Indian J Anim Health (2023), 62(2): 340-346
DOI: https://doi.org/10.36062/ijah.2023.15522

Research Article

Characterization of bacteriophages isolated against *Salmonella Enterica* spp. from Izatnagar, India

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Abstract

***Salmonella* spp. is a worldwide food borne pathogen, and chicken is regarded as a main reservoir for zoonotic pathogen. *Salmonella* Typhimurium, *S.* Enteritidis and *S.* Gallinarum are important serotypes implicated with poultry. In this study, two bacteriophages (φSEIz2 and φSGIz3) were isolated from sewage water samples against *S.* Enteritidis and *S.* Gallinarum, respectively. Transmission Electron Microscopy revealed that phages belonged to the family *Myoviridae* morphotype A and subdivision A1. The phages showed wide host range against different strains of *Salmonella* spp. The phages were found to be stable between pH 4 to 11 and temperature between 4°C to 70°C.**

Keywords: Bacteriophage, characterization, *Salmonella* Enteritidis, *Salmonella* Gallinarum, TEM

Highlights

- TEM revealed that bacteriophage φSEIz2 and φSGIz3 belong to the family *Myoviridae* morphotype A and subdivision A1.
- The bacteriophages revealed lytic activity against different strains of *Salmonella* spp.
- Bacteriophages survived at temperatures ranging from 4°C to 60°C, and the phages remained stable between pH 4 and 11.

INTRODUCTION

Salmonella remains one of the most important pathogens responsible for causing gastroenteritis, bacteremia and enteric fever. It is estimated that *Salmonella* causes 93.8 million human infections and 155,000 deaths annually worldwide (Hendriksen *et al.*, 2011). In India, salmonellosis pattern of distribution is hyper-endemic (Geetha and Palanivel, 2018). Globally, around 86% of salmonellosis cases are foodborne (Majowicz *et al.*, 2010). The foods commonly associated with salmonellosis are foods of animal origin, such as egg (Popa and Papa, 2021), poultry, pork (Wales *et al.*, 2011), beef and raw dairy products (Freitas *et al.*, 2010). Of different sources, chicken meat and eggs are the major ones (Gupta *et al.*, 2020). There is an urgent need for prevention and control of its occurrence.

Bacteriophages are the most widespread entities on the earth's surface, phages with total numbers estimated from 10^{30} to 10^{32} (Balcazar, 2014). Virulent phages cause bacterial host cell lysis and not only function to control bacterial populations but also as tools for identifying (typing) specific bacterial strains (Rogovski *et al.*, 2021).

A bacteriophage particle consists of a single or double-stranded (ss or ds) DNA or RNA molecule, encapsulated inside a protein or lipoprotein coat. Several methods such as electron microscopy to ascertain morphology, genome sizing, restriction enzyme profiling, proteomics etc. have been used to characterize bacteriophages.

All *Salmonella* phages reported thus far belong to the order *Caudovirales* (tailed phages) and represent three families: the *Siphoviridae*, *Podoviridae* and *Myoviridae*. De Lappe *et al.* (2009) found that 16 phages of *S.* Enteritidis belonged to these three families. Of these, only a small fraction is lytic (Kropinski *et al.*, 2007). Several *Salmonella* phages and prophages have been reported (e.g., FelixO1, Fels-1, P22, Gifsy-2), the diversity of *Salmonella* phages is still severely under-sampled, and our knowledge of the genomic diversity

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of *Salmonella* phages associated with different environments is minimal.

Bacteriophages have been exploited in several practical applications. Phages are used as indicators of bacteria, as typing reagents, for targeted biological control of human, animal, and plant bacterial diseases, as bio-control agents for the food industry, phage therapy (McLaughlin and King, 2008) and vaccination. Characterization of their unique biological properties is a prerequisite to exploring these potential (Carey-Smith *et al*., 2006). The primary objective of this work was to isolate the bacteriophages attacking *S*. Enteritidis and *S*. Gallinarum and characterize them for their morphologies, host ranges and growth characteristics.

MATERIALS AND METHODS

Bacterial strains: Bacterial strains (Table 1) used in the study were procured from the repository of the National Salmonella Centre (Veterinary), Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar. The isolates were tested for their purity, morphological, cultural and biochemical characteristics as per the methods of Edwards and Ewing (1972). *Salmonella* Enteritidis (SE) E-2487 and *Salmonella* Gallinarum (SG) E-76 are the two strains used for the isolation of bacteriophages.

Table 1. List of bacterial isolates used in the study

S.No.	Strain No.	Serotype
1	E-4321	*S.* Typhimurium
2	E-2478	*S.* Enteritidis
3	E-76	*S.* Gallinarum
4	E-1460	*S.* Virchow
5	E-1426	*S.* Saintpaul
6	E-4807	*S.* Typhimurium
7	E-2368	*S.* Gallinarum
8	E-810	*S.* Abortusequi
9	E-1472	*S.* Virchow
10	E-155	*S.* Abortusequi
11	E-157	*S.* Abortusequi
12	E-154	*S.* Pullorum
13	E-155	*S.* Pullorum
14	E-161	*S.* Enteritidis
15	E-4671	*S.* Enteritidis
16	IVRI-127	*Staphylococcus aureus*
17	E-413	*E. coli*

Isolation of bacteriophages: Bacteriophages attacking *S*. Enteritidis and *S*. Gallinarum were isolated in the present study. Sewage samples (45 mL) obtained from poultry farms, Central Avian Research Institute, Izatnagar, Bareilly were mixed with 5 mL of decca strength of New Zealand Casamino Yeast extract medium (NZCYM) broth along with 1 mL of the exponential growth phase cultures of *Salmonella* Enteritidis E-2487 and *Salmonella* Gallinarum E-76 in separate flasks. After 24 h incubation at 37°C, 10 mL of the suspension was centrifuged at 8000 g for 15 min, and the supernatant was filtered through a syringe filter of 0.22 µ. The spot test assay was performed by placing 10 µL of the supernatant on nutrient agar seeded with respective strains of *Salmonella* Enteritidis and *Salmonella* Gallinarum. The plates were observed for plaques after 24 h at 37°C. Bacteriophage titer was estimated by double agar layered method of Mohamed *et al.*, 2020.

Phage purification and lysate preparation: The purification of phage was done by consecutive isolation of single plaque until homogenous plaques were procured by the standard method described by Maszewska and Rozalski, 2019. The isolated single plaque of phage is further processed in NZCYM broth culture with its indicator organism, and was incubated further at 37°C for 24 h. The filtration of lysate was done through a 0.22µ syringe filter and was tested for the presence of phage against the host strains by double agar layered method. The process was repeated 3 times in order to purify the bacteriophage, and a stock of about 10 mL of each purified phage was prepared and stored at 4°C.

Phage concentration: The concentration of phage (titre having $\geq 10^{10}$ pfu/mL) in the phage stock was established as per the method of (Carroll-Portillo *et al.*, 2021) with some modifications. Briefly, NaCl (1M) and polyethylene glycol (PEG) 8000 (10% w/v) were added and kept at 4°C overnight. Then, pelleted by centrifugation at 11,000 g for 10 min, and pellet was resuspended in 0.01 original volume sterile with sodium chloride magnesium sulfate (SM) diluents (50 mL 1M Tris-HCl (pH-7.5), 5.8 g NaCl, 2 g $MgSO_4$, 2% gelatin) and stored at 4°C.

Phage count of phage stock: The phage φSEIz2 and φSGIz3 with high titre were stored as a suspension at 4°C and for lyophilized preparation at -20°C. The phage titers of the preserved bulks were monitored at regular intervals during the entire study period. At the beginning of the study, phage count was found to be 3×10^{11} and 5×10^{11} PFU/mL, respectively for φSEIz2 and φSGIz3 phage; no significant reductions in the counts were recorded during 1 year of storage at 4°C. The phage count of the freezedried lot was found to be 2.1×10^{10} and 3.4×10^{10} PFU/mL, respectively for φSEIz2 and φSGIz3 phage which remained stable throughout the study period.

Bacteriophage morphology: For the fixation of concentrated phage (10^{11} pfu/mL), 1/10^{th} volume of 2.5% buffered glutaraldehyde solution was added and kept at room temperature for few minutes. Negative staining of bacteriophages with 2% uranyl acetate and transmission electron microscopy (Morgagni 268D, FEI Company) operating at an 80-kV accelerating voltage was performed at All India Institute of Medical Sciences (AIIMS), New Delhi. Bacteriophage morphology and dimensions were recorded.

Determination of host range of phages: The lytic range of the phage was carried by spot test assay against 12 strains of *Salmonella* and one each of *E. coli* and *Staphylococcus aureus* (Table 1). Ten µL of bacteriophage preparation (~10^{11} pfu/mL) was spotted on the lawn cultures of the different *Salmonella* strains. The plates were observed for the appearance of clear zones after incubation at 37°C for 24 h.

Stability of bacteriophage on varying pH and temperature: To test the pH stability, the bacteriophage was incubated in NZCYM broth with pH varying from 1 to 12 (adjusted using NaOH and HCl) at 37°C for 24 h. To test heat stability, the bacteriophage was incubated in a water bath in test tubes at each temperature (-20°C, 4°C, 25°C, 37°C, 45°C, 50°C, 60°C, 70°C, 80°C, 90°C) for 60 min. The bacteriophage titer was conducted by double agar layered assay.

RESULTS

Bacteriophage isolation: Bacteriophages against *S.* Enteritidis and *S.* Gallinarum were successfully isolated in this study. *S.* Enteritidis phage plaques were about 2-3 mm in diameter and appeared as circular spot with double zone of lysis; inner zone was clear, and outer zone was slightly turbid with well-defined edges (Fig. 1), while *S.* Gallinarum plaques were about 4-5 mm diameter circular spot with clear double zone of lysis and well-defined edges (Fig. 1). The two phages were designated as φSEIz2 and φSGIz3 for *S.* Enteritidis and *S.* Gallinarum, respectively.

Host range of bacteriophages: It was observed that phage φSEIz2 was lytic to 6 of 12 isolates, whereas phage φSGIz3 was lytic to 8 of 12 isolates of *Salmonella* spp. tested. However, both the phages did not form any plaque against *S.* Saintpaul, *S.* Virchow and also against *Staphylococcus aureus* and *E. coli.*

Bacteriophage microscopy: The morphologies of the two phages were determined by transmission electron microscopy (TEM). Phage φSEIz2 had hexagonal head with diameter of 66.82±2 nm and long, straight contractile tail with length of 197.24±3 nm (Fig. 2). Phage φSGIz3 had hexagonal head with diameter of

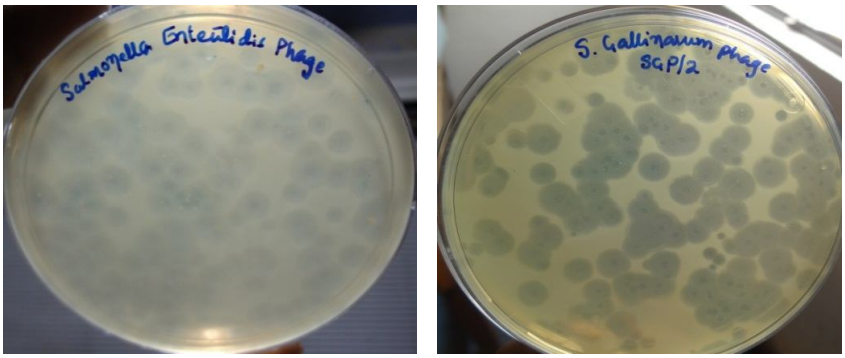

Fig. 1. Plaque morphology of phages φSEIz2 and φSGIz3; Bacterial lawn of A) *Salmonella* Enteritidis (φSEIz2) B) *Salmonella* Gallinarum (φSGIz3) on NZCYM agar showing differing plaque morphologies of phages

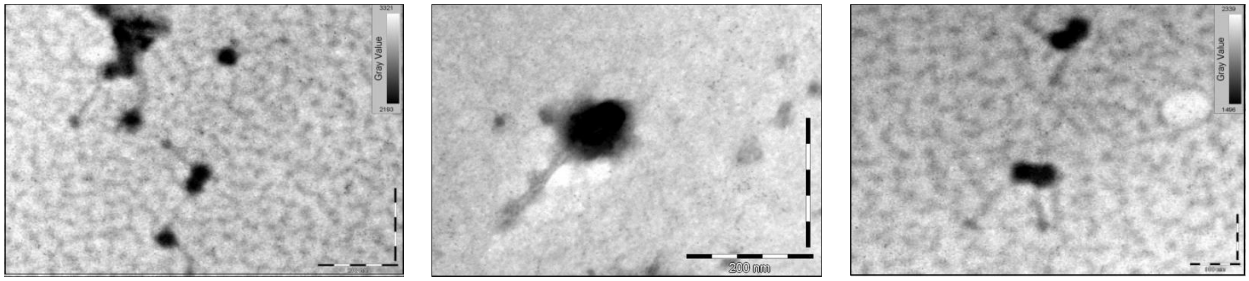

Fig. 2. Transmission electron micrograph of phages of *Salmonella* Enteritidis phage (φSEIz2)

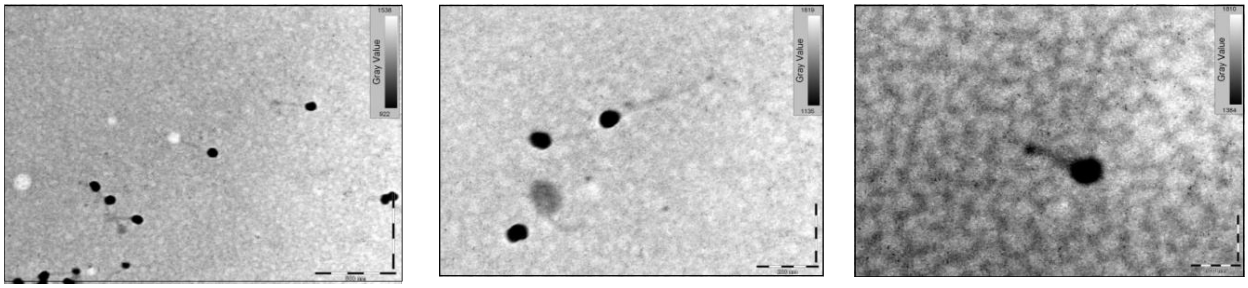

Fig. 3. Transmission electron micrograph of phages of *Salmonella* Gallinarum phage (φSGIz3)

64.23±1 nm, and long, straight contractile tail with length of 150.29±5 nm (Fig. 3). Morphologically, both phages resembled to members of family *Myoviridae* further classified into subcategory morphotype A, subdivision A1.

Effect of different physico-chemical conditions of pH and temperature on survivability of bacteriophages:

The phages φSEIz2 and φSGIz3 were almost stable from pH 4 to pH 11, indicating that the phage could survive wide variations of pH. Phage φSEz2 revealed that 16% of the phage were inactivated at pH 4 and 11% reduction was observed at pH 11 (Table 2, Fig. 4), while phage φSGIz3 showed about 12% reduction at pH 4 and 9% reduction at pH 11(Table 2, Fig. 4). In the acidic pH of

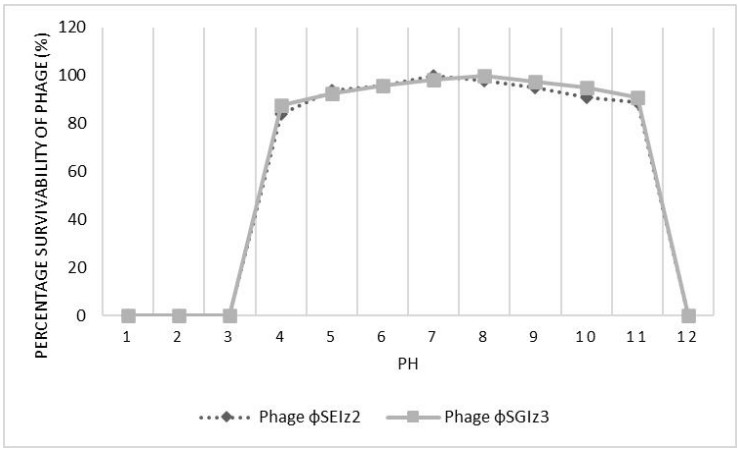

Fig. 4. Effect of pH on stability of *Salmonella* Enteritidis phage (φSEIz2) and *Salmonella* Gallinarum phage (φSGIz3)

Table 2. Stability of phages at different pH

	φSEIz2		φSGIz3	
pH of medium used	**Count of phage after exposure (PFU/mL)**	**Percentage survivability of phage (%)**	**Count of phage after exposure (PFU/mL)**	**Percentage survivability of phage (%)**
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	1.2×10^9	83.9	1.05×10^9	87.5
5	1.34×10^9	93.7	1.11×10^9	92.5
6	1.37×10^9	95.8	1.15×10^9	95.8
7	1.43×10^9	100	1.18×10^9	98.3
8	1.4×10^9	97.8	1.2×10^9	100
9	1.36×10^9	95	1.17×10^9	97.5
10	1.3×10^9	91	1.14×10^9	95
11	1.27×10^9	89	1.12×10^9	91
12	0	0	0	0

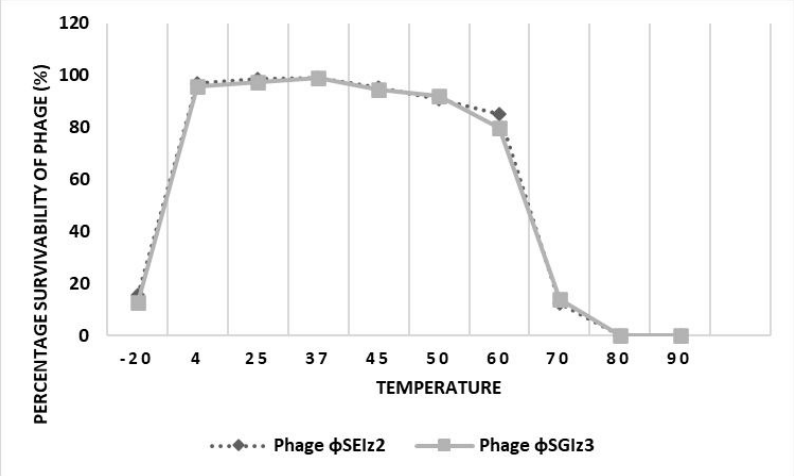

Fig. 5. Effect of temperature on stability of *Salmonella* Enteritidis phage (φSEIz2) and *Salmonella* Gallinarum phage (φSGIz3)

Table 3. Stability of phages at different temperature

Temp in degree celsius	φSEIz2		φSGIz3	
	Count of phage after exposure (PFU/mL)	Percentage survivability of phage (%)	Count of phage after exposure (PFU/mL)	Percentage survivability of phage (%)
-20	2.4×10^8	15.6	2.11×10^8	12.8
4	1.49×10^9	97	1.58×10^9	96
25	1.51×10^9	98.6	1.6×10^9	97.5
37	1.52×10^9	99.3	1.62×10^9	99
45	1.46×10^9	95.4	1.55×10^9	94.5
50	1.4×10^9	91	1.51×10^9	92
60	1.3×10^9	85	1.32×10^9	80
70	1.9×10^8	12.4	2.3×10^8	14
80	0	0	0	0
90	0	0	0	0

3 and alkaline pH of 12, no visible plaques were noticed in both the phages.

Thermal stability of phages revealed that 85% to 94% of the phages were still capable to infect host cells after 60 min of heat at 50°C to 60°C. The phages were found stable between temperatures 4°C to 60°C. Phage φSEIz2 revealed that 12.4% of phages survived after treatment at 70°C and 15.6% of phages survived at -20°C (Table 3, Fig. 5). However, phage φSGIz3 showed that only 14% phage survived at 70°C and 12.8% phage survived at -20°C (Table 3, Fig. 5). At a temperature of 80°C and 90°C the titer of the three phages was zero.

