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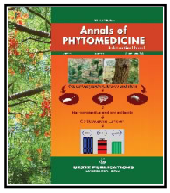

Original Article : Open Access

Isolation and characterization of a novel *Salmonella* polyvalent bacteriophage 'MediPhag' in Uzbekistan

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Abstract

Salmonella are pathogenic microorganisms that are transmitted through food. The use of *Salmonella* phages as biocontrol agents in modern medicine, has aroused great interest in recent years. Due to the high biodiversity of bacteria belonging to the *Salmonella* family, the detection of lytic *Salmonella* phages targeting their various serovars is one of the current challenges. In our research, a total of nine *Salmonella* phages were isolated and 86% (20/24), Asia_Sal_UZ6 87.5% (21/24), Asia_Sal_UZ8 87.5% (21/24) bacteriophages with the highest lytic activity were selected. Based on the selected phages, a polyvalent bacteriophage, MediPhag with strong lytic activity against *Salmonella* enteritidis, *Salmonella* moscow, *Salmonella* java, *Salmonella* paratyphi B, *Salmonella* typhimurium, *Salmonella* newport, *Salmonella* agama salmonella serovars was developed. Mediphag bacteriophage was also found to be resistant to high temperatures and pH points in different ranges. These results suggest that Mediphag bacteriophage can be used as a potential antibacterial agent to control *Salmonella* serovars in food samples.

1. Introduction

Salmonellosis is a disease that is transmitted to humans through contaminated foods including poultry and eggs (Abdelsattar *et al.*, 2021). About 20 per cent of the worlds poultry products are reported to be contaminated with *Salmonella* ssp. (Staes *et al.*, 2019). *Salmonella enterica*, *Salmonella enteritidis* and *Salmonella typhimurium* are the most important infectious serovars that can remain in the human and animal body for a long time. They account for a high percentage of foodborne illnesses in humans (Phongtang *et al.*, 2019). *Salmonella* strains resistant to many antibiotics used in the treatment of humans and animals are common among developing countries. Diseases caused by NTS, such as *S. enteritidis* and *S. typhimurium* often result from the consumption of contaminated poultry products (Choi *et al.*, 2020). *Salmonella* serovar strains are a serious threat to public and pet health and this necessitates the discovery of new antimicrobial agents that can mitigate resistance mechanisms or biofilm formation. Ninety per cent of antibiotics are given orally to chickens and hens in poultry farms. However, they are not completely absorbed in the intestinal tract of the chicken and are often excreted unchanged in the feces. The poultry industry produces large amounts of manure and litter used on agricultural lands. Contaminated poultry droppings can transmit zoonotic pathogens such as *Salmonella enteritidis* to the environment (Chen *et al.*, 2018). Biological control of *S. enteritidis* can reduce the risk of microbial transmission between animals and humans and these results can serve as new and effective strategies. The accumulation of antibiotic residues in the human and animal bodies results in the formation of antibiotic-resistant bacteria. The use of probiotics and bacteriophages as alternative treatments in such cases is of great interest. In recent years, bacteriophages have attracted great attention especially in the treatment of food-borne pathogens as an alternative to potential antimicrobial compounds to eradicate harmful bacterial infections. Bacteriophages with high lytic activity damage and multiply the cell of pathogenic bacteria and form new bacteriophage particles. Bacteriophages are common in nature and can be isolated from soil, body parts of healthy and sick animals and sewage (Kurtz *et al.*, 2017; Liu *et al.*, 2021). Bacteriophages affect natural microbiota populations of humans and animals. They are also widely used in the treatment and classification of bacterial strains including *Salmonella*. In medicine, the use of bacteriophages for prophylactic and phage therapy purposes is absolutely safe. Therefore, our study focused on the isolation and characterization of phages with new strong lytic activity from water samples of different forms and the formation of polyvalent phages based on them, thereby evaluating the antimicrobial effect against salmonella pathogenic strains.

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2. Materials and Methods

2.1 Bacterial strains and their culture conditions

A total of eight common serovars of *Salmonella* strains were used in this study. These strains were purchased from the Ministry of Health of the Republic of Uzbekistan, the National Collection of Human Infectious Microorganisms, the Scientific Research Institute of Epidemiology, Microbiology and Infectious Diseases (Uzbekistan, Tashkent). All *Salmonella* strains were stored in 25% glycerin at –80°C in the Luria-Bertani agar medium. Each salmonella serovars were identified using an agglutination test in accordance with ISO 6579-1 requirements.

2.2 Isolation and propagation of phages

Salmonella phages were isolated from water basins of different shapes with some modifications by the standard point and two-layer agar method (Shang *et al*., 2021). Each sample was mixed in 5 g of magnesium buffer. In the next step, it was centrifuged at 5000 × g for 10 min and the supernatant containing phage was collected through a 0.45 mm membrane. A 50 mM $MgSO_4$ buffer was then added to the filtrate and filtered through a 0.22 mm membrane. At the end of the filtration process, the membrane was divided into pieces and 50 ml of elution solution was placed in 3% meat extract, 3% Twen 80 and 50 mM NaCl solution and processed by homogenization using ultrasound (200 W, 40 kHz) for 10 min to complete elution (Twest and Kropinski, 2009; Shang *et al*., 2021). In the next step, phage concentrate filtered in a 2 ml 0.22 mm filter for phage enrichment was mixed with 40 µl logarithmic growth phase host bacteria (*Salmonella* serovars) in Luria Bertani medium (2 mM $CaCl_2$) and incubated at 37°C. The enriched culture fluid was then filtered through a 0.45 mm syringe filter to obtain a phage suspension (Tang *et al*., 2019; Sun *et al*., 2022). The enrichment phase was repeated at least three times to obtain high titers of phages.

Phage cleaning was performed using the two-layer agar method (Li *et al*., 2020). The suspension (SM) was successively diluted in the buffer. 100 ml of the logarithmic growth phase was mixed with the host bacterium and 5 ml with Luria Bertani containing 0.4% semi-liquid agar and then placed on a solid LB containing 1.5% agar. After incubation for 5-8 h at 37°C, single plaque was selected and re-suspended in a SM buffer. This purification process was repeated five times and the purified phages were stored in LB medium filled with 25% glycerin at –80°C for subsequent use (Sobhy *et al*., 2021; Islam *et al*., 2020).

2.3 Characterization of polyvalent bacteriophage Mediphag

2.3.1. Host range determination

The host range of these isolated phages was determined relative to eight different serovars of *Salmonella* strains (Li *et al*., 2020). A 10 µl phage solution (<10^{10} PFU/ml) was poured into each petri dish from the top of the *Salmonella* strains planted as lawn. The plaques were then found to be present in petri dishes after incubation at 37°C overnight. If clear zones of plaque have formed in the petri dishes, this indicates that the bacterium has been lysed (+) by the phage. If clear plaque zone does not appear, it indicates that bacterial strains have not been lysed by the phage (–). Each test was conducted in triplicate (Huang *et al*., 2018; Duc *et al*., 2020).

2.3.2 Transmission electron microscopy

For the morphological description of a bacteriophage sample by transmission electron microphotography, the phage was preliminarily filtered on a 0.22 µm filter and the titer was adjusted to 10^8 PFU/ml. The high titer phage lysate was centrifuged at 25,000 × g for 40 min.The precipitate formed was washed twice with 0.1 M ammonium acetate solution (pH 7.0).The precipitate sample was then transferred to a 10 µl carbon-coated film and stained with 2% uranyl acetate. The phage sample was then examined under a JEM-100C transmission electron microscope (JEOL LTD, Tokyo, Japan) at a magnification of 65,000 times.

