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Identification of a lytic bacteriophage against clinical isolates of *Salmonella typhimurium* in turkey poults

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Abstract

The poultry products are known as a source of zoonotic and multi-drug resistant pathogens, especially *Salmonella* spp. The objective of this study was using bacteriophages as an alternative anti-microbial agent against *Salmonella typhimurium* isolate from turkey poults. The antibiotic susceptibility test was used to identify the antibiotic resistance pattern of the isolates. The bacteriophage was purified, enhanced and titrated using the Spot test and double layer agar (DLA) techniques after being isolated from a chicken slaughterhouse and sewage treatment facility. By determining the morphological characteristics of resulting plaque, the specificity and host range of the phage were studied on *S. typhimurium* isolates. A total number of 22 suspected *Salmonella* isolates were confirmed biochemically positive in sample by cultures method. Nine of these isolates (40.90%) were identified as *S. typhimurium* by polymerase chain reaction. All of isolates (100%) were resistant to chloramphenicol, doxycycline, kanamycin, florfenicol, rifampin, and erythromycin. Seven isolates (77.77%) were resistant to amoxicillin and nalidixic acid. The plaques were present with 3.00 ± 0.22 mm in diameter on the culture of 6 out of 9 (66.66%) isolates of *S. typhimurium* on brain heart infusion broth using DLA method. The amount of phage titer was 7.60×10^7 phage forming unit mL^{-1} and its multiplicity of infection value was calculated as 5.06×10^{-2} based on obtained results. In place of antibiotics, the multi-drug resistant (MDR) *S. typhimurium* was successfully destroyed by the isolated bacteriophage from wastewater. *In vitro* settings were used in this investigation to identify the efficient bacteriophages against MDR *S. typhimurium*.

Introduction

A member of the Enterobacteriaceae family, the genus *Salmonella*, is well known as a foodborne pathogen. According to their antigenic characteristics, a huge variety of *Salmonella* serotypes with a diverse spectrum of animal and human reservoirs exhibit various problems. Beef, poultry meat, egg, and milk have been shown to carry *Salmonella* serotypes. Poultry is the main source of *Salmonella* and plays an extremely important role in the spread of this pathogen. *Salmonella* spp. have been identified as a significant human contamination source in poultry products.[1,2] Food contamination with *Salmonella* causes decreased productivity, pain, medical costs and finally death. Each year, non-typhoidal *Salmonella enterica* (NTS) causes 57,000 fatalities and around 153 million episodes of gastroenteritis worldwide.[3] Serotypes of *S. typhimurium* and *Salmonella enteritidis* are the most common causes of human salmonellosis in the majority of industrialized nations. The zoonotic potential and wide variety of hosts of *S. typhimurium* make it unique among *Salmonella* serovars.[2,4]

In addition to the disease-free intestinal carriage of *salmonella* serotypes by poults, infection of turkey poults within a few hours of hatching with a variety of serotypes can result in considerable morbidity and mortality depending on the strain involved. *Salmonella typhimurium* is one of the serotypes most isolated from poultry and, as was experimentally shown can be particularly virulent for turkey poults.[4-6]

Salmonella is becoming more and more resistant to "first-line" antibiotics globally. Salmonellosis poses a serious threat to both human and animal health, and the widespread misuse and overuse of antibiotics in poultry

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and animal husbandry is to blame for the global spread of multi drug resistant (MDR) of *S. enterica* serovars. This could result in a significant reduction in treatment options for both human and animal salmonellosis.[6,7]

