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Phage therapy against MDR and XDR *Salmonella enterica* serovars at Basrah Province, Iraq

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ABSTRACT

Salmonella enterica is a significant contributor to infectious diseases, and the proliferation of antibiotic-resistant bacteria has led to considerable public health challenges. Currently, the prevention and control of multidrug-resistant *Salmonella* contamination face enormous difficulties in food contamination management. Bacteriophages offer a promising approach to managing foodborne pathogens by lysis it. In this study, a lytic *Salmonella* phage, belonging to the genus *Felixounavirus*, family *Myoviridae*, and order *Caudovirales*, was isolated. This phage exhibited high lytic activity against multi- and extensively drug-resistant *Salmonella*, with a lytic activity rate of 85.71% (6 out of 7 strains). In comparison 14.29% (1 out of 7 strains) were resistant, showing highly significant differences ($P<0.001$). The results indicated that a dilution factor of 10^{-6} yielded the best countable number of plaques and optimal lytic activity at 37°C and pH 7, with decreased activity at pH 4, and no activity at pH 9. These findings suggest that this phage can be used as an antibacterial agent to control multi- and extensively drug-resistant *Salmonella*.

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Introduction

Salmonella has become one of the most prevalent foodborne pathogens globally. It is predominantly transmitted through contaminated foods, such as milk, lettuce, chicken, and beef, or through water (Brown et al. 2021; Shang et al. 2021; Sritha & Bhat, 2021; Zhang et al. 2023). Salmonellosis is responsible for approximately 90 million cases of food poisoning and 150,000 fatalities worldwide each year (Nale et al. 2021; Sritha & Bhat, 2021).

S. enterica is responsible for approximately 99% of salmonellosis cases in humans, posing a significant threat to public health. Symptoms of this infection include diarrhea, fever, and abdominal cramps (Jajere 2019). The primary method of transmission is through the consumption of foods derived from contaminated animals, such as dairy products, meat, eggs, and seafood (Popa & Papa 2021; Sheng & Wang 2021). The remarkable inhibitory properties of antibiotics make them a popular choice for the biological control of *Salmonella* contamination. However, the misuse of antibiotics is currently contributing to the rise in the prevalence of antibiotic-resistant bacteria (AL-Mazini & AL-Hajaj 2015; Pan et al. 2023).

Salmonella enterica (*S. enterica*) subsp. *enterica* is the human-restricted agent that causes enteric fever, a severe infectious disease. The *Salmonella* genus comprises two distinct species: *Salmonella bongori* and *S. enterica*, with a total of more than 2500 serotypes. There are 1500 serotypes of *S. enterica* subsp. *enterica* (Lamas et al. 2018). *S. enterica* subsp. *enterica* serovar Typhi (*S.* typhi) and *Salmonella paratyphi* A, B, and C

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are highly adapted to the human host, serving as their typical reservoir (Gal-Mor et al. 2014).

Phages, the most frequently encountered organisms in the biological field (Evran et al. 2022; Sun et al. 2022), are a promising and highly effective biocontrol agent for the prevention and management of *Salmonella* contamination, including drug-resistant strains (Pereira et al. 2016; Li et al. 2021a; Gao et al. 2022). Bacteriophages are viruses that can specifically attach and infect bacteria, possessing strong proliferation abilities and a wide range of sources (LeLièvre et al. 2018). They have garnered significant attention in recent years due to their effective bactericidal properties and the benefits of leaving no residue. Phages that are not detrimental to the normal flora and specifically eliminate target strains, regardless of target strain resistance, present an interesting alternative to or complement existing therapies (Alsaadi et al. 2022).

Bacteriophages have been employed to control *Salmonella* growth in a variety of dietary products, including chicken (Pelyuntha & Vongkamjan 2022), egg fluid (Li et al. 2020), milk (Li et al. 2021b), and lettuce (Islam et al. 2021). Many studies in Basrah province, Iraq, implicate the use of bacteriophages as a biocontrol for different species of bacteria (Yaqoob et al. 2016; Jalil et al. 2017, 2019; Jamalludeen 2021).