2.3.3 Phage titer and optimal multiplicity of infection

The phage titer was determined by the two-layer agar method after successive dilution of the phage solution and was calculated according to the following formula:

PFU/ml = number of plaques × dilution factor × 10 (Shang *et al*., 2020). The prevalence of infection is expressed as the ratio of phages to the number of host bacteria during infection. 10^6 colony-forming units (CFUs)/ml [Optical density at 600 nm ($OD_{600\ nm}$) = 0.18] were prepared with a correlation curve of host bacteria in the first logarithmic growth phase. The 100 ml of diluted phages were then mixed with 100 ml of host bacteria (10^6 CFU/ml) in a ratio of 0.001, 0.01, 0.1, 1, 10, 100 and at 37°C for 10 min. 5 ml of Luria Bertani (2 mM $CaCl_2$) medium was then added to the mixture and the mixture was incubated at 37°C for 12 h. It was then centrifuged at 12000 × g for 5 min and the sediment was removed. The supernatant was then filtered using a 0.45 mm syringe filter. To determine the phage titer with the highest titer of infection which was considered the optimal MOI was performed using the two-layer agar method. Each MOI test was performed three times in parallel (Wintachai *et al*., 2019; Luo *et al*., 2021; Rodwell *et al*., 2021).

2.3.4 One-step growth curve

The host bacterium, *Salmonella typhimurium* strain was grown in 25 ml of Luria Bertani medium at 37°C. The bacterial suspension was then diluted to 10^7 CFU/ml and centrifuged at 12000 × g for two min. It was re-suspended with 1 ml (10^5 PFU/ml) phage to achieve infection abundance (MOI). The resulting mixture was incubated at 37°C for 15 min. It was then centrifuged at 12000 × g. In the next step, the precipitate was re-suspended in 10 ml of Luria Bertani medium and incubated at 37°C by shaking at 200 rpm. During incubation, a 100 µl phage sample was taken at 5 min intervals to determine the phage titer using the two-layer agar method. The single-stage growth curve was described based on the relationship between the phagar titer logarithm (log_{10} PFU/ml) and the time of infection. This experiment was performed in triplicate. The latent period of the phages and the rupture of the pathogenic cell could be determined by a single-stage growth curve. The final number of phages in which the volume of phage cell rupture increased was proportional to the initial number of bacteria (Bao *et al*., 2015; Ajuebor *et al*., 2018; Kwon *et al*., 2020; Yang *et al*., 2020).

2.3.5 Stability studies

The ability of isolated bacteriophages to survive at different hot temperatures and at different pH values was studied. Phage suspension (10^5- PFU/ml) for thermal stability test incubation at 4°C, 20°C, 30°C, 37°C, 40°C, 50°C, 60°C, 70°C and 80°C is made and harvested. For the pH stability test, the phage suspension was added to the Luria Burtani medium with different pH values and adjusted in the range of 2 to 13 using HCI and NaOH until the final (10^5PFU/ml) concentration.The culture fluid was then incubated at 37°C for 1 h. After appropriate temperature and pH experiments, the phage titer was determined using the two-layer agar method (Yu *et al*., 2017; Harada *et al*., 2018).

2.4 Lytic activity of phages

The specificity of the isolated bacteriophages, the range and degree of lytic activity and the concentration of phage particles were determined using the method Otto.

2.4.1 Method Otto

Nutrient agar (HiMedia) was inoculated into the petri dishes containing the culture medium of the bacteria and instilled drop-by-drop from the marked phage. The petri dish was then raised to 45°C to one side and drained to the opposite side. The petri dish was then incubated in a thermostat for 16-18 h. If the phage is homologous and biologically active with the bacterium, then the place where the phage is instilled will be clear. The antimicrobial effect of the bacteriophage against the pathogenic bacteria is then evaluated as follows:

cl (conflict lysis-a zone cleared of bacteria when phages are used)

- fag ++++ scl (semi-confluent lysis - complete cleaning, but with a dull background)

- get phag +++ (opaque lysis - blurring of the whole cleared area)

- fag ++ sp (several small - small zones are observed)

- phag + and - (negative reaction)

++++ complete lysis of phage with culture (4 points)

+++ Subtle signs of culture growth in the test tube (3.5 points)

++ partial lysis (3 points)

+ slight growth of culture in the test tube (2 points)

± significant growth of culture in the test tube (1 point)

- complete absence of lysis (0 points)

2.4.2 Lytic activity of microdilution

The lytic activity of phages against *Salmonella* was investigated *in vitro*. *Salmonella typhimurium* was used as the host strain. To do this, the host bacterial culture in (10^6 CFU/ml) the 100 µl logarithmic growth phase was mixed with an equal volume of phage suspension (10^8-10^2 PFU/ml) in a 96-hole microplate to achieve MOI in ratios of 0.001, 0.01, 0.1, 1, 10, 100. The microplates were then incubated at 37°C for 24 h. At the end of the incubation time, $OD_{600\ nm}$ was measured at half-hour intervals. Cultures of non-phage-added host strains were used as a positive control. Phages mixed with Luria Bertani medium served as a negative control. All experiments were performed on the basis of three repetitions. $OD_{600\ nm}$ values were measured using.

2.5 Preclinical studies of the polyvalent bacteriophage mediphage against *Salmonella*

For clinical studies, liquid and capsular forms of the *Salmonella* polyvalent bacteriophage, 'Medifag' produced by "Asia Immunopreparat' (Uzbekistan, Tashkent) were used. White mice weighing 19-21 g were used in the study of acute toxicity of capsules and liquid preparation. A 34% solution (340 mg ± 1 ml of distilled water) was prepared from the *Salmonella* polyvalent bacteriophage, Mediphag capsule and administered to the experimental animals unilaterally enterally with a probe of 8500 mg/kg (0.5 ml) and 13600 mg/kg (0.8 ml) reception interval 30 min).The liquid preparation of Medifag was also administered enterally at 25 ml/kg (0.5 ml) and 40 ml/kg (0.8 ml) (with an interval of 30 min). The experimental animals were under constant supervision. The functional state, intensity, motor activity, character, number of movements, coordination of movements, response to external stimuli and skeletal muscle tone, frequency and depth of respiratory movements, consistency and amount of feces were taken into account. During the experiment, the clinical condition of the animals signs of poisoning the time of their appearance were observed. All experimental animals were kept in general ration under standard conditions provided with water and feed. All results are given in Tables 2 and 3.To study the local pathogenicity of liquid *Salmonella* polyvalent bacteriophage, Mediphag young, healthy, adult albino rabbits weighing 2 kg were used. To evaluate the tested drug was divided into 3 experimental and control groups. 0.9% sodium chloride solution was used as a control drug. Animals were examined before each experiment to identify lesions that could be observed in the gut such as tumors infectious lesions associated with infectious diseases and so on. For the experiment, a catheter with a 1 ml syringe attached to the end with a length of 6 cm was used.

1 ml of phage drawn into the catheter was sent to the intestines of the animals. The laboratory equipment used was used separately for each animal. This process was repeated once every 24 h for 5 days. After the first 24 h of injection, the appearance of the injected site, secretory discharge, erythema, were marked and recorded. Twenty four hours after the last injection of the test material, the animals were anesthetized with urethane (1 g/kg) and part of the rectum and distal part of the colon were cut lengthwise and examined for scratches, damaged epithelial layer or tissue necrosis. Rectal tissue of animals in the experimental and control groups was compared. Macroscopic changes in the appearance of rectal tissue in each animal differences in animals in experimental and control options were described and recorded (Abdelkader *et al*., 2019; Onsea *et al*., 2021).