Animal husbandry and the food business now utilize antibiotics, disinfectants, chemical preservatives and physical measures to prevent and control the spread of foodborne infections. The public health might be harmed by these drugs, bacterial drug resistance could emerge, or food quality could decline. Additionally, the use of chemical preservatives and antibiotics should be avoided due to the rising consumer demand for chicken products and the antibiotic residues found in such items. A straightforward, risk-free and affordable bio control method are also required to lessen the sources of pathogens in livestock and poultry, in addition to treating illnesses in people. Therefore, safe natural antibacterial options are needed. Lytic bacteriophages, which are viruses that attack and destroy bacteria, are the most promising of these natural antibacterial agents. Bacteriophages are common in the environment and do not damage helpful bacteria since they only target the host. They may also play a role in maintaining a healthy gut microbiota and reducing the host's bacterial population which makes them a source of natural antimicrobials that are inexpensive.[1,7] Hence, there are limitations for using the phages to control the infections. Lack of possible effect on intracellular bacteria, lack of proper penetration in the place of inflammation, stimulation of autoimmune diseases, their rapid elimination by the reticulo-endothelial system and not reaching the therapeutic level, the possibility of carrying resistance, virulence, toxins genes, role in general transduction, antibody production against phage antigens and bacterial resistance against phages are among the limitations and disadvantages of the use of phages.[8]

Also, bacteriophages may limit the usage of broad-spectrum antibiotics as part of an international effort to improve antibiotic monitoring or provide an appropriate treatment option in cases when organisms are resistant to front-line antibiotics. Studies on phage treatment in animals have shown that in certain circumstances it may aid in lowering bacterial population densities to levels that may enable the host immune system to build an effective defense and eradicate the illness.[1]

According to pre-slaughter research, feeding hens on a four-phage cocktail around 10^7 phage forming unit (PFU) g^{-1} for 14 days reduced *Salmonella* colonization in the caecum by 1.00 $\log_{10}$ colony forming unit g^{-1}. Similarly, when 5.00×10^9 colony forming unit (CFU) *S. typhimurium* and a 16-phage cocktail (5.00×10^9 PFU) were given together, the amount of *Salmonella* in the caecum, ileum and tonsils of weaned pigs was decreased by 2.00 - 3.00 $\log_{10}$ CFU g^{-1}.[2]

Salmonella bacteriophages may be isolated in the gastrointestinal tracts of animals and are extensively disseminated in soil, water, wastewater, animal and poultry husbandry litter, plants and septic systems.[1] Now, researches employing volunteer oral trials, animal simulation experiments and whole genome sequencing comparison analyses have reported the safety of the *Salmonella* bacteriophage in numerous nations including Russia, China, the United States and others. The findings indicate that the *Salmonella* bacteriophage is generally harmless. *Salmonella* phages have little impact on the other healthy bacteria in the body and are very sensitive and specific to this infection. Finally, phages are inexpensive, simple to make and easy to get. New lytic phages are screened and bred throughout a brief cycle that only require a single action. Additionally, they may be carried out in settings where it is simple to carry them before their application at normal temperatures.[1,9] This work included the isolation and characterization of a wide range lytic bacteriophage of *S. typhimurium*.

Materials and Methods

Sampling. From September 2019 to June 2021, a total number of 80 bronze breed turkey poults from 16 turkey husbandry in West Azerbaijan, Iran, with clinical signs of salmonellosis, were referred to the bacteriology laboratory and were examined. Liver, spleen gallbladder and intestine samples were aseptically collected from diseased turkey poults carcasses and immediately used for routine bacteriological examination.

Isolation of *Salmonella*. To isolate *Salmonella*, 1.00 g of the contents of the gastrointestinal tract and 1.00 mL of the contents of the gallbladder of turkey poults carcasses were prepared in aseptic conditions and placed separately in sterile tubes containing 10.00 mL of Selenite F broth medium (Quelab, Montreal, Canada) and incubated for 18 hr at 42.00 ˚C. Sub-cultures were thereafter made onto plates of XLD (Ibresco, Karaj, Iran) agar and the plates were incubated at 37.00 ˚C for 24 hr aerobically. *Salmonella*-typical colonies on the plates with black centers on xylose, lysin, deoxycholate (XLD) were cultured onto triple sugar iron (TSI) agar slants, Urea agar, Simmon citrate agar, sulfur indole motility (SIM) and inoculated in Indol, methyl red (MR), vogues- procure (VP), Glucose mediums (Quelab). Then they were incubated at 37.00 ˚C for 24 hr and confirmed.

