against untreated control group D (plane bar).

https://doi.org/10.15218/zjms.2024.042

indicate the existence of its matching-host bacteria because phages are obligate parasites of bacteria and their abundance and virulence vary according to their bacterial hosts.[22] Therefore, it is likely that wastewater channel in Farmanbaran includes a higher abundance of Salmonella than Bahrka, Shaqlawa, and Korey. In fact, the Farmanbaran neighborhood is located inside the city of Erbil where there is a much higher population density than the other three locations. The molecular mechanism of lytic efficacy in bacteriophage can largely vary among isolates depending on their membrane protein called holin as the phage's functional group in lysing bacterial cells.[23]

The application of bacteriophages in medicines requires a good understanding of phage-bacteria interactions. After a successful in-vitro trial, the role of the most virulent phage strain in combating *S.enteritidis* using duck chicks for an in-vivo trial was examined. It has been found that poultry farming is one of the key factors associated with the spread of infections caused by *Salmonella enteritidis* in many countries in the world.[24] Although *Salmonella* burden in the fecal samples was reduced by the two bacteriophage doses (low: 7 Log10 PFU and high: 12 Log10 PFU) during the experimental period, the group which received a higher dose of the phage had a significantly lower number of Salmonella on the second-day post bacteriophage therapy. Similarly, it has been reported that oral administration of *Salmonella enteritidis* phage dose ranging $2\times10^9$ PFU can prevent Salmonella infection in mice.[25] On the second and third days after phage admiration, bacterial colonization was sharply dropped (below 2.5 log10) for both treated groups, but later, the bacterial count started to slightly increase to a more stable abundance (i.e. 3.5 log10). The evolution of phage resistance by bacteria is considered to be one of the limitations of phage therapy, but the development of a phage cocktail is thought to overcome this issue.[21]

The present study concluded that Salmonella counts in cecum contents were similar in the two phage-treated groups while the bacterial count in the cecum of untreated experimentally infected birds was significantly higher than in the treated groups. This might suggest that untreated infected poultry represents a serious risk of Salmonella infections. It has been reported that the burden of typhoid fever is very high in Iraqi Kurdistan.[2] and the prevalence of multidrug-resistant Salmonella serotypes is continuously increasing in poultry farms.[8] Therefore, the results of the current study suggest that the application of Salmonella phage as a biocontrol agent could be the best alternative to antibiotics to control the source of pathogenic Salmonella in the region.

## Conclusion

In this study, broad host lytic bacteriophage isolates from different locations in Erbil were isolated and their plaque morphology and lytic activities were characterized. The results revealed that the phage isolates could successfully control the growth of Salmonella both in-vitro and in-vivo indicating that this phage could be a key candidate for the biological control of Salmonella in poultry. Further studies are recommended to prepare bacteriophage cocktail with a broad spectrum of activity in order to be tested against diverse Salmonella species including MDR strains.

## Funding

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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