2.6 Statistical analysis

Results were expressed as mean values ± standard deviations. GraphPad Prism 9.0.1 was used for statistical analysis. Two-way ANOVA with multiple comparisons was performed to determine differences between groups. A *p*-value less than (0.05) was considered statistically significant in all cases.

3. Results

3.1 *Salmonella* strains and their culture conditions

Salmonella strains were collected from the National Collection of Human Infectious Microorganisms, Institute of Epidemiology, Microbiology and Infectious Diseases (Uzbekistan, Tashkent). *Salmonella* strains were transplanted into petri dishes containing xylose lysine deoxycholate agar (XLD). The petri dishes were then incubated at 37°C for 24 h. In the next step, it was stored in Luria Bertani broth containing 20% glycerin at –20°C (Figure 1).

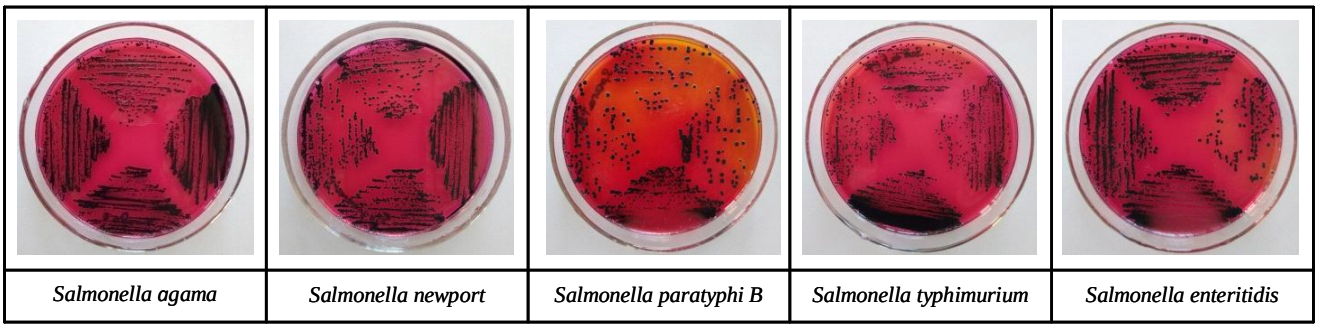

Figure 1: ***Salmonella*** **strains in xylose lysine deoxycholate agar (XLD) medium.**

3.2 Phage isolation and their host range

Different forms of water samples were used to isolate the phages (Figure 2). A total of nine *Salmonella* phages were isolated from these water samples. We tested the specific spectrum of these phages against 24 of the most common *Salmonella* serovars. According to the results of the experiment, Asia_Sal_UZ1 52.4% (10/24), Asia_Sal_UZ2 46.8% (8/24), Asia_Sal_UZ3 86% (20/24), Asia_Sal_UZ4 24.6% (6/24), Asia_Sal_UZ5 17.8% (4/24), Asia_Sal_U6 % (21/24), Asia_Sal_UZ7 12.6% (3/24), Asia_Sal_UZ8 87.5% (21/24) and Asia_Sal_UZ9 4.2% (1/24) lysis of salmonella serovars. Asia_Sal_UZ3, Asia_Sal_UZ6 and Asia_Sal_UZ8 have been identified as new bacteriophages with high lytic activity against common *Salmonella enteritidis, Salmonella moscow, Salmonella java, Salmonella paratyphi* B, *Salmonella typhimurium, Salmonella newport, Salmonella agama* serovars. These found bacteriophages have not been previously reported by other researchers. Based on these results, we selected Asia_Sal_UZ3, Asia_Sal_UZ6 and Asia_Sal_UZ8 phages for further experiments and formed a polyvalent bacteriophage based on their mixture. The resulting polyvalent phage was called *Salmonella* polyvalent bacteriophage, 'Mediphag' and was used in subsequent studies.

Figure 2: Different water samples used in the isolation of bacteriophages A - ditch, B - river, S - stream, D - pond water, E - river water.

3.3 Morphological characterization by TEM analysis.

Electron microscopy reveals the ultrastructure of bacteriophages. Using this method, it is possible to determine which family bacteriophages belong to. TEM analysis is an important criterion for the taxonomic classification of bacteriophages. This method provides information on the high-level classification of bacteriophages and compared to genome analysis, TEM analysis is cheaper and faster. In our experiment described above, bacteriophages with the highest lytic activity were selected, isolated from water samples of various forms and based on their combination, *Salmonella* polyvalent bacteriophage 'Mediphag' was created. TEM analysis was performed to identify each type of phage in the polyvalent bacteriophage. Based on the experiments, the following results were obtained. We have observed *Salmonella* polyvalent bacteriophage, Mediphag forming round plaques of approximately 2.4 - 2.5 mm in size on a two-layer agar plate.TEM imaging with a transmission electron microscope showed that Asia_Sal_UZ3 and Asia_Sal_UZ8 are species recurring typical features of the family Siphoviridae. TEM analysis showed that the bacteriophage has a hexagonal head. The width of the detected bacteriophage head was 77.6 ± 2.34 nm and the length was 101.4 ± 1.6 nm.The presence of a non-contractile tail with a length of 178.14 ± 1.64 nm was revealed. Also, the analysis of transmission electron microscopy of bacteriophage Asia_Sal_UZ8 showed that the species repeats the features of a member of the family Myoviridae. The results of analysis using a transmission electron microscope showed that the

species has an icosahedral head with a width of 78.5 ± 0.77 nm and a length of 103.4 ± 2.7 nm. At the same time, the presence of a tail with a length of 141.3 ± 1.1 nm, which has the property of shrinking was confirmed (Figure 3). The morphology of the *Salmonella* polyvalent bacteriophage, Mediphag and the formation of phage plaques on two-layer agar plates are shown in Figure 4.