The current study aims to isolate and characterize the bacteriophage against antibiotic-resistant *Salmonella* spp. recovered from Basrah Province, Iraq.

Materials and Methods

Bacteria strains and culture conditions

A total of seven multidrug-resistant (MDR) and extensively drug-resistant (XDR) *S. enterica* strains, representing two serotypes (*S. typhi* n=5, *S. typhimurium* n=1), were isolated from clinical samples (blood and stool of human) and used in this study, as shown in table 1. All strains were utilized for phage lytic range determination. *S. typhi* LC773420.1 was used as a host strain for phage isolation, propagation, and purification.

Bacteriophage Isolation and Purification

The method previously described by Kumari et al. (2009) and Golkar et al. (2013) was employed to isolate phages from poultry feces in Basrah, Iraq. Briefly, 200 mL of poultry feces (25g/L) were combined with 20 mL of bacteriophage broth (peptone (100g/L), beef extract (30g/L), yeast extract (50g/L), sodium chloride (25g/L), and potassium dihydrogen phosphate (80g/L)), 20 mL of MDR bacteria broth, and 20 mL of Brain Heart Infusion (BHI) broth. The mixture was subsequently transferred to a 1L sterile flask and incubated at 37°C for 48 hours with stirring at 120 rpm. After incubation, the mixture was centrifuged at 10,000xg for 10 minutes to remove solids. The supernatant was then filtered through a Millipore filter with a pore size of 0.45 μm.

Table 1 *Salmonella* isolates used in this study

Codes of isolates	Taxa
MJH1\ LC773417.1	*S.* typhi
MJH2\ LC773418.1	*S.* typhi
MJH3\ LC773419.1	*S.* typhi
MJH4\ LC773420.1	*S.* typhi
MJH7\ LC773421.1	*S. typhimurium*
MJH\ LC773422.1	*S. enterica*

Preparation of bacteriophage stocks

Bacteriophage stock preparation was conducted with following with Kropinski et al. (2009). A stock was generated by incubating 100 μL of bacteria with 1 mL of bacteriophage at 37°C for 20 minutes. After incubation, 4.5 mL of soft agar (0.7%) was added, and the mixture was incubated at 37°C for 18 hours. Using a sterile L-shape, the top layer of the BHI agar plate was removed, and 2 mL of SM buffer was added. The plate was then incubated in 10 mL vials at 4°C for 2-3 hours. Subsequently, 0.2 μL of chloroform was introduced, and the mixture was centrifuged at 4500xg for 15 minutes. The stock was maintained at 4°C after being filtered through a 0.45 μm Millipore filter.

Determination of host range

The efficacy of the *Salmonella* isolates phage was evaluated against seven *S. enterica* isolates using spot assays. Initially, 100 μL of the bacteria in BHI broth was added onto BHI agar plates and then incubated at 37°C for 24 hours. The culture was further incubated at 37°C for 24 hours after adding 10 μL of phage filtrate, except for the control which was left untreated. The plates were then examined, and the presence of a clear zone on the bacterial lawn was recorded as complete lysis (Karumidze et al. 2012). The specificity lysis profile of the isolated phage was determined using various isolates, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.

The plaque assay was conducted according to Kropinski et al. (2009), using serial dilutions (10^{-1} to 10^{-12}) and the agarose overlay method to determine the bacteriophage titer. For this, 900 μL of normal saline was combined with 100 μL of phage filtrate. Subsequently, 100 μL of each dilution was transferred to 100 μL of bacterial growth and incubated at 37°C for 20 minutes. The bacterial growth was then mixed with 4.5 mL of top agarose (7.0 g/L) and promptly poured onto BHI agar plates. After allowing the plates to dry for 30 minutes,

they were incubated at 37°C for 24 hours (Kropinski et al. 2009). Phage titer or plaque-forming unit (PFU) for each dilution was calculated using the following formula: Phage titer (PFU/mL) = (Number of plaques / Dilution factor) x volume of diluted phage added to the plate (mL). The dilution factor that gave the best countable number of plaques was selected and used for all other experiments.

Transmission electron microscopy (TEM)

The phage morphology was analyzed using a transmission electron microscope (Zeiss EM10C TEM) according to Ács et al. (2020).

Characterization of bacteriophages

Thermal stability testing was conducted according to the methodologies outlined by Harley & Prescott (1993) and Karumidze et al. (2012). The purified phage was exposed to varying temperatures of 50°C, 60°C, and 37°C (control) for durations of 10, 20, 30, 40, 50, and 60 minutes. Additionally, the phage's sensitivity to acidity and alkalinity was assessed by subjecting the purified phage to pH levels of 4.0, 7.0 (control), and 9.0 (Harley & Prescott 1993; Karumidze et al. 2012).

Phage DNA extractions

Phage DNA was extracted using the QIAprep Spin M13 kit (QIAGEN, Germany) according to the manufacturer's instructions from 100 mL of purified phage stocks with a titer of 10^6 plaque-forming units (PFU)/mL

Random amplified polymorphic DNA (RAPD) PCR analysis

Random amplification of polymorphic DNA was carried out according to the method described by Gutierrez et al. (2011) using four primers (5′-ACGCAGGCAC-3′; 5′-AACGCGCAAC-3′; 5′-CCGCAGCCAA-3′; 5′-AACGGGCAGA-3′). Purified phage DNA was subjected to RAPD PCR analysis using a thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, Germany) under the following conditions: four cycles at 94°C for 45 s, 30°C for 120 s, and 72°C for 60 s; 26 cycles at 94°C for 5 s, 36°C for 30 s, and 72°C for 30 s (with the extension step increased by 1 s for each new cycle); and a final step of 10 minutes at 75°C (Gutierrez et al. 2011).

Agarose gel electrophoresis

The amplicons were subjected to electrophoresis on a 0.8% agarose gel at a voltage of 80V for a duration of 55 minutes. The PCR marker with a size of 10,000 base pairs was subjected to electrophoresis to confirm the size of the amplified DNA fragment.

Sequencing of bacteriophage DNA

The PCR bands that were successfully amplified were forwarded to Macrogen Inc. (Macrogen Korea) for sequencing. Multiple alignments of each sample sequence with the NCBI database were conducted using the Basic Local Alignment Search Tool (BLAST) computer program for molecular identification of the samples. ***Statistical analysis*** The data were analyzed using SPSS version 28. The statistical significance of the difference in data was assessed by Chi-square. *P* values ≤ 0.05 were considered statistically significant. **Results** Phage was successfully isolated and showed high lytic activity against MDR and XDR *Salmonella* serovars (Typhi and Typhimurium).

Morphology of Bacteriophages

The morphology of the purified phage of *Salmonella* serovars (Typhi and Typhimurium) was identified. The phage was classified in the *Caudovirales* order and the *Myoviridae* family (figure 1). The software Image J 1.50i was used to measure the diameters of the phage. According to the microscopy analysis, the *Salmonella* phage possessed a long, inflexible contractile tail measuring 130.409×35.152 nm and an icosahedral head with a diameter of 111.4×99.431 nm.

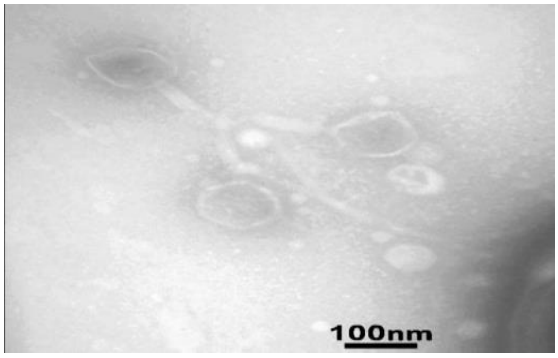

Fig 1. Negative stain TEM of *Salmonella* serovars phage (Scale= 100nm)

Host range of bacteriophage

The effectiveness of the lytic ability of *Salmonella* phage was tested against MDR and XDR *Salmonella* by spot test assay. The *Salmonella* phage showed a different host range, it was able to lyse 6 out of 7 strains (85.71%), while 1(14.29%) strain (MJH\ LC773422.1) was resistant with highly significant differences (P< 0.001). Based on the specificity of lysis, *Salmonella* phage had high lytic specificity against *S. typhi* and *S. typhimurium,* whereas there was no lytic activity against other species including *P. aeruginosa, K. pneumoniae,* and *S. aureus,* and bacterial growth was seen on the entire culture medium.

Bacteriophage Titration

A plaque assay was used to determine phage titration. The high titrations (PFU/mL) were 296×10^6 of *Salmonella* phage, as shown in table 2. These results revealed that a dilution factor of 10^{-6} of *Salmonella* phage was the best countable number of plaques. A *myoviridae* phage for *Salmonella* formed clear small sized plaques approximately 1-2 mm in diameter when spread on bacterial lawn of MDR and XDR *Salmonella* serovars.

Characterization of bacteriophage

Thermal Tolerances

The capacity of the *Salmonella* phage was evaluated at 37°C (as a control), 50°C, and 60°C (table 3). The results indicated that the number of plaques progressively decreased over time until reaching zero at 30 minutes at 50°C and 20 minutes at 60°C. This observation was based on plaque formation. Statistical analysis showed highly significant differences ($P<0.000$) in phage titers at 37°C compared to phage titers at 50°C and 60°C. Additionally, there were significant differences ($P< 0.000$) between the phage titers at 50°C and 60°C.

Resistance to acidity and alkalinity Sensitivity and resistance to pH ranges of 4.0, 7.0, and 9.0 were assessed. Table (4) demonstrated that both phages were capable of surviving within the pH range of 4.0 to 7.0. However, the phage of *Salmonella* serovars exhibited sensitivity and no activities at pH 9.0, with highly significant differences ($P<0.000$).

Molecular Characterization of Salmonella phageRAPD-PCR Analysis

DNA bands of the RAPD-PCR products of *Myoviridae* (figures 2 and 3) show the RAPD-PCR products performed using four different primers. Two of these primers produced several bands (table 5) on 0.8% agarose gel when compared to the DNA ladder (10.000 bp).

Sequencing of Salmonella phage

The bands of the *Myoviridae* phage were submitted for sequencing. The two bands of the *Salmonella* phage were successfully sequenced and registered in NCBI under accession numbers LC807147.1 and LC807148.1. Molecular identification of the two bands of *Salmonella* phage was performed by multiple alignments of each sequence with the NCBI database using the BLAST computer program.

Discussion

Salmonella is a significant foodborne pathogen that substantially impacts global food producers and public health systems (Besser 2018). Typhoid fever remains a major and persistent public health concern, with the World Health Organization (WHO) estimating 27.3 million cases in 2010 (Date et al. 2014).

As MDR and XDR bacteria become more prevalent and antibiotics prove ineffective in eliminating these pathogens, it is crucial to explore alternative antimicrobials. This highlights the importance of phage therapy as an effective tool for combating antibiotic-resistant bacteria. Bacteriophages are a viable alternative

