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Isolation and Partial Characterization of Bacteriophage against *Salmonella gallinarum*

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A B S T R A C T

A lytic bacteriophage (SGP-1) against *Salmonella gallinarum* (*S. gallinarum*) was isolated by an enrichment protocol from the sewage water of a poultry farm. The presence of bacteriophage was observed by spot test over the bacterial lawn and double agar overlay assay. Isolated phage was able to produce clear, circular plaques of 2-3mm diameter on *S. gallinarum* lawn culture. This Bacteriophage also had a lytic effect on *Salmonella typhimurium*. The bacteriophage SGP-1 was found to be stable at a temperature range between 4°C to 60°C for 30 min and able to survive in a wide range of pH between 2 to 11. The characterization of the bacteriophage would help establish a basis for adopting the application of the most effective bacteriophage therapeutics to control bacterial infection of poultry.

Introduction

Fowl typhoid is an acute and severe septicemic disease of adult chicken caused by *Salmonella enterica* subsp. *enterica* serovar Gallinarum (*S. gallinatum*). Infection in chicken is characterized by severe spleenohepatomegaly, liver bronzing, anemia, and septicemia (Shivaprasad, 2000). Fowl typhoid is specifically limited to avian species and rarely causes food poisoning in humans (Pomeroy *et al.*, 1984). Among diseases of poultry, salmonellosis is of great concern and has been responsible for serious economic losses to the poultry producers (Gast and Shivprasad, 2003; Kabir, 2010). The emergence of antibiotic resistance among *S. gallinatum* against currently available antibiotics is increasing day by day due to the indiscriminate use of antimicrobials in poultry farming. Restricted use of antibiotics has increased the need for novel and effective control strategies for fowl typhoid (Joerger, 2003). Bacteriophages have been successfully used to treat bacterial diseases in animals (Atterbury *et al.*, 2003; Huff *et al.*, 2005; Tanji *et al.*, 2005).

Bacteriophages are viruses that infect and kill bacteria (Connerton and Connerton, 2005). They are the most abundant life entity on the planet, widely distributed in soil, hot springs, deep seas, and water (Hendrix, 2003). Phage therapy is currently suggested as possible alternatives to antibiotics for the treatment of bacterial diseases in humans and animals and widely used to minimize the bacterial loads in food products of animal and plant origin. Phages have a wide variety of advantages over antibiotics or other chemical agents as they target only the pathogens of interest, not affecting normal microflora. There is no adverse effect on the human or animal immune system. The bacteriophages were also used in the early 20th century to control various diseases including cholera, dysentery, and *Salmonella* infections (García *et al.*, 2008; Summers, 2012). Phage therapies have been recently remerging as potential alternatives to antibiotics. Biocontrol using phages can also be applied through food, agriculture, and medical fields (Lu and Collins, 2009).

In an experiment, Taylor and Silliker (1958) reported that treatment of birds experimentally infected with *S. gallinatum* with bacteriophages controls avian typhoid caused by *S. gallinatum*. The ability of *Salmonella* bacteriophage to reduce the *S. typhimurium* burden in orally challenged susceptible mice was also tested (Boury, 2005). It was reported that bacteriophages can be used to reduce the caecal colonization of *S. enterica* serotypes Enteritidis and Typhimurium in commercial broiler chickens (Atterbury *et al.*, 2007). It was found out that the phage treatment, either by aerosol spray or drinking water; significantly reduces *Salmonella* infection in poultry (Borie *et al.*, , 2008). These findings encourage further work on the use of phages as an effective alternative to the antibiotic to reduce *Salmonella* infection in poultry (Toro *et al.*, 2005). Given the above findings, the present work was undertaken for isolation and characterization of bacteriophage against *S. gallinatum*.

Materials and Methods

Bacterial strains

The bacterial strains used were obtained from the Division of Biological Standardization, ICAR-IVRI, Bareilly. Stock culture of *S. gallinatum* isolate was revived by inoculation into Brain Heart Infusion (BHI) broth and overnight incubation at 37^{o}C. The broth cultures were streaked on the Hektoen Enteric Agar (HEA, Himedia) and plates were incubated overnight at 37^{o}C. The identity and purity of culture were confirmed by morphological, cultural, and molecular examination.

Bacteria identity validation

Smooth, transparent, black-centered colonies with greenish periphery were picked up and confirmed biochemically as per Edwards and Ewing (1972). For molecular characterization, the DNA was extracted by a snap-chill method. The PCR targeting *inv*A gene (genus-specific) was standardized as per the method given by Rahn *et al.*, (1992) with certain modifications. The identity of the strain was further validated by amplification of *I137_08605* and ratA ROD genes (serotype-specific) by multiplex PCR as per protocol described by Xiong *et al.*, (2018) with slight modifications. The PCR mixture consisted of 12.5 µl of 2x SapphireAmp fast PCR master mixtures (Takara), 1 µl (10pmol/ µl) of each primer (Table 1), 2 µl of DNA template, and nuclease-free water to make final volume up to 25 µl. The cycling condition comprised an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 55°C for

45sec, elongation at 72°C for 1 min and finally a single step extension at 72°C for 10 min. The PCR products were resolved by agarose gel electrophoresis (1.5%) with Redsafe (Infobio) and visualized under a gel documentation system (Azure biosystems 200).

Sample collection and enrichment

To isolate the bacteriophage specific to *S. gallinatum* sewage water samples suspected to contain bacteriophage were collected in 50ml wide-mouthed samplers bottle from different poultry farms of ICAR-Central Avian Research Institute (CARI), Bareilly and were transported on ice to the laboratory for further processing. