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Major Article

Phage Therapy as an Approach to Control *Salmonella enterica* serotype Enteritidis Infection in Mice

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Abstract

Introduction: *Salmonella enterica* serotype Enteritidis (*S.* Enteritidis) is a cause of food-borne human illness. Given the prevalence of antibiotic resistance of *Salmonella* Enteritidis and the lack of antibiotic efficacy in future years, its replacement with other agents is necessary. One of the most useful agents is bacteriophages. **Methods**: *S.* Enteritidis was identified using a multiplex polymerase chain reaction assay. The effective bacteriophages were isolated from hospital wastewater samples. The effects of the bacteriophages were evaluated both *in vitro* and *in vivo*. **Results**: The phage SE20 belonged to the Podoviridae family, and the genome size was 40 kb. The evaluation of phage SE20 at variable pH ranges showed its susceptibility to pH < 3 and pH > 12. The animal model showed that mice infected with *S.* Enteritidis developed hepatomegaly and splenomegaly, but did not experience gastrointestinal complications after receiving the bacteriophages. **Conclusions**: The results of this study suggest that phage SE20 is a promising candidate for controlling salmonellosis caused by *Salmonella* Enteritidis.

Keywords: *Salmonella enterica* serotype Enteritidis. Phage SE20. Pulsed-field gel electrophoresis. Mice. Sewage.

INTRODUCTION

Foodborne diseases are one of the most important economic and health problems in industrialized and developing countries. In recent years, *Salmonella* has been one of the most common causes of foodborne diseases[1]. Salmonellosis is an infectious disease that is often transmitted through contaminated food, especially food products derived from meat, chicken, eggs, animal feed, milk, and sometimes vegetables[2,3]. However, estimating the prevalence of salmonellosis is challenging in developing countries, as no comprehensive research has been conducted in this area[4-8]. In addition, non-typhoidal salmonellosis is a disease caused by *Salmonella* serotypes other than Typhi, Paratyphi A, Paratyphi B, and Paratyphi C. Numerous studies have been carried out globally on the prevalence of *Salmonella* serotypes in different regions[9-11]. *Salmonella enterica* subsp. *enterica* serovar Enteritidis and Typhimurium are two common serotypes that have been identified in Iran and other countries[12-15].

Over the past two decades, the prevalence of antibiotic-resistant *Salmonella* has become a serious global challenge. Moreover, the extensive use of antibiotics, for instance, as a standard component of domestic animal feed, has led to antibiotic resistance[16]. In recent years, multidrug-resistant (MDR) strains have become major global concerns, and many studies in Iran and other countries have reported the high resistance of *Salmonella* strains to several antibiotics[9,22]. Antibiotics are among the most commonly used drugs in human medicine and are used unnecessarily for up to 50% of

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patients[17,18]. These agents are also used in animal feed to prevent, control, and treat diseases and improve animal growth[17,18].

In contrast, phages are viruses that can infect bacteria. The most important characteristic of lytic phages is their specific host range. In other words, they only kill strains of a specific bacterium; therefore, they are recognized as strong antimicrobial agents. Unlike broad-spectrum antibiotics and regardless of the target specificity, phage therapy has some other advantages, such as its minimum side effects, phage multiplication, site-specificity (concentration of phages at the infection site), and singular prescription[19,20].

Therefore, this study aimed to isolate an effective titer of phages against *Salmonella* Enteritidis and determine the phage characteristics. In addition, the use of a proper dose for the treatment of infection was investigated in an animal model.

METHODS

S. Enteritidis was isolated from 46 salmonellosis outbreak samples. After the primary diagnosis via biochemical tests, bacterial cells were cultured on Hektoen enteric agar (HE) medium and incubated at 37°C. After 18 hours, several colonies were inoculated in tryptic soy broth (TSB) medium (Difco Laboratories, Detroit, MI, USA). After three hours of incubation, the colonies were then stored at −20°C in the presence of 30% glycerol.