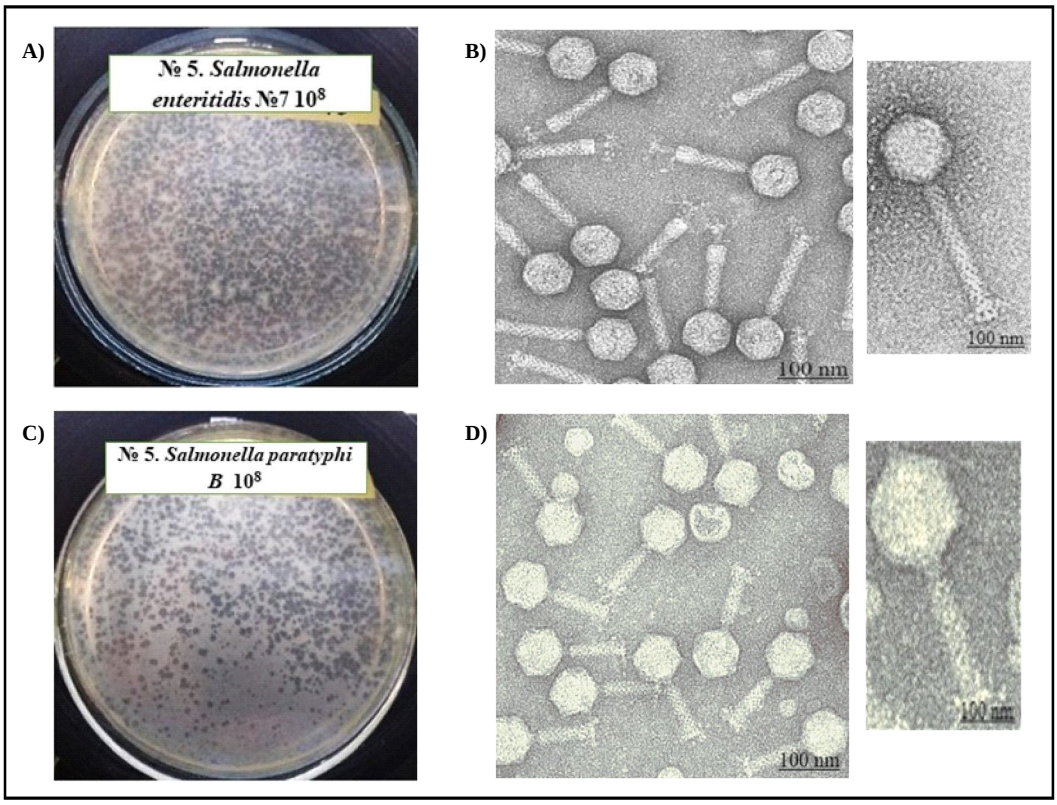

Figure 3: Photomicrograph of plaques produced by phage *Salmonella* polyvalent bacteriophage 'MediPhag' on lawns of *Salmonella enteritidis* [1]7 and *Salmonella paratyphi* B. Morphology of phage *Salmonella* polyvalent bacteriophage. Transmission electronmicrograph of negatively stained phage Asia_Sal_UZ6 and Asia_Sal_UZ8. The bar indicates 100 nm.

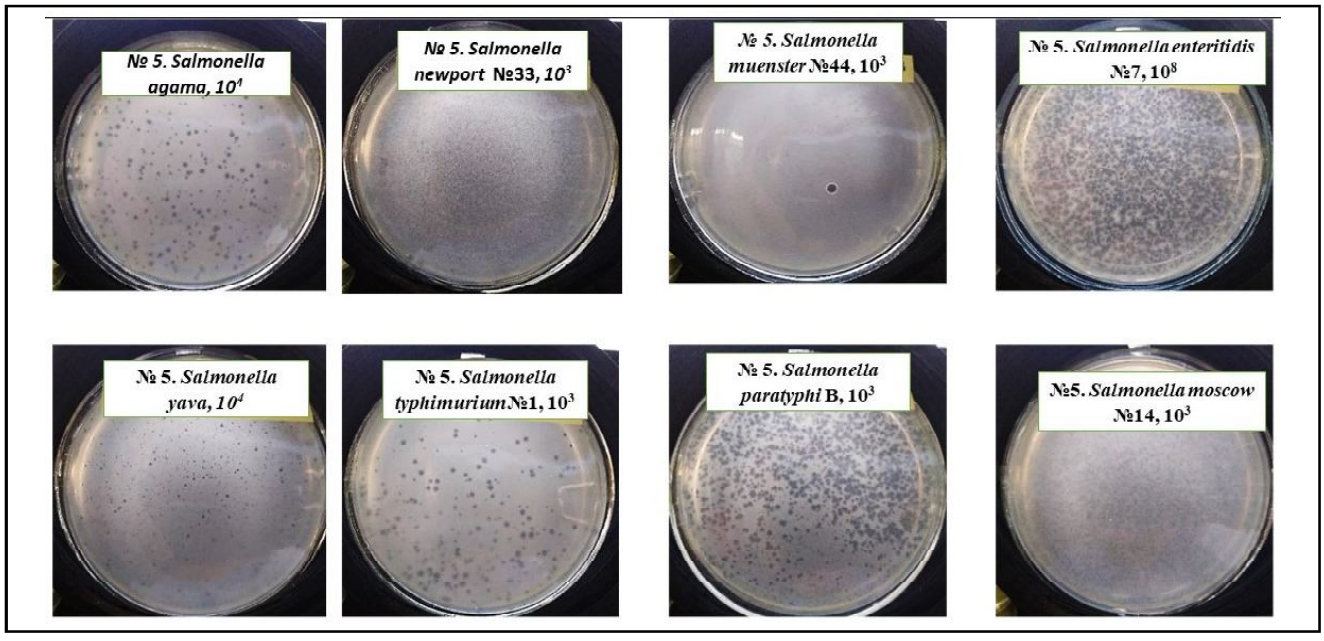

Figure 4: Morphology of *Salmonella* polyvalent bacteriophage Mediphag. Phage plaques formed on double-layer agar plates.

3.4 Optimal multiplicity of infections and latent period

The phage titers were similar in different MOI as shown in the figures below. However, *Salmonella* polyvalent bacteriophage was found to have a lower effect of MOI on the Medifag titer in experiments. Therefore, we chose MOI = 0.01 because the fag provided the highest titer, of 10^8 $\log_{10}$ PFU/ml. *Salmonella* polyvalent bacteriophage Medifag was found to be latent and ascending cycles of 20-30 min in the medium of Luria Bertani with *Salmonella typhimurium* selected as the host strain in the single-stage growth curve. The mean cell rupture volume was found to be 10^7 PFU/cell (Figure 5).

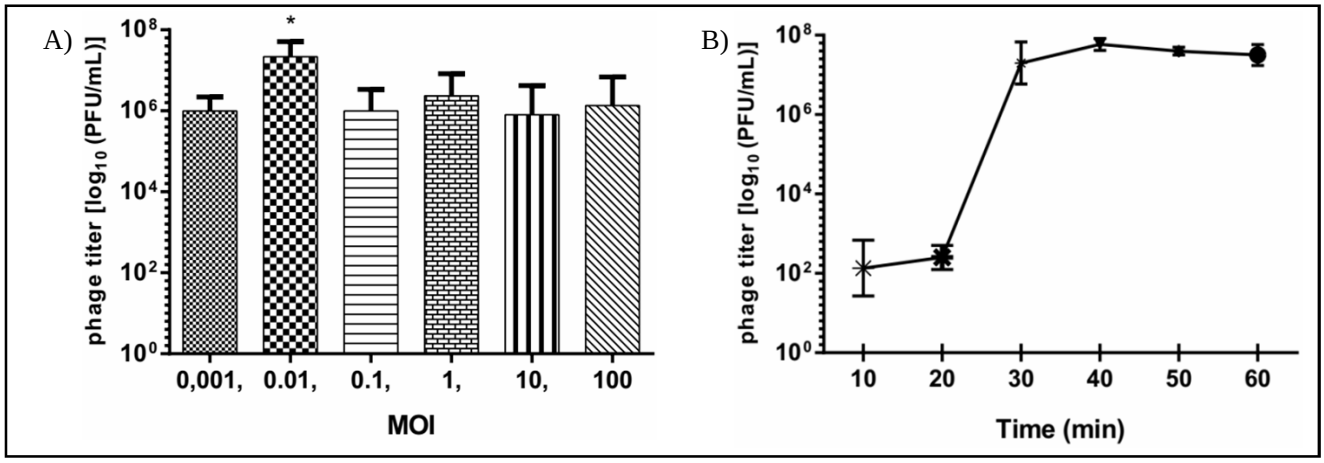

Figure 5: (A) Phage titers were measured in different infections (MOI) (0.001 to 100). (B) *Salmonella* polyvalent bacteriophage MediPhag was found to be 0.01 in the *Salmonella typhimurium* MOI on the single-stage growth curve.