***Salmonella typhimurium* confirmation by PCR.** The PCR technique was used to confirm *S. typhimurium* from other *Salmonella* isolates. All isolates were inoculated in tryptic soy broth (Quelab) and incubated with shaking at 200 rpm at 37.00 ˚C for 24 hr. For DNA extraction, a bacterial genomic DNA extraction kit (Yekta Tajhiz, Tehran, Iran) was used. The quality of the extracted DNA was assayed using a Nanodrop 2000c (Termo Fisher Scientific, Waltham, USA). The target gene and primers used in this study were determined based on the molecular

method of Ćwiek *et al*. Primer pair, forward (*SdiA1*-5-AATATCGCTTCGTACCAC-3) and reverse (*SdiA2*-5- GTAG-GTAAACGAGGAGCAG-3) which are specific for the *SdiA* gene of *S. typhimurium* with 273 bp, were used to amplify and identify this gene.[10] The PCR tubes with a final volume of 25.00 µL and a master mix (Sinaclon, Tehran, Iran), forward and reverse primers, depth water (Pishgam, Tehran, Iran) and, extracted DNA samples were used to amplify the *SdiA* gene by PCR reaction. Thermal cycles included a preliminary denaturation at 94.00 ˚C for 5 min, a second stage with 35 cycles, each of which consisted of denaturation at 94.00 ˚C for 30 sec, annealing at 52.00 ˚C for 40 sec, and elongation at 72.00 ˚C for 30 sec, followed by a final extension at 72.00 ˚C for 7 min. The PCR products were then electrophoresed in tris borate EDTA (Pishgam) buffer (0.50X, pH = 8.00) on agarose gel (Pishgam) using 10.00 µL of the PCR products and the bands were then captured on camera under UV light (Fig. 1).

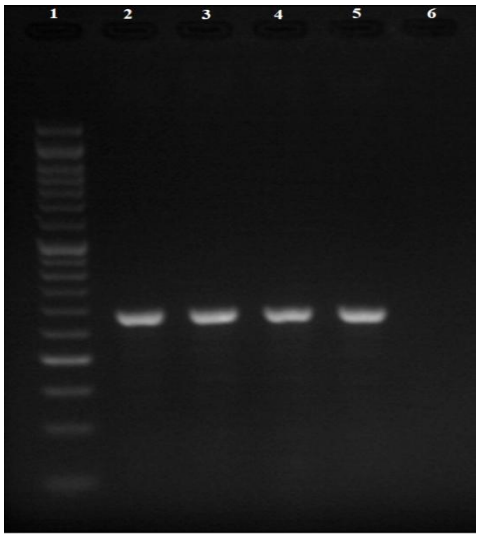

Fig. 1. Agarose gel image of amplified fragment of *Salmonella typhimurium SdiA* gene (273 bp) using PCR. Lane 1: 100-bp molecular ladder (Smobio Technology Inc., Hsinchu Taiwan). Lanes 2, 3 and 4: Positive samples for *S. typhimurium*. Lane 5: Positive control for *Salmonella* (Accession No. ON954830). Lane 6: Negative control.