A multiplex-PCR assay was used to determine *Salmonella* serotypes. The reference primer used has been described elsewhere[21]. The amplification reaction tests were performed using a PEQLAB thermocycler (Germany) at a final volume of 25 µL, containing 1X PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 9), 2.5 mmol/L of $MgCl_2$, 0.2 mmol/L of each primer, 1 U of Taq DNA polymerase, and 2 µL of sample DNA. The PCR conditions for amplification were as follows: five minutes of initial denaturation at 95°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, an extension at 72°C for 60 seconds, and a final extension at 72°C for two minutes. *S.* Enteritidis ATCC 13076 was used as the positive control[22,23].

Twenty samples from municipal wastewater and two samples from hospital wastewater were collected and sent to the laboratory immediately. The samples were stored at 4°C for 24 hours to precipitate their suspended solids. Then, 15 mL of the samples were centrifuged at 5000 g for 10 minutes to remove the bacterial cells. Next, the supernatant was passed through a 0.22 µm membrane filter (Millipore, USA). In order to enrich the bacteriophage, the supernatant was added to an equal volume of brain-heart infusion (BHI) broth, containing 10^6 CFU/mL of *S.* Enteritidis (*Salmonella* isolated from outbreaks), and incubated at 37°C for 24 hours. The broth culture was centrifuged at 5000 g for 10 minutes. Next, the supernatant was again passed through a 0.22 µm filter. The double-layer agar (DLA) method was used to confirm the presence of lytic phage in the supernatant[23]. Briefly, 50 µL of the filtered liquid was added to 450 µL of log-phase *S.* Enteritidis, incubated at 37°C for 15 minutes, and shaken at 120 rpm. After pouring the solution into 4.5 mL of 0.7% BHI agar, it was vortexed rapidly, added to 1.5% BHI agar, stored at room temperature (22°C) for 30 minutes, and finally incubated at 37°C for 20 hours.

To purify the phages SE20, the agar containing the phage plaque was cut using a sterile scalpel. The plaque was then removed using a Pasteur glass pipette, added to the *S.* Enteritidis broth suspension (1×10^6 CFU/mL), and incubated at 37°C for 24 hours. Afterward, the suspension was centrifuged at 4500 g for 10 minutes and passed through a 0.22 µm filter. The DLA method was performed three times until pure plaques were obtained. The bacteriophage was then purified by adding 5 mL of SM buffer (For 1 liter: 50 mL, 1 M Tris-HCl [pH 7.5]; 5.8 g, NaCl; 2 g, $MgSO_4.7H_2O$; 5 mL, 2% gelatin) to the plate. Next, the bacteriophage was incubated and shaken at room temperature (22°C) for four hours. Finally, the buffer containing the bacteriophages was removed from the plate, centrifuged at 4500 g for 10 minutes, and passed through a 0.22 µm filter.

To determine the size and morphology of the phages, the isolated phage SE20 was tested via transmission electron microscopy (TEM; Zeiss-Em10c-80k). For this purpose, copper-coated carbon was used, and 50 µL of freshly purified phage pellets suspended in SM buffer, was added. The phages were stained with 2% uranyl acetate and detected by TEM at an acceleration voltage of 80 kV.

Some phage infections create lysogens and do not result in a lytic infection. Because only lytic phages can be used in the treatment process, lysogens were removed by spreading phage-infected cultures of the host bacteria (100 ml) onto Luria-Bertani Miller (LB) agar plates containing kanamycin (50 µg ml^{-1})[35]. The bacteriophage host range was determined using the SPOT test[24]. Different bacteria were selected to determine the bacteriophage SE20 specificity, including *Yersinia enterocolitica* (ATCC9610), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Staphylococcus aureus* (ATCC2392), *Enterococcus faecalis* (ATCC29212), *Shigella flexneri* (ATCC12022), *Listeria monocytogenes* (ATCC19114), *Cronobacter sakazakii* (ATCC29544), and *Salmonella* species. The bacteria were cultured overnight in LB broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 mL of 2 N NaOH per liter). Next, 100 µL of culture and 4 mL of 0.7% BHI were vortexed and covered with 1.5% BHI agar. Then, 10 µL of the bacteriophage suspension was added to the surface of the agar, which was then incubated at 37°C overnight. In addition, sensitivity to bacteriophages was determined by measuring the clear zone. All experiments were performed in triplicate.

