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# Isolation and Characterization of Lytic Bacteriophage (φSTIz1) against *Salmonella enterica* serovars Typhimurium

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**One new bacteriophage against *Salmonella* Typhimurium designated φSTIz1 was isolated from sewage of poultry farm located at Bareilly, Uttar Pradesh. Ultrastructural (Transmission Electron Microscopy; TEM) analysis revealed that phage had round head with diameter of 52.95±3 nm, and long, noncontractile tail with length of 190.28±5 nm indicating that it belonged to the order *Caudovirales* and family *Siphoviridae* morphotype B2. The isolated bacteriophage was found to be double-stranded DNA. SDS-PAGE profile of bacteriophage revealed four structural proteins ranging from 59 KDa to 16 KDa. Host range analysis indicated that the bacteriophage was lytic to 9 of the 12 isolates of *Salmonella* tested and did not show lytic activity against *S.* Saintpaul, *S.* Virchow, *Staphylococcus aureus* and *E. coli.* The phage was found to be stable between the pH 4 to 11 and temperature between 4°C to 60°C.**

**Key words**: Bacteriophage, host range, Protein analysis, *Salmonella* Typhimurium, stability

Salmonellosis is one of the major causes of food borne disease worldwide and poultry is the main reservoir for its transmission to human. In India, salmonellosis is reported to be hyperendemic affecting both man and animal (Prakash *et al.*, 2005). To control *Salmonella* in animals and foodstuffs preventive measures should be applied from farm to fork. The first approach is to reduce the prevalence of *Salmonella* in farms and subsequently decrease its transmission through food chain. Thus, there is a need to find an acceptable, cost effective way of preventing infection of poultry with *Salmonella.* There is a renewed attention in phages in recent years as a possible antibiotic alternative, as biocontrol agent in food industry and as therapeutic and prophylactic agent against bacterial infections.

Bacteriophages are natural viral pathogens of bacteria. They are ubiquitous and found abundantly in environment and can be isolated from water, sewage and soil. The phages are non living entity outside the host cell and they require host cell for replication and other metabolic processes (Carlton, 1999). All phages consist of protein and nucleic acid. The nucleic acid may be either DNA or RNA but not both, either they may be single stranded or double stranded. Lytic bacteriophages infect bacterial cells multiplying until the bacteria lyses discharging new

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bacteriophages particles (Toro *et al.,* 2005). Host specificity has advantage that it does not kill other bacteria but demerit with narrow host range that it cannot be applied as biocontrol agent (Greer and Dilts, 1990). Phages have been successfully applied as biocontrol agent for *Salmonella* and other pathogens in human and animal showing advantages over antibiotics (Barrow and Soothill, 1997; Matsuzaki *et al.,* 2005; Petty *et al.,* 2007; Skurnik *et al.,* 2007). However, sometimes high specificity of phages produced in their natural host (Bielke *et al.,* 2007) may be a problem due to release of cell debris, both endotoxins and exotoxins and also the presence of live cells in the crude phage lysate (Clark and March, 2006; Skurnik and Straunch, 2006). This can be prevented by using a non pathogenic host instead of pathogenic bacteria (Bielke *et al.,* 2007). Most bacteriophages are host specific, which limits its application as biocontrol agent (Greer and Dilts, 1990). Therefore, the objective of the present study was to isolate and characterize bacteriophage against *Salmonella* with broad host range.

## MATERIALS AND METHODS

### Bacterial strains

The strain of *Salmonella enterica* serovars Typhimurium (E-4231) used in the study for isolation of bacteriophage was taken from the repository of the laboratory. Other strains (Table 1) used for host range study were also available in the laboratory. All the strains were tested for their purity, morphological, cultural and biochemical characteristics as per the methods of Edwards and Ewing (1972). The strains were maintained on the nutrient slant.

### Isolation of bacteriophage

Sewage samples were obtained from poultry farms, cattle farm, sheep and goat farm, Izatnagar, Bareilly. Sewage samples (45 ml) and 1ml of the exponential growth phase culture of *S.* Typhimurium were mixed with 5 ml of decca strength of NZCYM broth and incubated with shaking at 37°C. After 24 h incubation 10 ml of the suspension was centrifuged at 10,000 g for 15 min and supernatant was filtered through syringe filter of 0.22 µ. The supernatant was tested by a spot test assay by placing 10 µl on nutrient agar seeded with different strains of *S.* Typhimurium. The positive sample was further tested by plaque assay.