Antimicrobial susceptibility test of *salmonella* isolates. The antibiogram test was performed based on clinical laboratory standard institute (CLSI) guidelines using the standard disk diffusion method on the Mueller-Hinton medium (Ibresco) to determine the antibiotic resistance of the isolates. Antibiotic susceptibility of isolates to chloramphenicol, doxycycline, erythromycin, kanamycin, enrofloxacin, nalidixic acid, gentamicin, rifampin, amoxicillin and florfenicol were evaluated. Isolates resistant to four antibiotics or more were considered as MDR bacteria.[11]

A standard suspension of bacteria with 1.50 × 10^8 CFU mL^{-1} was made to conduct the antibiotic susceptibility test. Spreading bacteria onto pellets containing Mueller-Hinton (Ibresco) agar medium was used to cultivate the bacteria which were then left at room temperature for 10 min. The necessary antibiotic discs were then positioned on the agar medium using sterile forceps at a spacing of 25.00 mm between each disc and the plate wall and 18 hr at 37.00 ˚C. The inhibitory zone was then measured using a caliper and assessed in accordance with the CLSI recommendations and documented. As a quality control strain, *Escherichia coli* ATCC 25922 were used (Fig. 2).[11]

Fig. 2. Antibiogram test on Mueller-Hinton medium showed multidrug-resistant isolates.

Phage isolation and enrichment. To isolate the bacteriophage effective against *S. typhimurium*, several untreated wastewater samples were taken from the septic tank of the poultry slaughterhouse of and sewage treatment plant in 500 mL sterile containers and quickly transported to the bacteriology laboratory on ice. To help the suspended sediments settle, the samples were kept at 4.00 ˚C for a whole night. The 50.00 mL of samples were centrifuged at 8,000 *g* for 10 min using the sources listed above. To get rid of the germs and particles, a 0.45 µM filter syringe was used to filter the supernatant. *Salmonella typhimurium* was grown overnight at 37.00 ˚C in 5.00 mL of BHI broth (Ibresco) to achieve pure bacterial cultures in the exponential phase, and then added to 45.00 mL of filtrated lysate for bacteriophage enrichment, separation and purification. In addition, the mixture received 5.00 mL of double-strengthened BHI broth (Ibresco) and 1.00 mM $MgSO_4$ (Merck, Darmestadt, Germany) before being incubated overnight at 37.00 ˚C with 200 rpm of shaking. To lyse the bacteria, 5.00% chloroform (Quelab) was added to the mixture, vortexed for 15 min and then allowed to sit at room temperature for an hr. Following an 8,000 × *g* centrifugation for 10 min, the samples were separated into the supernatants which were then filtered through a 0.22 µM filter syringe and kept at 4.00 ˚C as enriched phage.[12] Spot-test method was used to confirm the presence of phage in the filtered enriched lysate. To perform a Spot-test method, 100 µL of overnight bacterial culture with minimum inhibitory concentration (MIC) 1.50 × 10^8 CFU mL^{-1} was poured into a sterile tube containing 4.50 mL of molten BHI agar medium (Ibresco; containing 0.70% agar and 1.00 mM $MgSO_4$, kept at 48.50 ˚C) and vortexed for a few moments and immediately poured on

the bottom BHI agar (Ibresco) plates (containing 1.50% agar and 1.00 mM $MgSO_4$) and was spread with circular movements. The top layer of agar on the plate was allowed to solidify for 20 min at room temperature. The top agar was then spotted with 10.00 µL of the enriched lysate using a micropipette and it was incubated at 37.00 ˚C for an overnight period. After 24 hr plaques (circular clear zones that show phage presence and bacterial lysis) started to appear (Fig. 3).

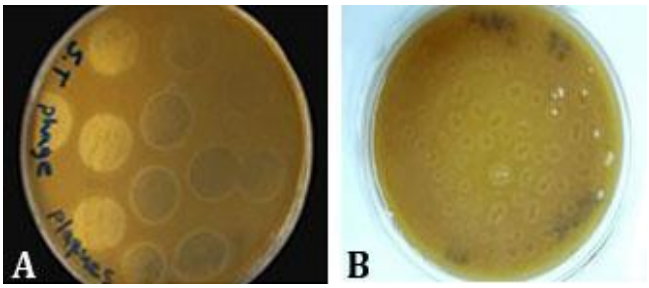

Fig. 3. A) A spot test of phage on *Salmonella typhimurium* lawn on BHI agar and **B)** The formation of plaques on the culture of *S. typhimurium* in the double layer agar method.