To determine phage sensitivity to temperature, the aliquots of phage SE20 suspensions were incubated in titers of 1×10^8 PFU/mL at 4°C, 25°C, 37°C, 50°C, 60°C, and 70°C (pH 7), respectively, for 60 minutes. Then, the phage titer was tested using the DLA method.

To determine phage sensitivity to pH, the aliquots of phage SE20 suspensions were incubated in titers of 1×10^8 PFU/mL at 4°C (pH 3-12) for 60 minutes. Then, the phage titer was tested according to the DLA method.

To conduct the one-step growth test, a modified version of the Ellis and Delbruck method[25] was used. Briefly, phage SE20 was added to the liquid culture medium containing *S.* Enteritidis with a multiplicity of infection (MOI) of 0.1. It was then incubated at 37°C for 15 minutes to induce phage adsorption by bacteria. Then, it was centrifuged at 12000 g for 10 minutes to remove the unbound phage particles. In the next stage, the supernatant was removed, and the pellet was added to LB broth. It was resuspended in 100 mL of LB medium and incubated at 37°C with shaking. The aliquots were removed at 10-minute intervals for 100 minutes, and the phage titer was determined using the DLA method. All experiments were repeated in triplicate.

To determine the effect of isolated phage on *Salmonella* Enteritidis *in vitro*, the aliquots of *S.* Enteritidis were added to fresh LB broth and incubated at 37°C while shaking for six hours at 150 rpm. Then, the bacteria were poured into four tubes (2×10^9 CFU/mL). The phage SE20 (2×10^8, 2×10^9, and 2×10^{10} PFU/mL) was also added to the four tubes. In addition, phosphate-buffered saline (PBS) was added to the fourth tube as the control. Bacterial growth was monitored by measuring the OD600 at 30-minute intervals.

PFGE was used to determine the size of genomes, according to the Erika Linghor method[26] Briefly, the phage SE20 suspension was dialyzed, and the cassette plug was assembled. To prepare the plug agarose, 0.5 mL of the phage suspension was used. After it was placed in a 54°C water bath, 400 µL of the purified phage were transferred to microcentrifugal tubes, which were then heated to 50°C in a temperature-controlled bath. Next, 400 µL of molten agarose plaque was added to a heated microtube containing the phage and mixed thoroughly via pipetting. The mixture (250 µL) was immediately added to the wells of the appropriate plug-in cassette. The plug was stored at 4°C for 15 minutes until rigidity. The plug was transferred into a tube containing 5 mL of phage lysis buffer (50 mM Tris, 50 mM EDTA, and 1% SDS w/v) and 25 µL of Proteinase K (20 mg/mL) and placed in a hot-water bath at 54°C for two hours with shaking. Then, the buffer was aspirated and added to the tube containing the plug and 5 mL of sterilized 1X TE buffer (10 mM Tris and 1 mM EDTA, pH= 8.0), followed by incubation at 54°C for 15 minutes. The plug was stored at 4°C and analyzed using a PFGE system (CHEF DRIII Chiller, BioRad, USA) at 6 v/cm for 20 hours with an incremental pulse of 2.2-54.2 s at 14°C. The gel was stained with 1X ethidium bromide solution and analyzed after washing with deionized water under UV light.

To evaluate the effect of phage SE20 on Salmonellosis, all the procedures involving animals were approved by the Animal Ethics Committee of Tehran University of Medical Sciences (Ethics Approval Code: IR.TUMS.REC.1394.2090). Five-week-old female BALB/c mice (mean weight, 20 g) were purchased from the Faculty of Pharmacology, Tehran University of Medical Science, and then weighed. The animal tests were carried out in accordance with the recommendations of the Ethics Committee of Tehran University of Medical Sciences. *Salmonellosis model:* Twenty-four mice were divided into three groups; the control group A, 200 µL of PBS was gavaged; group B, 200 µL of *S.* Enteritidis (1.5×10^7 CFU/mL) was gavaged; group C, 200 µL (1.5×10^7 CFU/mL) of *S.* Enteritidis was gavaged on the first day, and a single dose of phage SE20 was gavaged (2×10^8 PFU/mL) after 24 hours. To prevent damage to the phages in the digestive tract of the animal, anti-acid was gavaged.

Histopathology of the liver and spleen: After six days of infection, four mice from each group were anesthetized. Next, the spleen and liver were removed and fixed in 10% formalin for 24 hours at the refrigerated temperature (6°C). The samples were then sent to the Pathology Department of the Parsian Hospital in Tehran for hematoxylin and eosin (H&E) staining.

Data were analyzed in SPSS version 22 and GraphPad Prism version 6. For analyzing the results, the sphericity assumption test, Greenhouse-Geisser test, Levene's test, Mauchly's test, and posthoc tests were performed.

RESULTS

The Multiplex-PCR assay was used to confirm the serotypes of *S.* Enteritidis. The serotypes were isolated from five samples. One of the samples was randomly selected for the subsequent stages.

A lytic phage against *S.* Enteritidis was isolated from the hospital wastewater and purified. The size of the plaque was 2-3 mm. The TEM images showed that the phage had a head (about 64±3 nanometers) and a very small tail, which was hardly visible. The morphology showed that this phage belonged to the Podoviridae family (**Figure 1**).

The size of the bacteriophage genome was determined based on the PFGE method. The ladder used in this study was *S. enterica* subsp. *enterica* serovar Braenderup (ATCC BAA-664TM). Based on the results of this ladder, the genome size was 40 kb.

The SPOT test was used to determine the specificity of the bacteriophage. The results showed that the phage could infect *S.* Enteritidis and *S.* Typhimurium, which were sensitive to the phage. However, no lytic effect was observed in other bacteria. It was concluded that *S.* Enteritidis and *S.* Typhimurium have a common receptor for this phage, and the receptor was not present in the other tested bacteria.

Evaluation of the phage and its activity *in vitro* showed that pH plays an important role in its function. The results in a pH range of 3-12 showed that the phage was susceptible to pH < 3 and pH > 12 and was stable at a pH of 3-11. The optimal pH for this phage was 6-8. The results of the phage test at various temperatures (pH 7) showed that the phage was stable up to 60°C. However, the highest level of stability was reported at 4°C. On the other hand, the phage lost its stability at 70°C (**Figure 2**). The single-stage growth curve showed that the phage had a latent period of 30 minutes; after that, the number of phage particles in the medium increased rapidly, indicating that the bacteria were lysed, and viruses were released into the environment.

To determine the phage capacity to lyse the host bacteria *in vitro*, the bacteriophage titers (2×10^8, 2×10^9, and 2×10^{10} PFU/

mL) were added to four tubes to evaluate the host bacterial growth in the presence of the phage. The isolated bacteriophage showed a latent period of about 30 minutes. The burst time occurred between the 30th and 80th minutes. The differences between the groups gradually became insignificant after six hours of incubation. The optical density of the phage-inoculated groups was much lower than that of the control group (**Figure 3**).

In this study, a total of 24 mice were examined. The mice were infected with 1.5×10^7 CFU/mL of *S.* Enteritidis via oral gavage. The mice grew well in the control group (group A), whereas group B showed symptoms after the infection, including a significant weight loss within 14 days (p-value < 0.005). In this group, the appearance of the mouse hair also changed. The bacterial count was determined from stool and liver samples. The average number of *S.* Enteritidis was 1.63×10^8 CFU/mL in the stool sample (1 g) and 2.1×10^7 CFU/mL in the liver sample (1 g). The mice in group B as a result of infection with bacteria died at the end of the study period. Hepatomegaly and splenomegaly were observed after the clinical evaluation of the liver and spleen. Abscesses were also observed in the pathology of liver tissues (**Figure 4**). The size of the spleen was approximately three times larger in group B than in group A. Twenty-four hours after infecting the animals in group C, the specific bacteriophage with an MOI of 10 was gavaged. In

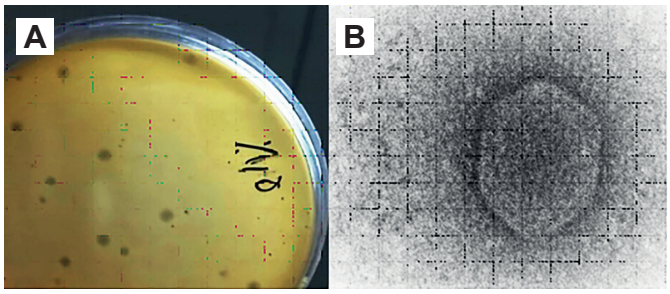

FIGURE 1: (A) A bacteriophage plaque and **(B)** electron micrograph of the isolated phage.

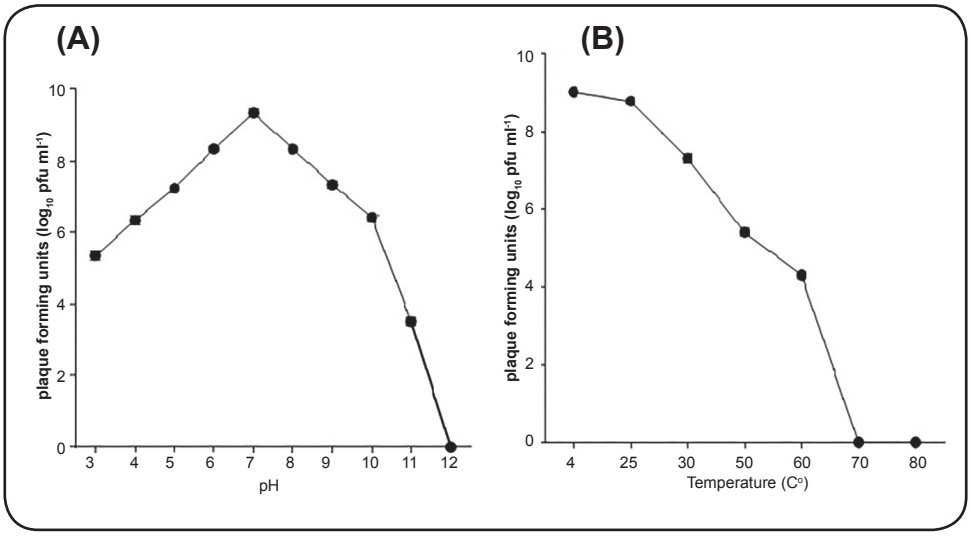

FIGURE 2: The stability of the specific bacteriophages under different **(A)** pH and **(B)** temperature conditions.

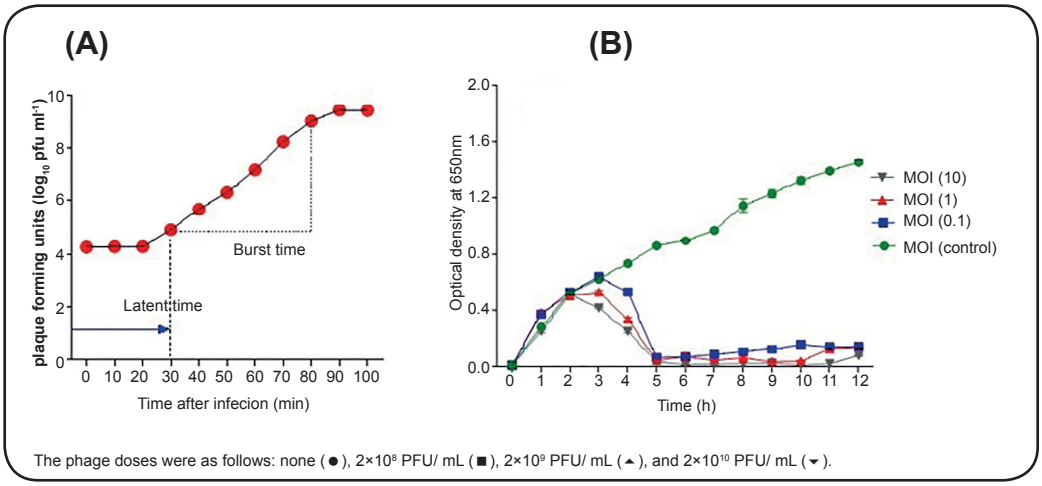

FIGURE 3: (A) The one-step growth curve of the *S.* Enteritidis bacteriophage. The isolated bacteriophage showed a latent period of about 30 minutes. The burst time occurred between the 30th and 80th minutes. **(B)** The function of the isolated lytic phage *in vitro*. *S.* Enteritidis (2×10^9 PFU/mL) was infected with different phage MOIs. The optical density was also measured for each sample.