DISCUSSION

The two phages isolated in this present study were designated as φSEIz2 and φSGIz3 isolated against *S.* Enteritidis and *S.* Gallinarum, respectively. Bacteriophage against *S.* Gallinarum (φSGIz3) was lytic to 8 of 12 *Salmonella* isolates tested but did not produce plaque for two *S.* Abortusequi isolates, 2 isolates of *S.* Virchow and 1 isolate of *S.* Saintpaul. Phage φSEIz2 showed restricted host range as it showed lytic activity against 6 out of 12 *Salmonella* isolates tested. Interestingly, both phages did not reveal any activity against either *Staphylococcus aureus* (IVRI-127) or *E. coli* (E-413). Similar findings were obtained by (Sun *et al.*, 2022) in which the phage (PSDA-2) was unable to show lytic effect against *E. coli* and some strains of *Salmonella*. *Salmonella* bacteriophages are generally host specific, and they are seen to infect only single bacterial species or only single serotype within a species (Lu *et al.*, 2020). However, there are studies conducted on phages which revealed lytic effect on wide range of bacterial species and even crossing the genus barrier

(Lu *et al.*, 2020). In contrast to our findings, there are reports of *Salmonella* phage that infects multiple genera (Bielke *et al*., 2007).

Transmission electron microscopy vistualized that phage φSEIz2 had hexagonal head with diameter of 66.82±2 nm, and long, straight contractile tail with length of 197.24±3 nm, and phage φSGIz3 had hexagonal head with diameter of 64.23±1 nm, and long, straight contractile tail with length of 150.29±5 nm. Morphologically both phages revealed similarity with the members of the family *Myoviridae* classified into subcategory morphotype A, subdivision A1. This was in accordance to the result obtained by many workers (De Lappe *et al.*, 2009; Santos *et al.*, 2010; Whichard *et al.*, 2010; Lee *et al.*, 2011; Anjay *et al.*, 2022; Bryan *et al.*, 2023). The bacteriophages having large tail viruses belong to the order *Caudovirales* (Ackermann, 1998), 44 *Salmonella* phages belonged to family *Myoviridae*, 68 to *Siphoviridae* and 64 to *Podoviridae* (Whichard *et al.*, 2010).

Both the phages (φSEIz2 and φSGIz3) were almost stable between pH 4 to 11, indicating that the phage could survive in wide variations of pH. Phage φSTEz2 revealed 16% of the phage was inactivated at pH 4, and 11% reduction was observed at pH 11, while phage φSGIz3 showed about 12% reduction at pH 4 and 9% reduction at pH 11. Similar results were recorded by Sun *et al.* (2022), who reported that the phage (PSDA-2) also survived a wide pH range between 3 to 10. Their stability in a wide range of pH can be described by the existence of abiotic and biotic factors in the sewage where the pH can oscillate from acidic to basic. The factors responsible for acidic nature of the sewage may be due to the abundance of aerobic and anaerobic bacterial communities present in the sewage water and their metabolic activities. Similarly, the new addition of sewage from households and industries may influence the pH bias towards basic. This oscillation in the pH might have induced the phages to adapt it to survive in wide pH environments (Sridhar *et al.*, 2013). The affinity for an alkaline environment may also depend on its source of isolation (Siragusa *et al.*, 2008).

Thermal stability of phages revealed that 85% to 94% of the phages were still capable of infecting host cells after 60 min of heat treatment at 50°C to 60°C. The phages were found stable between temperatures 4°C to 60°C.

Response of phages on exposure to varying temperatures is considered as a key model which suggests the ability of the organism under question to adapt to novel environments (Sun *et al.*, 2022). This was in accordance with the results obtained by Augustine *et al*. (2012), who obtained that phage was able to withstand high temperature of 80°C without affecting phage viability.

Data reported in this study indicates that owing to its lytic nature, broad host range, thermal and pH stability, the two bacteriophages can be further exploited to be used as therapeutic, bio-control agents and immunization agents for control of salmonellosis.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: PK: Collection of samples, microbiological work; RKA, MR: Conception and design; HZ, MSK, LJ: Checking the manuscript and data interpretation.

ACKNOWLEDGEMENTS

The authors are grateful to the Director, Indian Veterinary Research Institute, Izatnagar (U.P) for providing all the necessary facilities to carry out the above research work.

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Received- 15.12.2022, Accepted- 26.07.2023, Published-12.09.2023 (Online), 01.12.2023 (Print)
Section Editor: Dr. I. Samanta, Associate Editor