Phage purification. In order to purify isolated phages, Sambrook *et al*. method was used. A good isolated lytic plaque was quickly extracted from each plate using a sterile Pasteur pipette, then placed in a tube containing 5.00 mL of LB broth (Quelab), 50.00 µL of the host bacterium exponential phase culture, and shaken at 37.00 ˚C for 24 hr (150 rpm). Following a 24-hr incubation period, the lysate was centrifuged (10,000 *g* for 10 min at 4.00 ˚C), sterilized via a 0.22 µM filter and treated with 1.00% (v/v) chloroform to eliminate bacterial contamination. The filtrate lysate was then tested using the double layer agar (DLA; Quelab) technique to see whether it formed homogenous plaques. [12]

Phage concentration. For the concentration of the purified phage polyethylene glycol (PEG) sedimentation was used. Briefly, 10.00% (w/v) of PEG-8000 (Merck) was added to the supernatant and dissolved and then the suspension was incubated at 4.00 ˚C for 1 hr. 0.58 g of NaCl was added to 10.00 mL of phage suspension (final concentration: 1.00 M), stirred until completely dissolved and then placed on ice for 1 hr. The precipitate was diluted in 1.00 mL of SM buffer ($MgSO_4$,$7H_2O$ 2.00 g, NaCl 5.80 g, 50.00 mL l.00 mol L^{-1} Tris-HCl (pH 7.50; Pishgam), and 0.01% gelatin in 1.00 L.) and incubated at room temperature for an hr after bacteriophage precipitation was conducted by centrifugation (10,000 *g* for 10 min at 4.00 ˚C). An equivalent amount of chloroform was added to the solution, vortexed for 30 sec, then centrifuged (5,000 *g* for 15 min at 4.00 ˚C) to remove PEG and cell debris. The aqueous phase containing the bacteriophages was then collected.[12]

Phage titration. To ascertain the stock titer, after *S. typhymurium* had been activated in an LB medium (Quelab) and 100 µL of the suspension (with MIC 1.50 × 10^8 CFU mL^{-1}) was added. This mixture was then transferred to a plate and allowed 15 min to harden. Following the creation of the serial dilution from phage, 10.00 µL of the phage solution was put to the medium surface, spread out and incubated for 24 hr at 37.00 ˚C. The number of phages in a solution was assessed and the phage titer was calculated using a sample plate containing 30 to 300 countable plaques (Fig. 4). The following calculation was used to calculate how many phage particles were present in the suspension:

$$\text{Phage titer (PFU mL}^{-1}) = \frac{\text{Plaque count}}{\text{dilution factor} \times \text{aliquot volume}}$$

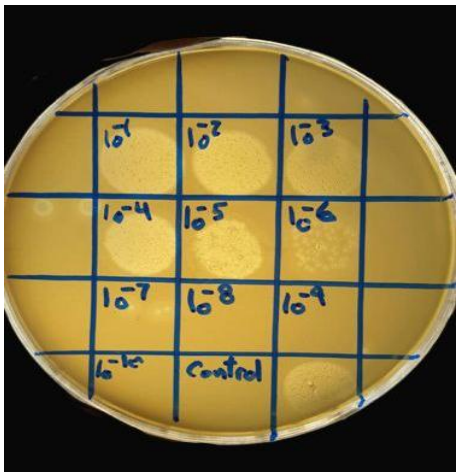

Fig. 4. Preparing serial dilutions of phage and using the Spot test method for titration.