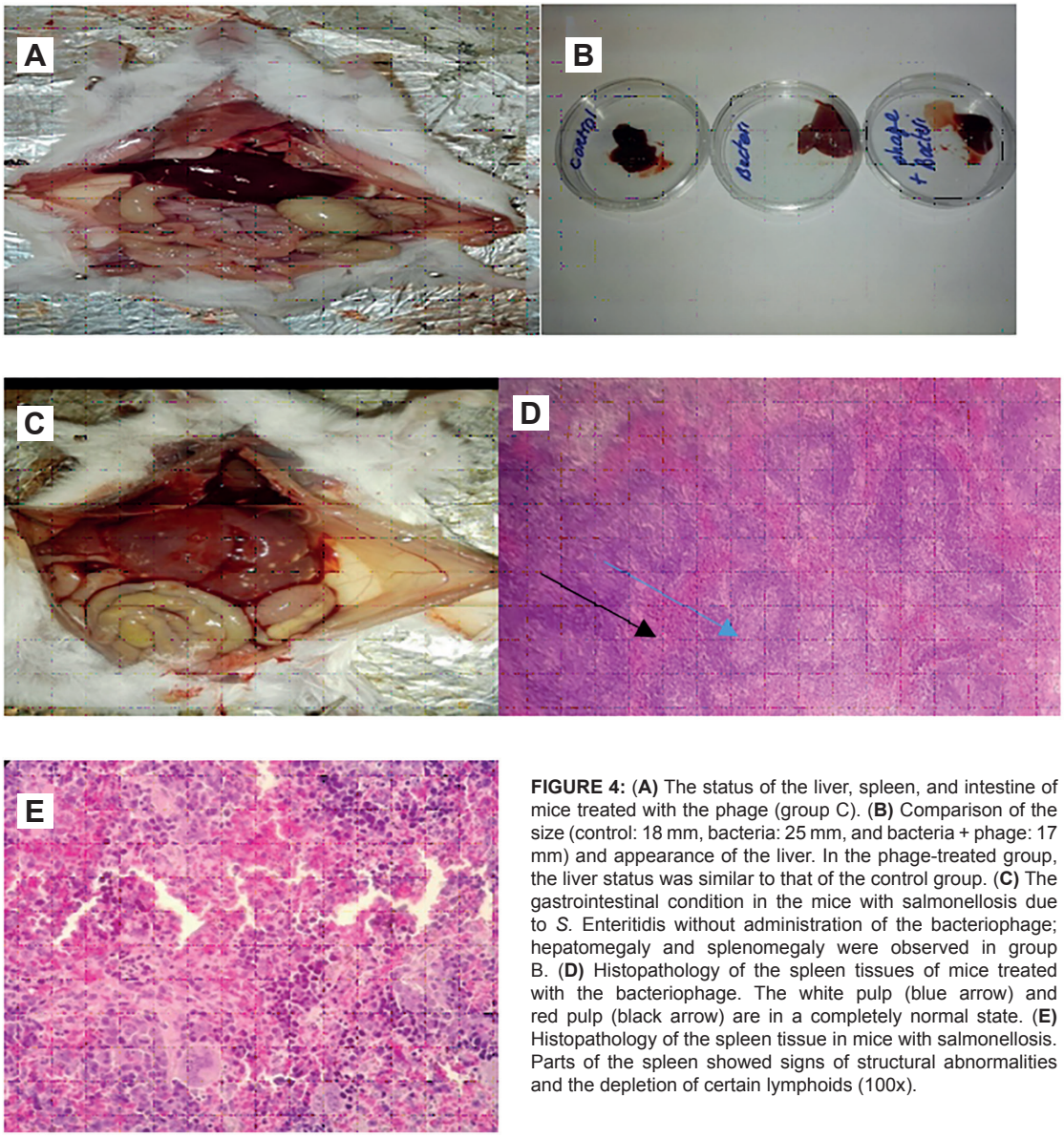

FIGURE 4: (**A)** The status of the liver, spleen, and intestine of mice treated with the phage (group C). (**B)** Comparison of the size (control: 18 mm, bacteria: 25 mm, and bacteria + phage: 17 mm) and appearance of the liver. In the phage-treated group, the liver status was similar to that of the control group. (**C)** The gastrointestinal condition in the mice with salmonellosis due to *S.* Enteritidis without administration of the bacteriophage; hepatomegaly and splenomegaly were observed in group B. (**D)** Histopathology of the spleen tissues of mice treated with the bacteriophage. The white pulp (blue arrow) and red pulp (black arrow) are in a completely normal state. (**E)** Histopathology of the spleen tissue in mice with salmonellosis. Parts of the spleen showed signs of structural abnormalities and the depletion of certain lymphoids (100x).

this group, the animals grew well and showed normal activity similar to the mice in group A. The appearance of their hair was completely normal, and no cases of death were reported. Moreover, a bacterial count of the liver was performed on the 7th and 10th days, and *S.* Enteritidis was not isolated. After autopsy, the clinical findings of the spleen and liver did not show any pathological changes under normal and pathological conditions.

DISCUSSION

There are over 2300 known serotypes of *S. enterica* with remarkable differences in their pathogenicity[27,28]. Considering the high prevalence of antibiotic resistance in *S. enterica* isolates, third-generation fluoroquinolones and cephalosporins are used as antibiotics to treat severe forms of *S. enterica* infection[29]. Therefore, it is necessary to replace antibiotics with other antimicrobials. Bacteriophages are a suitable alternative to antibiotics. In a report published by the National Institute of Allergy and Infectious Diseases in 2014, phage therapy was recognized as one of the seven methods integrated in a coordinated approach to address the threats of antibacterial resistance; this highlights the importance of phage therapy as an effective tool for combating resistant bacteria[30,31].

In this study, we detected a bacteriophage that could adequately control the growth of *S.* Enteritidis, showed intermediate resistance to ciprofloxacin *in vitro*, and prevented complications caused by *Salmonella* infection in an animal model. In this study, the isolated bacteriophage was evaluated to determine the proper hosts and their sensitivity to the phage among different bacteria, including *Salmonella* genera (*S.* Typhimurium, *S.* Enteritidis, and *S.* Infantis) and non-*Salmonella* genera (*Y. enterocolitica*, *E. coli*, *P. aeruginosa,* and *S. aureus*). It was shown that *S.* T*yphimurium* and *S.* Enteritidis were susceptible to this bacteriophage, while other bacteria did

not show any sensitivity. In another study, after determining *Salmonella* as the host for bacteriophage iEPS5, no growth zone was observed around the colonies of some *S.* Typhimurium strains. Moreover, in some strains, the lack of growth was observed with more opacity. Some strains of *S.* Enteritidis were susceptible to this phage; *S. paratyphoid* A was also susceptible to this phage. However, other species and genera, such as *S.* Typhi*, S.* Paratyphi B, *S.* Paratyphi C, *S.* Dublin, *E. coli*, *Shigella boydii*, *Vibrio fischeri*, *P. aeruginosa*, *C. sakazakii*, *E. faecalis*, *S. aureus*, *S. epidermidis*, *Bacillus subtilis*, *B. cereus*, and *L. monocytogenes,* were not reported; this indicates the specificity of this bacteriophage in some serotypes of *Salmonella*[24].

One of the objectives of this study was to evaluate the phage function at different temperatures. Therefore, the function of the isolated bacteriophage was evaluated at 4°C, 25°C, 37°C, 50°C, 60°C, 70°C, and 80°C. A temperature of 4°C was considered optimal for the survival of this bacteriophage, which is lower than the ambient temperature. The bacteriophage also survived at 37°C and in a temperature range of 50-70°C, although its count decreased significantly, and it was killed at 80-90°C. In another study, an isolated bacteriophage was evaluated for its sensitivity to different temperatures in the range of 50-90°C. The bacteriophage survived at 50-70°C, and its count decreased gradually with temperature; in other words, no bacteriophage was detected at 80-90°C[32].

The first step of phage infection is the attachment of phage receptors to the surface of a susceptible bacterial cell. In this study, an MOI of 0.1 was used to study the growth curve for 100 minutes.

A phage count was performed every 10 minutes. The growth curve showed that the number of bacteriophages exponentially expanded from the 20th minute. Therefore, the virus was absorbed by the bacteria in the first 20 minutes. From the 70th to 100th minutes, the phage count reached almost a constant level. In another study, to determine the absorption of phages by bacterial cells, the cells were cultured in a nutrient broth to reach the exponential phase. Then, these cells were contaminated with SAL-PG phage and placed at room temperature) 25°C). The samples were centrifuged every five minutes for 40 minutes. The supernatant was evaluated for titration of the absorbed phages. According to this study, after five minutes, more than 50% of the phage was absorbed by the bacterial cells, and 95% was absorbed within 15 minutes[33].

In another previous study, the effect of a bacteriophage was evaluated on the control of *S.* Enteritidis. The results showed that the isolated phage controlled the pathogenicity of bacteria, and *S.* Enteritidis was not isolated from the animal stool after phage administration[23]. In addition, Coombes et al. infected mice with *S.* Typhimurium at doses of 10^6 to 10^8 CFU/mL. After being infected, the mice showed decreased activity and weight. In the pathological study, meningitis and thrombosis were diagnosed; inflammation was also observed in the subarachnoid space of the brain[34]. In the present study, mice in group B that were infected with *S.* Enteritidis had softer feces on the third day and experienced slight diarrhea. A substantial decrease was observed in the animals' activity, as they remained immobile in the cage for a long time and lost weight. Finally, seven mice died on the 12th day. In this group, the observed signs included hepatomegaly, splenomegaly, signs of meningitis, turgidity, and intestinal dilatation. In addition, liver necrosis and abscess were observed.

In conclusion, the results of this study suggest that oral phage administration is effective in the treatment of salmonellosis and other infections caused by *S.* Enteritidis. The safety of the phage and its activity against biofilm formation need to be evaluated in future studies.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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