3.5 Thermal and pH stability

The thermal viability feature of *Salmonella* polyvalent bacteriophage MediPhag recorded a specific result at each temperature. For example, at 4°C 10^6 $\log_{10}$ PFU/ml, at 20°C 10^7 $\log_{10}$ PFU/ml and at 30-60°C 10^8 $\log_{10}$ PFU/ml an average of the differences were statistically significant was not recorded (p>0.05). It was also found that the survival rate of phage after incubation at 70-80°C for one hour averaged 10^2 $\log_{10}$ PFU/ml. However, the *Salmonella* polyvalent bacteriophage MediPhag provided the highest titer in the pH range of 7.5-8.0 (10^2 $\log_{10}$ PFU/ml). A significant decrease in phage titer ($p<0.05$) was also observed in the pH range of 2-3. At pH 12, the viability of the phage sharply decreased due to the highly alkaline nature of the medium (Figure 6).

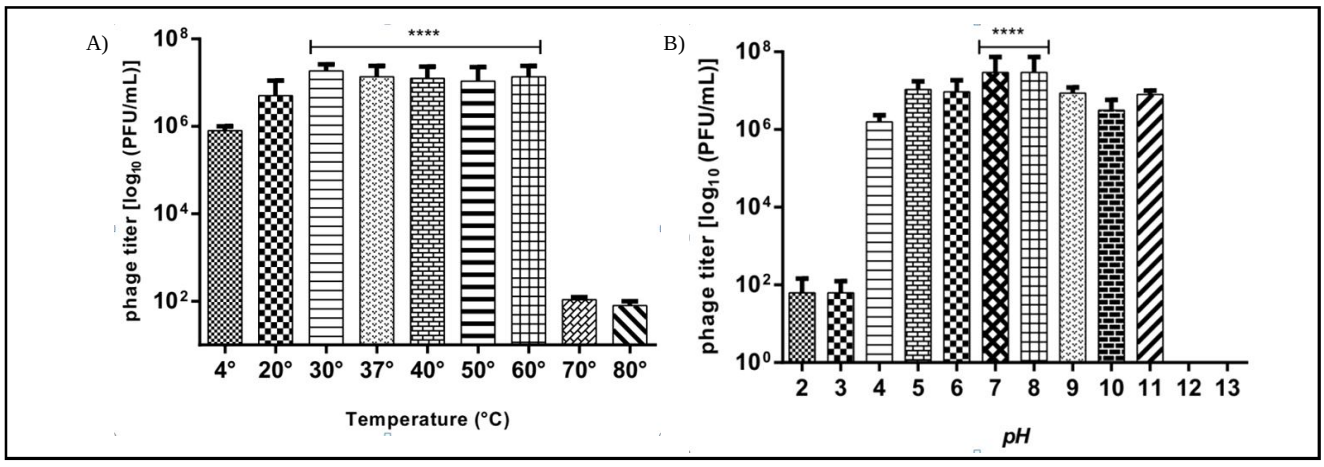

Figure 6: (A) Effect of temperature on phage survival. Results obtained by incubating MediPhag for one hour at 4°C, 20°C, 30°C, 37°C, 40°C, 50°C, 60°C, 70°C and 80°C. (Â) Effect of pH in various ranges on the viability of MediPhag. Data are expressed as mean ± standard deviation and are visible at ****p<0.05.

3.6 Bacteriolytic activity of phages

Salmonella serovars identified as the main producing strains were used to confirm that the isolated sterile filtrates contained bacteriophages capable lysing of *Salmonella*. *Salmonella enteritidis* [17], *Salmonella moscow* [114], *Salmonella java*, *Salmonella paratyphi* B, *Salmonella typhimurium* [11], *Salmonella newport* [1 33], *Salmonella agama* (Table 1 and Figure 7).

As a result of the adaptation of the initially isolated phage particles by the corresponding strains, all properties associated with their lytic activity improved significantly compared to the expected result. The specificity of *Salmonella* polyvalent bacteriophage *S. enteritidis* [17], *S. moscow* [114], *S. newport* [133], *S. agama* strains was increased to (++++). With strains of *S. yava*, *S. paratyphi* B, *S. typhimurium* [11] an increase of - (+++) was achieved (Figure 8).

Table 1: Determination of the susceptibility of water sample filtrates to *Salmonella* cultures

Sl.No.	Salmonella strains	Sample codes				
		No. 1	No. 2	No. 3	No. 4	No. 5
1	*Salmonella enteritidis* No. 7	ϕ 3,5+	ϕ 3+	ϕ 3+	ϕ 3+	ϕ 1+
2	*Salmonella moscow* No. 14	ϕ 4+	ϕ 1+	ϕ 1+	ϕ 2,5+	ϕ 1+
3	*Salmonella yava*	ϕ 3,5	ϕ 0,25+	ϕ 0,25+	ϕ 4+	ϕ 4+
4	*Salmonella paratyphi* B	ϕ 2+	ϕ 2+	ϕ +	ϕ 3+	ϕ 3+
5	*Salmonella typhimurium* No. 1	ϕ 3+	ϕ 2,5+	ϕ 2,5+	ϕ 3+	ϕ 3+
6	*Salmonella newport* No. 33	ϕ 4,5+	ϕ 2+	ϕ 2+	ϕ 3+	ϕ 2,5+
7	*Salmonella muenster* No. 44	ϕ 2,5+	ϕ 1,5+	ϕ 1+	ϕ 1,5+	ϕ 0,5+
8	*Salmonella àgama*	ϕ 2+	ϕ 2+	ϕ 2+	ϕ 2+	ϕ 4+

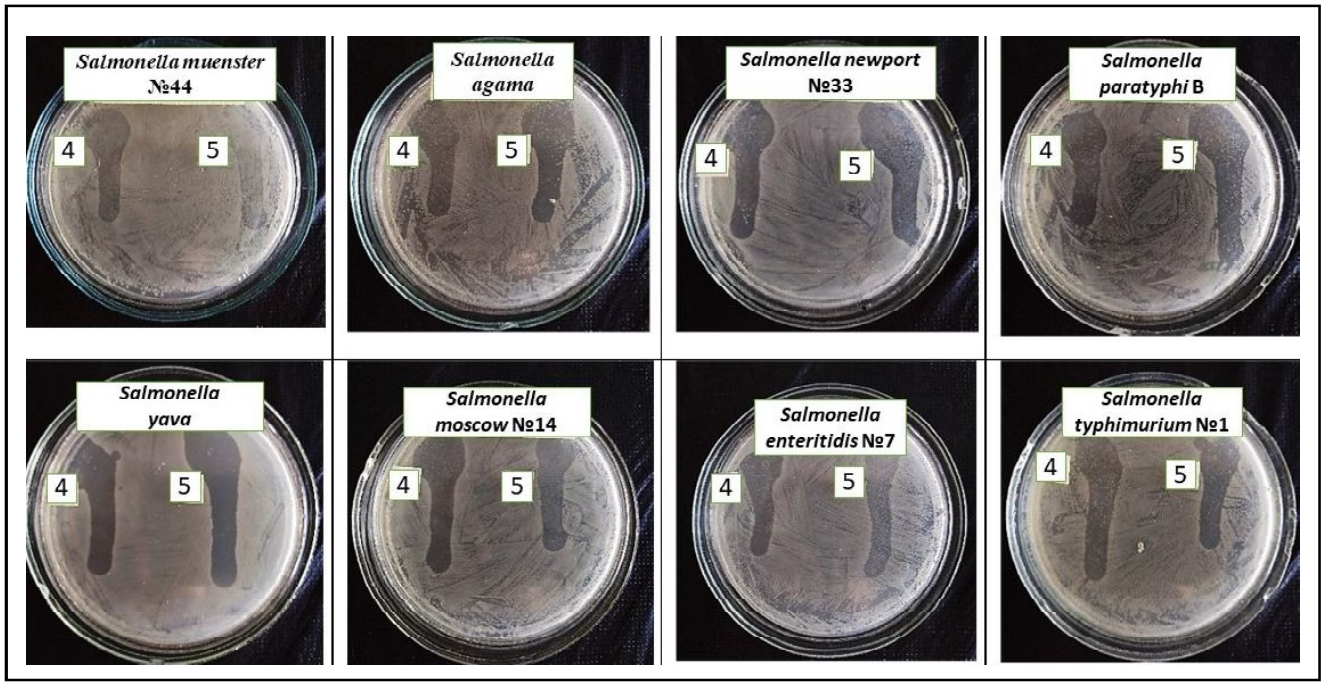

Figure 7: Results of determining the susceptibility of water sample filtrates to *Salmonella* strains.

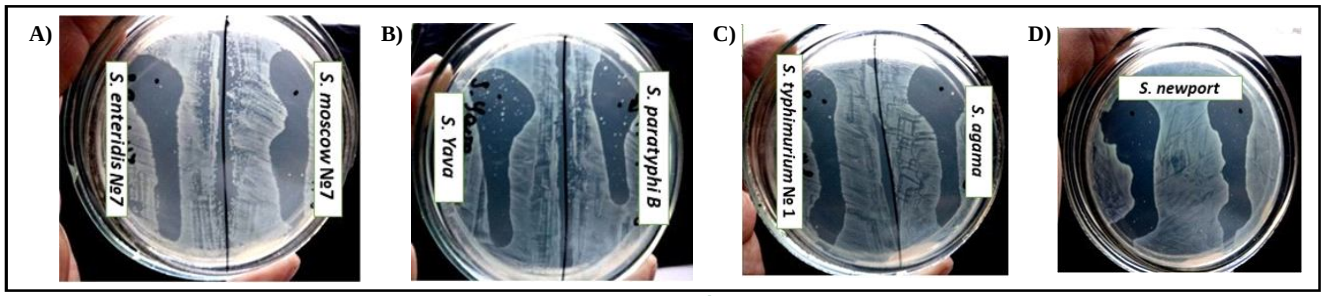

Figure 8: Lytic activity of MediPhag against *Salmonella* serovars. À) *S.enteritidis* No. 7 and *S.moscow* No. 14; B) *S.yava* and *S.paratyphi* B; C) *S. typhimurium* No. 1 and *S. agama*; D) *S. newport*

3.7. Lytic activity of microdilution

The ability of polyvalent bacteriophage MediPhag to inhibit the dynamic growth of *Salmonella* strains was determined by the microdilution method. According to the experimental results, we observed that when exposed to bacteriophage, the growth of *Salmonella* reaches its logarithmic phase in one hour and then increases sharply. The OD_{600nm} values of the Luria Bertani medium used as negative control remained unchanged. During the experiment, MOI was used in different ratios (MOI = 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100). $OD_{600\ nm}$ values of MediPhag filled cuvettes were found to be less than the positive control ($p<0.05$). These results suggest that polyvalent MediPhag effectively inhibits the growth of *Salmonella* strains (Figure 9).

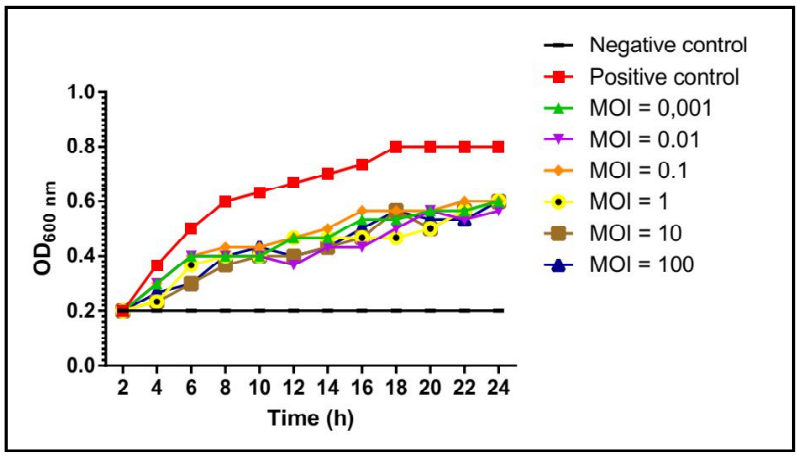

Figure 9: Lytic activity of bacteriophage MediPhag against pathogen *S. paratyphi* B in the Luria Bertani medium in MOI at different ratios (0.001-100) at 37°C. Cultures of host strains without phage were used as a positive control. Phages mixed with Luria Bertani medium served as a negative control. All experiments were performed on the basis of three repetitions. OD_{600nm} values were measured using.

3.8 Preclinical studies of polyvalent bacteriophage, Mediphag

When the white mice used in the experiment were exposed to the liquid and capsular forms of the *Salmonella* polyvalent bacteriophage 'Mediphage', the general condition and behavior of the mice were stable, no changes were observed. The tone of the skeletal muscles was normal, no epilepsy were observed, no reactions to sound and light stimuli were detected, the frequency and depth of movements of the musculoskeletal system, the heart rate corresponded to physiological norms. The condition of the wool and skin is good, the color and size of the mucous membranes have not changed from the norm, water consumption has not increased, body weight is stable, the amount and concentration of feces have not changed. The experiment was conducted for 14 days. No mouse deaths were observed during the experiment (Tables 2 and 3).

Table 2: Determination of acute toxicity of *Salmonella* polyvalent bacteriophage 'Mediphag' liquid drug. Manufacturer (LD_{50}) 'Asia Immunopreparat' LLC, Uzbekistan

Number of experimental options No.	Weight (g)	Quantity, ml / kg	Method of reception	The result
1	20	25	enteral	no death
2	21	25	enteral	no death
3	19	25	enteral	no death
4	20	25	enteral	no death
5	19	25	enteral	no death
6	20	25	enteral	no death
1	20	40	enteral	nodeath
2	20	40	enteral	no death
3	19	40	enteral	no death
4	20	40	enteral	no death
5	20	40	enteral	no death
6	20	40	enteral	no death
LD_{50}	>40 ml / kg			

Table 3: Determination of acute toxicity of *Salmonella* polyvalent bacteriophage 'Mediphag' capsule drug. Manufacturer (LD_{50}) 'Asia Immunopreparat' LLC, Uzbekistan

Number of experimental options No.	Weight (g)	Quantity, ml / kg	Method of reception	The result
1	20	8500	enteral	no death
2	21	8500	enteral	no death
3	19	8500	enteral	no death
4	20	8500	enteral	no death
5	21	8500	enteral	no death
6	20	8500	enteral	no death
1	20	13600	enteral	no death
2	19	13600	enteral	no death
3	19	13600	enteral	no death
4	20	13600	enteral	no death
5	19	13600	enteral	no death
6	20	13600	enteral	no death
LD_{50}	>13600 ml / kg			

Based on the results obtained, the following conclusion can be drawn. *Salmonella* polyvalent bacteriophage 'Mediphage' in liquid and capsule form produced in Uzbekistan by Asia Immunopreparat LLC which has undergone clinical trials, did not show signs of toxicity in experimental animals when studying the acute toxicity of Mediphage. According to the results of the study of the local excitatory properties of the liquid preparation, no changes were detected on the surface on which the preparation was applied 24 h after the first injection and 5 days before the next experiment. Secretory separation and damage was not observed. Within 24 h after the last administration of the drug, the animals were anesthetized and examined under a microscope. The following was revealed: part of the rectum and the distal colon were not scratched, the epithelial layer was not damaged, necrosis of the rectal tissue was not detected. No difference was observed in the rectal tissue of the animals in the experimental and control groups.