Host range and specificity. Spot testing was used to assess the phage host range with six distinct Gram-positive and Gram-negative bacterial standard strains (*Streptococcus aureus*, *Proteus vulgaris*, *Pasteurella multocida*, *E. coli*, and *Klebsiella pneumonia*). Briefly, using the above-mentioned method, 4.00 mL of solid LB (bottom agar) was overlaid with 100 µL of bacterial culture and 4.00 mL of LB (top agar). After allowing the solid LB (bottom agar) to dry out, it was spotted with 10.00 µL droplets of serially diluted phage suspension from the existence of obvious lysis (+) or no-lysis (-) zones where the phage suspension was observed and bacterial sensitivity to the phage was deduced. The effectiveness of plating was assessed in bacteria where lysis zones were seen (Fig. 3).

Results

Isolation of *Salmonella* spp. was attempted from field samples (liver, gallbladder, spleen, ileum, and caecum) and was detected by morphological and bacteriological examination in 27.50% of 80 samples collected from 16 turkey poult farms in West Azerbaijan. Post-mortem examination of the collected samples from freshly dead birds showed nodular myocarditis, pericarditis and

hepatitis. The isolated bacteria were Gram-negative, non-spore-forming and short rod-shaped single or paired in an arrangement under the microscope.

Salmonella suspected isolates showed smooth red colored colonies with a black center on XLD agar. These isolates were subjected to further biochemical identification where suspected *Salmonella* isolates were positive for TSI, carbon utilization in Simmon's citrate agar but negative to Urea and Indole tests as in figure.[1] Results of biochemical identification revealed that 22 suspected *Salmonella* isolates were biochemically positive. Nine of these isolates (40.90%) were confirmed as *S. typhimurium* by PCR molecular method.

Based on the results of antimicrobial susceptibility tests that were performed for the isolates obtained as *S. typhimurium*, all of them (100%) were resistant to chloramphenicol, doxycycline, kanamycin, florfenicol, rifampin, and erythromycin. Seven isolates (77.77%) were resistant to amoxicillin and nalidixic acid. None of the isolates showed resistance to ciprofloxacin, gentamicin, sulfamethoxazole and trimethoprim. Therefore, based on these findings, the obtained isolates were considered as MDR.

Spot tests were used to check an isolated phage for *S. typhimurium* contamination. Due to the phage lytic activity, a clean zone over a bacterial lawn was seen, measuring around 16.00 mm in diameter. On DLA plates, this phage formed tiny, clear plaques with a similar shape that were around 3.00 ± 0.22 mm in diameter. Bacteriophage enrichment was carried out using the repeated plaque purification technique for further characterization.

The phages titration was determined using the DLA technique. After overnight incubation at 37.00 ˚C, plaques were counted and the isolated phage titer was found to be 7.60×10^7 PFU mL^{-1}.

The phage host range was determined using *S. typhimurium* strains isolated from turkey poults as well as standard strains of *S. aureus, Streptococcus viridians, P. vulgaris, P. multocida, E. coli,* and *K. pneumonia*. Six *S. typhimurium* isolates of a total of nine (66.66%), were sensitive to obtained phage with multiplicity of infection (The ratio of host cells and phages) equal to 5.06×10^{-2}, formed plaques and showed clear zones in the spot test. The plaques formed on the lawn culture of the other 3 (33.40%) *S. typhimurium* isolates were very weak. Phage was not effective on other mentioned used bacteria.

Discussion

Salmonella contamination in milk products, fruit juice, tomatoes, fish, shrimp, vegetables, and yeast has been reported. Among all food sources, poultry and poultry-associated products are commonly recognized as the most remarkable sources for human salmonellosis.[13,14]

Antimicrobials used in veterinary practice are considered a crucial factor in the emergence of drug-resistant *Salmonella*, and multidrug resistance was commonly seen in isolates from food animals rather than from human clinical cases.[14,15]

For the purpose of controlling *Salmonella* outbreaks, the amount of contamination and antibiotic resistance of the bacteria must be identified. Additionally, NTS is a serious issue for young turkeys and it causes a substantial yearly economic burden. The rise of bacterial strains that are resistant to antibiotics is seen to pose a serious threat to the chicken industry and to human health.[5,16]