### Phage purification

The phage was purified by successive single plaque isolation until homogenous plaques were obtained by the standard procedure described by Sambrook and Russell (2001). A single plaque of phage isolate was transferred by picking it with help of sterile Pasteur pipette to NZCYM broth culture of its indicator organism, and was incubated further at 37° C for 24 h. The lysate was filtered through 0.22 µ syringe filter and was tested for the presence of phage against the indicator strain by double agar layered method. The process was repeated 3 times in order to purify the bacteriophage and a stock of about 10 ml each purified phage was prepared and stored at 4°C.

### Bacteriophage stock preparation

One hundred µl of log phase broth culture of *S.* Typhimurium and 100 µl phage was added into 5 ml of soft NZCYM agar (0.75% agar) at 45°C water bath and immediately poured onto hard nutrient agar plate with gentle mixing. Plates were incubated at 37°C for overnight. The phage lysate was harvested after eluting with 4 ml SM diluents (50 ml 1M Tris-HCl (pH-7.5), 5.8 g NaCl, 2 g $MgSO_4$, 2% gelatin) and kept at 4°C for 6 h. The harvested suspension was clarified by centrifugation (10,000 g 15 min at 4°C), decanted, and filtered through 0.22 µ PVDF filters and stored at 4°C. Bacteriophage titer of final stock was determined by 10 fold serial dilution of phage by double agar layered method of Adams (1959).

### Phage concentration

Phage stock having titre $10^{11}$ plaque forming unit/ml (pfu/ml) was concentrated according to the method of Yamamoto *et al.* (1970) 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 11,000x g for 10 min and pellet was resuspended in 0.01 original volume sterile with SM diluent and stored at 4°C.

### Enumeration of phage in concentrated Stock

The phage count of the sterile stock was determined by agar overlay technique. Phage concentrate was serially diluted (10 fold) in PBS (7.2) and 100 µl of each dilution was mixed in 5 ml soft NZCYM agar (0.75% agar) held at 47°C in water bath, seeded with log phase culture of *S.* Typhimurium. After mixing properly, the soft agar

in each tube was plated on hard nutrient agar (3% agar) plates and left for solidifying. All plates were then incubated at 37ºC for 24 h. Number of plaques was counted and the phage count of the concentrate was determined.

**Bacteriophage morphology**

The concentrated phage ($10^{11}$ pfu/ml) was fixed by adding 1/$10^{th}$ volume of 2.5% buffered gulteraldehyde solution for minutes at room temperature. 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. Morphological characteristics were used to classify phage following the guidelines of the International Committee on Taxonomy of Viruses (Ackermann, 2000; O' Flynn *et al.*, 2004).

**Determination of host range of phages**

The lytic range of the phage was determined by spot test 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 bacterial 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 determine the pH stability, phage φSTIz1 was incubated in NZCYM broth with pH varying from 1 to 12 by adding either 0.1 N NaOH and 0.1 N HCl). A 100 µl of the (2x$10^{10}$ pfu/ml) was added into 900 µl of NZCYM broth of different pH values in microcentrifuge tubes and kept at 37ºC for 24 h. For thermal stability test, phage φSTIz1 was treated at different temperatures at (25º C, 37º C, 45º C, 50º C, 60º C, 70º C, 80º C, 90ºC). It was done by adding 100 µl of phage φSTIz1(2x$10^{10}$ pfu/ml) was added to 900 µl of NZCYM broth in mirocentrifuge tubes and heating at respective temperatures in water bath for 60 min and then cooled on ice for 30 sec before proceeding for plaque assay. The isolated phage was also tested for stability at -20ºC and 4 ºC for varying period upto one year.

**Extraction of bacteriophage nucleic acid**

Extraction of nucleic acid from bacteriophage was performed according to the method of Sambrook and Russell (2001) with few modifications. The bacteriophage was treated with RNase A and DNase I and incubated for 15 min at 37ºC. It was centrifuged at 10,000 g for 10 min and supernatant was resuspended with 0.5 M EDTA, 5M NaCl and 5% CTAB and proteinase k (100 mg / ml final concentration) and incubated at 65ºC for 10 min. Proteins were removed by two extractions with phenol/chloroform/ isoamyl alcohol and DNA was precipitated with isopropanol. After washing in 70% ethanol, pellets was resuspended in TE (10 mM Tris pH 8.0, 1m M/EDTA).