4. Discussion

We know that bacteriophages are multimicroorganisms that are widely distributed in the environment. It is surprising to all of us that bacteriophages have a dry soil weight of 10^{10} g. Bacteriophages are widely used in the food industry to combat pathogenic bacteria. However, the main limitation of the use of bacteriophages as biological control is the narrow range of hosts. This is because the main limitation in the use of most isolated bacteriophages as biological control for *Salmonella* is the narrow host range as most isolated phages are typically specific to *S. enteritidis* or *S.typhimurium* (Goodridge *et.al*., 2018; Abhisingha *et al*., 2020; Abdelsattar *et al*., 2022). Expanding the host range of bacteriophages requires genetically novel advanced technologies and approaches. That is why the search for bacteriophages resistant to a number of *Salmonella* serovars with a high lytic ability is one of the most urgent tasks today (Besser *et al*., 2020; Choi *et al*., 2020). In our study, a polyvalent bacteriophage was formed based on a total of 3 bacteriophages. Eight common *Salmonella* serovars were used as hosts to determine the lytic activity of the generated polyvalent bacteriophage. MediPhag has been identified as a new polyvalent bacteriophage that reliably infects salmonella serovars such as *Salmonella enteritidis* ¹7, *Salmonella moscow* ¹14, *Salmonella java*, *Salmonella paratyphi* B, *Salmonella typhimurium* ¹1, *Salmonella newport* ¹ 33, *Salmonella agama*. In a one-step growth curve of the *Salmonella* polyvalent bacteriophage, it was found that the latent and ascending cycles are approximately 15 - 20 min. The volume of decay of pathogenic bacterial cells of the bacteriophage was estimated at 10^7 PFU cells. The latent period of MediPhag is short and can effectively inactivate bacteria during this period.The thermal and pH stability of bacteriophages is important for the biological control of pathogens. MediPhag has shown relatively high thermal stability, which means that it is compatible with pasteurization in the processing of practical food products. (Fierer and Guiney, 2001; García-Anaya *et al*., 2020; Gurney *et al*., 2020). MediPhag has also been found to maintain high activity with a wide pH range which allows it to be used in food matrices with different pH values (Guenther *et al*., 2012; Dallal *et al*., 2019; Wessels *et al*., 2021). *Salmonella* polyvalent bacteriophage in liquid and capsule form has been shown to have low acute toxicity and no local pathogenicity in all experiments performed in experimental animals under Mediphag laboratory conditions. To date, changes in the lytic activity of bacteriophages have been observed under special conditions. The main reason for this was to study the stability of their ability to lyse infectious disease causing bacteria when used as a drug. Based on the above data, this drug can be recommended for trials in clinics for the treatment and prevention of patients with salmonellosis. Further work will be done to study the mechanism and interaction of recognition and lysis between phages and their hosts as well as to characterize other phages.

5. Conclusion

In general, bacteriophages with strong lytic activity were isolated from water samples in Uzbekistan and on their basis polyvalent

bacteriophage, MediPhag against *Salmonella* was formed. In this study was formed a polyvalent bacteriophage with strong lytic activity. This bacteriophage is specific to the serovars of *Salmonella* (*Salmonella enteritidis* 17, *Salmonella moscow* 114, *Salmonella java*, *Salmonella paratyphi* B, *Salmonella typhimurium* 11, *Salmonella newport* 1 33, *Salmonella agama*) and can lyse a wide range. However, of the morphology, lytic range, latent period, pathogen cell rupture volume, pH and thermal stability of this identified bacteriophage were tested. The *Salmonella* polyvalent bacteriophage 'MediPhag' can also exhibit its properties as biological control agent for *Salmonella* even in the difficult conditions of the food industry.

Conflict of interest

The authors declare no conflict of interest relevant to this article.

References

Abdelkader, K.; Gerstmans, H.;Saafan, A.; Dishisha, Tand Briers, Y. (2019). The preclinical and clinical progress of bacteriophages and their lytic enzymes: The parts are easier than the whole.Viruses, **11**(2):96.

Abdelsattar, A. S.; Safwat, A.; Nofal, R.;Elsayed, A.; Makky, S and El-Shibiny, A. (2021). Isolation and characterization of bacteriophage ZCSE6 against *Salmonella* spp.: Phage application in milk. Biologics, **1**(2):164-176.

Abdelsattar, A.; Dawoud, A.;Makky, S.; Nofal, R.; Aziz, R. and El-Shibiny, A. (2022). Bacteriophages: From isolation to application.Current Pharmaceutical Biotechnology, **23**(3):337-360.

Abhisingha, M.; Dumnil, J. and Pitaksutheepong, C. (2020). Efficiency of phage cocktail to reduce *Salmonella typhimurium* on chicken meat during low temperature storage. LWT, **129**:109-580.

Ajuebor, J.; Buttimer, C.; Arroyo-Moreno, S.; Chanishvili, N.; Gabriel, E. M.; O'Mahony, J. and Coffey, A. (2018). Comparison of *Staphylococcus* phage K with close phage relatives commonly employed in phage therapeutics. Antibiotics, **7**(2):37.

Bao, H.; Zhang, P.; Zhang, H.; Zhou, Y.; Zhang, L. and Wang, R. (2015). Biocontrol of *Salmonella enteritidis* in foods using bacteriophages. Viruses,**7**(8):4836-4853.

Besser, J. M. (2018). *Salmonella* epidemiology: A whirlwind of change. Food Microbiology, **71**:55-59.

Chen Y.; Sun E.; Song J.; Tong Y. and Wu B. (2018). Three *Salmonella enterica* serovar *Enteritidis* bacteriophages from the *Siphoviridae* family are promising candidates for phage therapy. Can. J. Microbiol., **64**(11):865-875.

Choi I.Y.; Park D.H.;Chin B. A.; Lee C.; Lee J. and Park M.K. (2020). Exploring the feasibility of *Salmonella typhimurium* specific phage as a novel bioreceptor. J. Anim. Sci. Technol., **62**(5):668-681.

Choi, I. Y.; Park, D. H.; Chin, B. A.; Lee, C.; Lee, J. and Park, M. K. (2020). Exploring the feasibility of *Salmonella typhimurium*-specific phage as a novel bioreceptor. Journal of Animal Science and Technology, **62**(5):668.

Li, C., Yuan.; X., Li, N., Wang, J.; Yu, S.; Zeng, H. and Ding, Y. (2020). Isolation and characterization of *Bacillus cereus* phage vB_BceP-DLc1 reveals the largest member of the Ö29-like phages. Micro organisms, **8**(11):1750.

Dallal, M. M. S.; Nikkhahi, F.; Alimohammadi, M.; Douraghi, M.; Rajabi, Z.; Foroushani, A. R. and Fardsanei, F. (2019). Phage therapy as an approach to control *Salmonella enterica* serotype *Enteritidis* infection in mice. Revista da Sociedade Brasileira de Medicina Tropical, pp:52.