Salmonella spp. were found in 27.50% (22/80) of the turkey poults in our investigation, which is higher than the findings of Sodagari *et al*. who found the bacteria in 21.60% of the poultry livers. *Salmonella* was found in samples of turkey liver and heart with a frequency of 8.60% and 06.70%, respectively, according to a different investigation conducted in Iran. Additionally, according to the study by Jahantigh *et al.*, 14.80% of turkeys tested were positive for *Salmonella*. Also, a prior research conducted in Iran indicated that the prevalence of *Salmonella* spp. infection in chicken liver, heart and gizzard were lower than that discovered in the current study, at 18.00%, 6.00%, and 4.00%, respectively. These figures and their comparison show that the frequency of this infection in poultry has significantly been increased in recent years.[5,15,17]

Naturally, it should be remembered that data on the prevalence of *Salmonella* in various studies is difficult to compare because variations observed between the reported *Salmonella* prevalence in prior investigations around the world may be caused by a number of factors, including different sampling methods, the age of the flocks sampled, isolation methods used to detect *Salmonella*, various breeds of birds, different climates, and etc.[17]

Salmonella contamination rates discovered in this research implied that chicken products might be the cause of human salmonellosis. Although the poultry sector has previously been exposed to good manufacturing practices and hazard analysis key control point principles, managing poultry production incorrectly might result in *Salmonella* being present.

In the current investigation, 40.90% (9/22) of the positive isolates were *S. typhimurium*, indicating that it was quite common compared to other *Salmonella* serotypes. The most common serotypes reported by public health labs in the United States, according to the centers for disease control and prevention (CDC), are *S. typhimurium*, *S. enteritidis*, and *Salmonella newport*.[9]

Therefore, it is crucial to control this serotype in particular in turkey poults and the products they produce. Studies on chickens conducted all around the globe have shown striking variances in *Salmonella* resistance to a broad variety of antimicrobial agents. All *S. typhimurium*

isolates in our investigation showed multi-drug resistance. *Salmonella* isolates multi-resistance profiles point to the need for efficient control measures and more responsible antibiotic usage which is a serious issue for the general public health.[5,11]

Salmonellosis therapy is complicated by the improper use of these antibiotics in veterinary medicine which results in the worrying resistance profiles of *Salmonella* isolates against widely used antibiotics.[15,16]

A large number of MDR isolates were found in our most recent experiment which was consistent with the findings of Oral *et al*. and Al *et al*. The deletion(s) in the phase II flagellin locus that prevent the expression of the second flagella antigen $FljB_8$, the genes linked to heavy metal resistance, and increasingly widespread MDR are key characteristics of this pandemic pathogen which was initially referred to as the European Clone due to its suggested origins.[3,15,18]

Since there is currently no effective vaccination against this infection, antibiotics are available therapy for gastroenteritis brought on by *Salmonella*. However, due to the rise of numerous antibiotic resistances in these bacteria, alternative or combination treatment strategies must be used to manage and limit the usage of antibiotics in order to address this worldwide concern. One of the best options is to use the biological control of this pathogen by lytic bacteriophages. There are many reasons for this because bacteriophages act specifically on the target host and are abundantly found in the environment, easy to prepare, a cheap bio control method, and based on studies, improve the immune system that usually do not cause adverse effects on the body. The immune-modulatory effects, the ability to prevent tumor development and the absence of horizontal gene transfer from phage lytic life cycle are some of the other salient features of phages[11,16] Lytic phages are preferred over lysogenic phages for managing bacterial infections because of their superior bacteriolytic activity and lack of capacity to transduce or transmit genetic material.[16]

Antibiotics have been found to be less stable across a range of pH, biotic habitats and ambient conditions than phages which are biological organisms. Along with stability, phages reproduce and create more infectious particles when the target bacterial pathogens are present providing ongoing anti-infective dose delivery (auto-dosing) at infection sites. Furthermore, phages do not affect other commensal microbiota while selectively eradicating target bacteria.[2]