Table 2 Titration of *Salmonella* serovars phage

Volume of phage plated (mL)	Dilution factor (DF)	Plaque per plate	Plaque forming unit (PFU/mL)
1	10^{-1}	Complete lysis	-
1	10^{-2}	Complete lysis	-
1	10^{-3}	Complete lysis	-
1	10^{-4}	Complete lysis	-
1	10^{-5}	Uncountable	-
1	10^{-6}	296	296×10^6
1	10^{-7}	90	9×10^8
1	10^{-8}	58	58×10^8
1	10^{-9}	40	4×10^{10}
1	10^{-10}	28	28×10^{10}
1	10^{-11}	26	26×10^{11}
1	10^{-12}	10	1×10^{13}
P value	-	0.000*	0.000*

***Statistical significance**

Table 3: Thermal tolerance of bacteriophage

Time (min.)	Volume of phage plated (mL)	Temperature			Dilution factor of *Salmonella* phage	*P* value of 37 °C	*P* value of 50 °C	*P* value of 60 °C
		Salmonella phage						
		37°C	50°C	60°C				
		Phage titer (PFU/mL)						
10	1	296×10^6	5×10^6	2×10^6	10^{-6}	0.000	0.000	0.000
20	1	296×10^6	1×10^6	0	10^{-6}	0.000	0.000	0.001
30	1	296×10^6	0	0	10^{-6}	---	---	
40	1	296×10^6	0	0	10^{-6}	---	---	
50	1	296×10^6	0	0	10^{-6}	---	---	
60	1	296×10^6	0	0	10^{-6}	---	---	
P value			0.219 (NS)*					

Table 4: Acidity and alkalinity sensitivities of bacteriophage

pH value	Phage titer (PFU/mL)
4.0	3×10^6
7.0	25×10^7
9.0	0
P value	0.000*

* **Statistical significance**

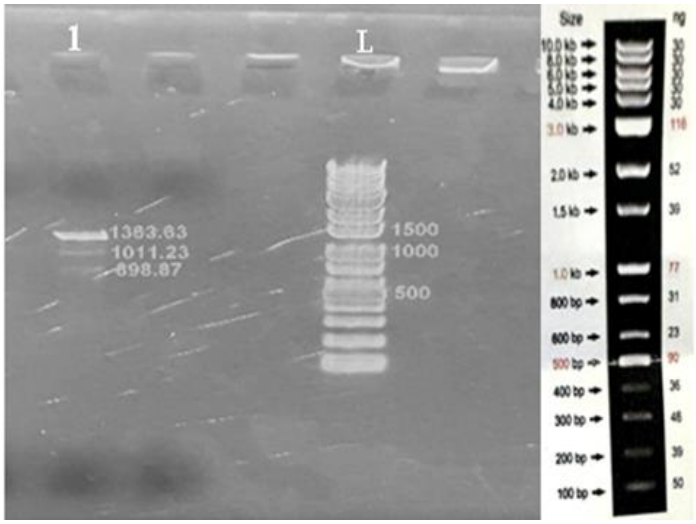

Fig 2. RAPD-PCR products of the Myoviridae phage. Lane L corresponds to a 10,000 base pair DNA ladder, while lane 1 displays bands of primer number 3.

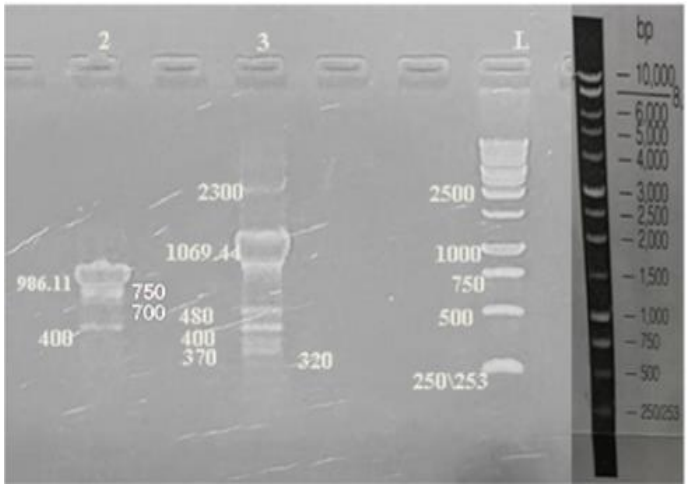

Fig 3. RAPD-PCR products of *Myoviridae* phage. Lane L corresponds to a 10,000 base pair DNA ladder, while lane 2 displays bands of primer number 3 and lane 3 displays bands of primer number 4.

Table 5 Amplified fragments size of RAPD-PCR products of *Salmonella* phage.

Family	Primer	Lane	Bands		
	3	1	898.87	1011.23	1383.63
Myoviridae	3	2	400	700	750,986.1
	4	3	320	370	400
	4	3	480	1069.44	2300

to antibiotics. The National Institute of Allergy and Infectious A 2014 report by the National Institute of Allergy and Infectious Diseases recognized phage therapy as one of seven methods in a coordinated approach to address antibacterial resistance (Rattanachaikunsopon & Phumkhachorn 2006; Viertel et al. 2014).

The present study demonstrates the lytic efficacy of specific bacteriophage against multidrug-resistant strains of *Salmonella* serovars. Phage therapy holds significant potential as an alternative or complementary approach to antibiotics, as this study identified bacteriophage that effectively suppressed the growth of MDR and XDR *Salmonella* serovars. Numerous studies have confirmed the diversity and abundance of bacteriophages. Recently, *Salmonella* phages have been isolated from municipal wastewater plants (Hong et al. 2018; Huang et al. 2018; El-Dougdoug et al. 2019), commercial broiler houses (Andreatti Filho et al. 2007), raw chicken skin and gizzard (Duc et al. 2018), and other sources. It has been reported that phages isolated from farm environments exhibit considerable phenotypic and genetic diversity and can lyse various *Salmonella* serovars associated with human salmonellosis (Switt et al. 2013). The studied phage, which exhibited distinct virulence characteristics, showed a significant decrease in efficacy at 60°C for 30 minutes compared to other bacteriophages, as observed in a study by Dallal et al. (2019).

The host range of phages refers to the specific categories of organisms, such as genera, species, subspecies, or strains that a bacterial virus can infect. This trait is crucial in identifying and distinguishing a particular phage (Kutter 2009). The *Salmonella* phage was capable of lysing 85.71% of the tested strains (6 out of 7), while 14.29% (1 strain) showed resistance, highly significant difference ($P< 0.001$). The lack of efficacy of the *Salmonella* phage against all MDR and XDR *Salmonella* isolates may be attributed to changes in receptors that result in a decrease in the strength of their binding (Nikkhahi et al. 2017). The selectivity of bacteriophages for host cells is strongly linked to the affinity of binding between the receptors on the host and the receptor-binding proteins on the bacteriophages (Nzouankeu et al. 2010; Choi et al. 2013). Modifications in the receptors on the surface of host cells cause bacteriophage resistance, leading to a decrease in the ability of the phages to lyse the cells (Ellis & Delbruck 1939; Bonyadian et al. 2007).

Conclusion

The phage obtained in this investigation has a significant degree of lytic activity against multi-drug resistant (MDR) and extensively drug resistant (XDR) *Salmonella* serovars, specifically Typhi and Typhimurium. This phage demonstrates a strong level of selectivity, since it does not display any action against *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*. This particular characteristic allows it to be utilized for the authentication of diagnoses of these distinct *Salmonella* serovars (Typhi and Typhimurium). The lytic activity of the phage is maximized at a pH of 7, and it remains effective within the pH range of 4 to 7. The RAPD PCR approach is widely regarded as the most effective technique for diagnosing phages in unfamiliar environmental samples.

Ethical approval and consent to participate

The study was approved by the Ethics Committee of Basrah Health directorate\ Training and Development Division in document No. 141\2021 dated 29 \12\2021 and complied with guidelines and principles of the Declaration.

Conflict of interest

The authors declare that they have no conflict of interest.

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