Duc, H. M.; Son, H. M.; Yi, H. P. S.; Sato, J.; Ngan, P. H. and Masuda, Y. (2020). Isolation, characterization and application of a polyvalent phage capable of controlling *Salmonella* and *Escherichia coli* O157:H7 in different food matrices. Food Res. Int. **131**:108-977.

Fierer, J. and Guiney, D. G. (2001). Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection. J. Clin. Investig. **107**:775-780.

García-Anaya, M. C.; Sepulveda, D. R.; Sáenz-Mendoza, A. I.; Rios Velasco, C.; Zamudio Flores, P. B. and Acosta Muñiz, C. H. (2020). Phages as biocontrol agents in dairy products. Trends Food Sci. Technol. **95**:10-20.

Goodridge, L.; Fong, K.; Wang, S. and Delaquis, P. (2018). Bacteriophage-based weapons for the war against foodborne pathogens. Curr. Opin. Food Sci., **20**:69-75.

Guenther, S.; Herzig, O.; Fieseler, L.; Klumpp, J. and Loessner, M. J. (2012). Biocontrol of *Salmonella typhimurium* in RTE foods with the virulent bacteriophage FO1-E2. Int. J. Food Microbiol., **154**:66-72.

Gurney, J.; Brown, S. P.; Kaltz, O. and Hochberg, M. E. (2020). Steering phages to combat bacterial pathogens. Trends Microbiol., **28**:85-94.

Harada, L. K.; Silva, E. C.; Campos, W. F.; Del Fiol, F. S.; Vila, M.; D1browska, K. and Balcão, V. M. (2018). Biotechnological applications of bacteriophages: State of the art. Microbiological Research, **212**:38-58.

Huang, C.; Shi, J.; Ma, W.; Li, Z.; Wang, J.; Li, J. and Wang, X. (2018). Isolation, characterization, and application of a novel specific *Salmonella* bacteriophage in different food matrices. Food Research International, **111**:631-641.

Islam, M. S.; Hu, Y.; Mizan, M. F. R.; Yan, T.;Nime, I.; Zhou, Y. and Li, J. (2020). Characterization of salmonella phage LPST153 that effectively targets most prevalent salmonella serovars. Micro organisms, **8**(7):10-89.

Kurtz, J. R.; Goggins, J. A. and McLachlan, J. B. (2017). Salmonella infection: Interplay between the bacteria and host immune system. Immunology Letters, **190**:42-50.

Kwon, J.; Kim, S. G.; Kim, H. J.; Giri, S. S.; Kim, S. W.; Lee, S. B. and Park, S. C. (2020). Isolation and characterization of *Salmonella* jumbo-phage pSal-SNUABM-04.Viruses, **13**(1):27.

Li, Z.; Ma, W.; Li, W.; Ding, Y.; Zhang, Y.; Yang, Q.and Wang, X. (2020). A broad-spectrum phage controls multidrug-resistant *Salmonella* in liquid eggs. Food Research International, **132**:109011.

Liu, R.; Li, Z.; Han, G.;Cun, S.; Yang, M. and Liu, X. (2021). Bacteriophage ecology in biological wastewater treatment systems. Applied Microbiology and Biotechnology, **105**(13):5299-5307.

Luo, D.; Li, C.; Wu, Q.; Ding, Y.; Yang, M.; Hu, Y. and Zhang, J. (2021). Isolation and characterization of new phage vB_CtuP_A24 and application to control *Cronobacter* spp. in infant milk formula and lettuce. Food Research International, **141**:110109.

Onsea, J.; Post, V.; Buchholz, T.;Schwegler, H.;Zeiter, S.;Wagemans, J. and Metsemakers, W. J. (2021). Bacteriophage therapy for the prevention and treatment of fracture-related infection caused by *Staphylococcus aureus*: A preclinical study. Microbiology Spectrum, **9**(3):e01736-21.

Phongtang, W.; Choi, G.P.;Chukeatirote, E. and Ahn J. (2019). Bacteriophage control of *Salmonella typhimurium* in milk. Food Sci. Biotechnol., **28**(1):297-301.

Rodwell, E. V.;Wenner, N.; Pulford, C. V.; Cai, Y.; Bowers-Barnard, A.; Beckett, A. and Perez-Sepulveda, B. M. (2021). Isolation and characterisation of bacteriophages with activity against invasive non-typhoidal *Salmonella* causing bloodstream infection in Malawi.Viruses, **13**(3): 478.

Shang, Y.; Sun, Q.; Chen, H.; Wu, Q.; Chen, M.; Yang, S. and Zhang, J. (2021). Isolation and characterization of a novel *Salmonella* phage vB_SalP_TR2. Frontiers in Microbiology, **12**:14-52.

Sobhy, H.; Askora, A.; Wareth, G. and Ahmed, W. (2021). Isolation, characterization, and efficacy of three lytic phages infecting multidrug-resistant *Salmonella* serovars from poultry farms in Egypt. Archives of Razi Institute, **76**(3):507.

Staes, I.; Passaris, I.; Cambre, A. and Aertsen, A. (2019). Population heterogeneity tactics as driving force in *Salmonella* virulence and survival. Food Res. Int., **125**:108-560.

Sun, Z.; Wen, H.; Ma, L. and Chen, Z. (2022). Isolation, characterization and application of bacteriophage PSDA-2 against *Salmonella typhimurium* in chilled mutton. PloS One, **17**(1):262-946.

Tang, F.; Zhang, P.; Zhang, Q.;Xue, F.; Ren, J.; Sun, J. and Dai, J. (2019). Isolation and characterization of a broad-spectrum phage of multiple drug resistant *Salmonella* and its therapeutic utility in mice. Microbial Pathogenesis, **126**:193-198.

Twest, R. V. and Kropinski, A. M. (2009). Bacteriophage enrichment from water and soil. In: Bacteriophages (pp:15-21). Humana Press.

Wessels, K.; Rip, D. and Gouws, P. (2021). *Salmonella* in chicken meat: Consumption, outbreaks, characteristics, current control methods and the potential of bacteriophage use. Foods, **10**:17-42.

Williamson, K. E.; Fuhrmann, J. J.;Wommack, K. E. and Radosevich, M. (2017). Viruses in soil ecosystems: An unknown quantity within an unexplored territory. Annu. Rev. Virol., **4**(1):201-219.

Wintachai, P.;Naknaen, A.;Pomwised, R.;Voravuthikunchai, S. P. and Smith, D. R. (2019). Isolation and characterization of *Siphoviridae* phage infecting extensively drug-resistant *Acinetobacter baumannii* and evaluation of therapeutic efficacy *in vitro* and *in vivo*. Journal of Medical Microbiology, **68**(7):1096-1108.

Yang, M.; Liang, Y.; Huang, S.; Zhang, J.; Wang, J.; Chen, H. and Tan, Z. (2020). Isolation and characterization of the novel phages vB_VpS_BA3 and vB_VpS_CA8 for Lysing *Vibrio parahaemolyticus*. Frontiers in Microbiology, **11**:259.

Yu, P.; Mathieu, J.; Lu, G. W.;Gabiatti, N. and Alvarez, P. J. (2017). Control of antibiotic-resistant bacteria in activated sludge using polyvalent phages in conjunction with a production host. Environmental Science and Technology Letters, **4**(4):137-142.

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