Regarding the antibacterial properties of phages, considerable achievement has been attained in the field of animal husbandry. The present work was the first to identify and describe lytic phages against strains of *S. typhimurium* in turkey poults that are multidrug resistant. The successful separation of phages from human urban sewage samples and poultry slaughterhouse septic samples raised the likelihood of a tight connection between human and poultry *S. typhimurium* strains. Six of nine *S. typhimurium* isolates (66.66%) showed phage sensitivity, illustrating the remarkable specificity of phages in fighting different bacterial strains and serotypes.

According to a former research, phage activity varied depending on temperature and for the majority of the phages tested, it was somewhat quicker at 37.00 ˚C than at 25.00 ˚C. Although these findings showed enhanced phage activity at the ideal host development temperature, other authors have shown that this does not prevent phage application at low temperatures.[19] Therefore, in our study, the performance evaluation of the isolated phage was considered at 37.00 ˚C which was the optimum temperature for the growth of *Salmonella*.

The results of this study showed that phage isolated from the wastewater of poultry slaughterhouse and wastewater treatment plant with 0.01 MOI was sufficient to perform excellent lytic activity against 66.66% of MDR *S. typhimurium* isolates. These findings demonstrated amplified phage ability to stop *Salmonella* from growing at a given dose.

The reasons that the isolated phage was not able to produce complete and clear lysis in 33.40% of the isolated *S. typhimurium* could probably be due to the lack of specificity of the phage to these bacteria, low phage concentration or the presence of mechanisms to create resistance against phage in these *salmonella* isolates. The host cell surface and extra-cellular modifications such as receptor adaptations, outer membrane vesicles and quorum sensing as well as the intra-cellular modifications such as abortive infections, phage exclusion, RM systems and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems are what cause bacteria to develop phage resistance. Though phages have defense mechanisms and overcoming phage resistance is not an impossible challenge. Phages that have the capacity to pick up novel receptors may alter the proteins that bind to those receptors. Therefore, phages can detect changes in receptor structure and prevent disruptions in phage adsorption receptors when a host receptor transforms into a mutant form.[16,20]

Additionally, phage-infected bacteria with CRISPR-Cas immunity do not survive, yet, since phage replication is decreased as a result of this system and the bacterial population is partly protected.[20]

On the other hand, when a lytic phage enters a host cell, it multiplies and lyses the cell there that is an outburst of viral particles. Thus, with every host cell lysis, there is an increase in the phage numbers. However, a high titer of phage is sufficient to lyse bacteria without multiplication. In lysis without multiplication, phages adsorb to the surface of the host cell at high MOI (> 100) and puncture the host cell at multiple cell surface regions leading to host death.[13,14] Even in the presence of CRISPR/Cas, phage

infection results in considerable decreases in both membrane integrity and cellular metabolism.[20]

Consequently, it is necessary to choose phages with a wide host range in order to create efficient phage treatment cocktails. Candidates for a phage must be virulent and able to spread through the lytic cycle within the host. An optimal phage cocktail, according to worldwide specialists, should include phages from several families, have the best adsorption capacity and be resilient to a variety of physicochemical circumstances. Phages expressing integrase or recombinase as well as virulence or anti-microbial resistance genes are not the best candidates for effective therapeutic applications.[16] The study by Lu *et al.* concluded that the phages isolated from turkeys were effective on various *Salmonella* serovars commonly associated with foodborne Salmonellosis.[21]

The results of the present investigation supported the possibility that phages might be useful in treating *S. typhimurium* infections in turkey poults when antibiotics were ineffective owing to the establishment of multidrug-resistant strains. In conclusion**,** bacteriophage-based treatment may be used to prevent *salmonella* infection in turkey poults and has the potential to take the place of antibiotics and chemical agents in the effort to provide healthier food for people and healthy flocks of turkeys.

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Conflict of interest

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

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