**Nucleic acid characterization**

The genomic nucleic acid of bacteriophage was treated with DNase I, RNase A and S1 Nuclease (Thermo-scientific). Each digestion was performed in 20 µl volume, which consisted of ~250 ng nucleic acid and 1 µl of DNase I, RNase A and S1 nuclease Enzyme (5 U/ µl), respectively. The reaction was kept at 37°C for 30 min, subsequently reaction was terminated by addition of EDTA (10 mMol/L final concentration) and digested product was separated on 0.7% agarose gel electrophoresis.

**SDS PAGE profile of bacteriophage**

SDS-PAGE of the bacteriophage was carried out according to the method of Laemmli (1970) with some modification. One ml of purified bacteriophage and 4 ml of ice cold acetone was kept overnight at -20ºC, then centrifuged at 13,000 g for 15 min, then supernatant was discarded and the pellet was air dried. The pellet was resuspended in Laemmli's sample buffer (with ß-mercaptoethanol and SDS) and heated for 5 min at 95-100oC. The protein extracted was separated onto polyacrylamide gel at 70 V for 2 h. Protein was stained with 0.25% Coomassie Brilliant Blue R-250 stain then destained and visualized by gel documentation system (Syngene, USA).

## RESULTS AND DISCUSSION

**Bacteriophage isolation**

Phages are ubiquitous, being found wherever bacteria are present (Biebricher and Gardiner, 1997; Marks and Sharp, 2000). Phages are generally isolated from environments that are habitats for the respective host bacteria e.g. sewage, soil, water (Vinod *et al.*, 2006). In the

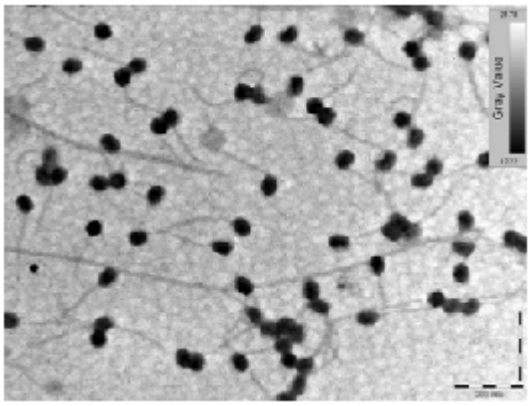
present study phage was isolated from sewage sample collected from poultry farm, Izatnagar, Bareilly. Phage was screened for their lytic activity on the basis of clear plaque formation. The isolated bacteriophage was exhibiting potent lytic activity with clear and transparent zone, medium size (1-2

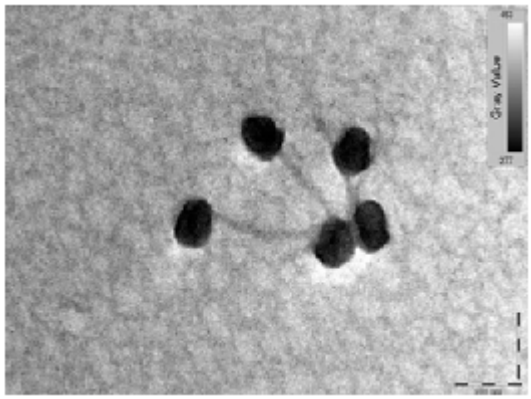

**Fig. 1**. Plaque morphology of *S. Typhimurium*

**Table 1.** Host range of φSTIz1 phage

| S. No | Bacterial isolates | Lytic activity |
|---|---|---|
| 1. | *S. virchow* (E-1460) | - |
| 2. | *S. saintpaul* (E-1426) | - |
| 3. | *S. Typhimurium*(E-4807) | + |
| 4. | *S. Gallinarum* (E-2368) | + |
| 5. | *S. abortus* equi (E-810) | + |
| 6. | *S. virchow* (E-1472) | - |
| 7. | *S. abortus* equi (E-155) | + |
| 8. | *S. abortus* equi (E-157) | + |
| 9. | *S. pullorum* (E-154) | + |
| 10. | *S. pullorum* (E-155) | + |
| 11. | *S. enteritidis* (E-161) | + |
| 12. | *S. enteritidis* (E-4671) | + |
| 13. | *Staphylococcus aureus* | - |
| 14. | E. coli | - |

+ lytic effect -no lytic effect





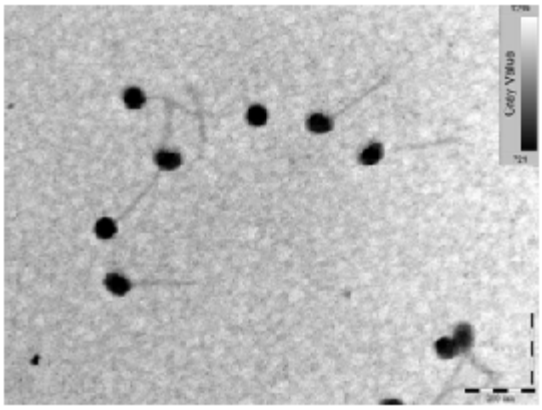


**Fig. 2.** Transmission electron micrographs of phages φSTIz1. Scale bar represents 200 nm **A**: Showing clusters of φSTIz1 **B**: Overveiw of φSTIz1. Scale bar represents 100 nm **C**: Close up view of φSTIz1. Scale bar represents 100 nm.

mm in diameter) plaques with well defined edges (Fig. 1). It was named as φSTIz1and further characterized.

**Phage count of phage stock**

High titre of phage φSTIz1 was stored as a suspension at 4°C and, as lyophilized preparation at -20°C. The phage titers of the preserved bulks were monitored at regular intervals during the entire period of study. The initial phage count was found to be $3 \times 10^{11}$ PFU/ml, and no significant reduction in the counts was recorded during 1 year storage at 4°C. The phage count of the freeze dried lot was found to be $3x10^{10}$ PFU/ml which remained stable throughout the study period.

**Bacteriophage morphology**

Transmission electron microscopy revealed that *S.* Typhimurium phage (φSTIz1) had round head with diameter of 52.95±3 nm, and long, noncontractile tail with length of 190.28±5 nm. Based on morphological studies, phage φSTIz1 was assigned to the order *Caudovirales* and family *Siphoviridae* morphotype B2 (Fig. 2). Morphology of phage ÆSTIz1 was similar to *Salmonella* phage st104a and st104b (O'Flynn *et al.*, 2006; and Phage wksl3 (Kang *et al.*, 2013). Also morphologically similar to phage FGCSSa2 (Carey-Smith *et al.,* 2006) and *Salmonella* paratyphi B phage Jersey (Demczuk *et al.*, 2004). Phage φSTIz1 differed from

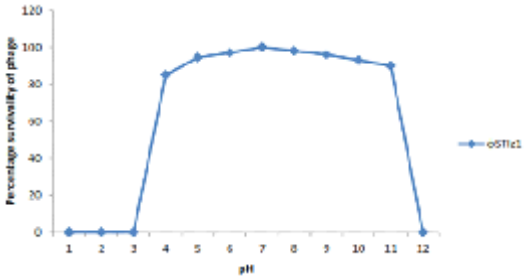



**Fig. 3.** Effect of pH on the stability of φSTIz1. At pH 3 and pH 12, the titer of the bacteriophages was zero

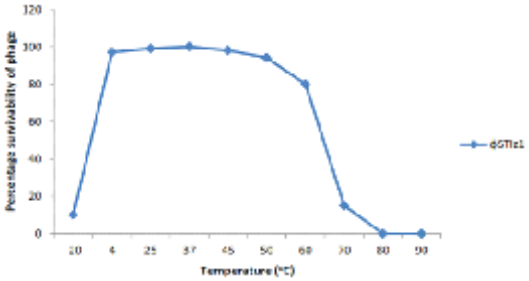
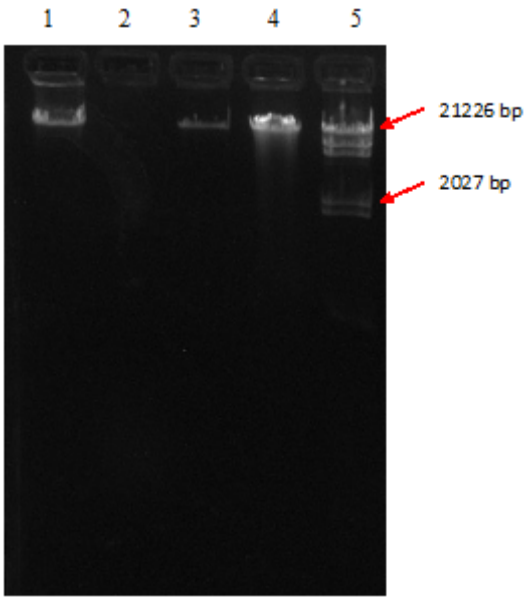
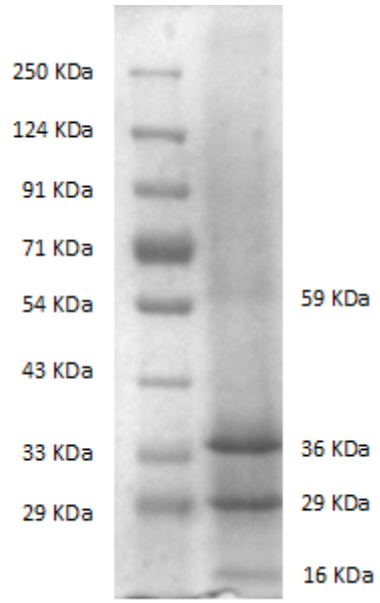



**Fig. 4.** The heat stability of φSTIz. At 80 and 90°C the titer of the bacteriophage was zero




M: λDNA/EcoR1+ HindIII 1: Genomic DNA of φSTIz undigested 2: Digested with DNase I 3: Undigested with RNase A 4: Digested with S1 Nuclease

**Fig. 5.** Agarose gel electrophoresis analysis




M: Protein molecular weight marker 1: Protein content of φSTIz1 phage

**Fig. 6.** SDS PAGE profile of φSTIz1 phage

*Salmonella* Typhimurium-specific bacteriophage φSH19 (Hooton *et al.,* 2011).

**Host range**

Host range was determined using different strains of *Salmonella* spp. (12), *E. coli* (1) and *S. aureus* (1) (Table 1). The results indicated that the φSTIz1 was lytic to 9 of the 12 isolates of *Salmonella* tested. However, it did not form any plaques against *S*. Saintpaul, *S*. Virchow and also against *Staphylococcus aureus* and *E. coli.* The variation in host range may be due to the environmental origin of bacteria tested in which loss of bacteriophage receptors may occur due to co-evolution between bacterium and bacteriophage. It may be also associated with the prevention of adsorption by bacterial receptor mutations or with degradation due to restriction or modification of the resistance bacteria system (Buckling and Rainey, 2002).

**Stability of bacteriophage on varying pH and temperature**

The phage φSTIz1was almost stable between the pH 4 to 11 indicating that the phage could survive wide variation of pH (Fig. 3). Only about 15% of the phage particles were inactivated at pH 4 and 10% reduction was seen at pH 11. In the acidic pH of 3 and alkaline pH of 12, no visible plaques were observed. Similar results were observed for *Salmonella* Typhi phages STP A and STP B (Sridhar *et al.,* 2013). The stability of phage in wide range of pH may be due to abiotic and biotic factors present in the sewage, which causes the variation from acidic to basic. This fluctuation in pH might have induced the phage to adopt itself to survive in wide pH environment.

The phage φSTIz1 was found stable between temperatures 4° C to 60° C and 15% of phage particles survived after treatment at 70°C and 10% of phage particles survived at -20°C. At a temperature of 80°C and 90°C the titer of the bacteriophage was zero (Fig. 4). Similar results have been reported for the *Salmonella* phage PSPu-95 and PSPu-4-116, which survived highest temperature of 70°C (Bao *et al.,* 2011). Inability of the phage to withstand higher temperature might be due to the presence of envelop and excess heat may have caused irreparable damage to the envelop making it ineffective against bacteria at higher temperature (Chandra *et al.,* 2011).

**Nucleic acid characterization**

The nucleic acid of phage φSTIz1 could be digested with DNase I but were resistant to digestion with RNase A and S1 Nuclease. The result revealed that the nucleic acid of phage φSTIz1 was double stranded DNA (Fig. 5).

**SDS PAGE profile of bacteriophage**

SDS page profile of bacteriophage reveled four structural proteins ranging from 59 KDa to 16 KDa, of which two were major proteins (36 and 29 KDa) and two minor proteins (59 and 16 KDa) (Fig. 6). In earlier study,phage SaP1 showed similar band at 36 KDa and phage SaP2 showed band at 56 KDa (Ngangbam and Devi, 2012).

## CONCLUSIONS

It can be concluded from the study that the isolated phage against *S*. Typhimurium belongs to family *Siphoviridae* morphotype B2 and it had double stranded DNA. It was found to be stable in environment with varying temperature and pH. It revealed wide host range and owing to its lytic nature can be further exploited as an antibiotic alternative, biocontrol agent, therapeutic and prophylactic agent against *Salmonella* spp. to prevent contamination of food and food borne disease.

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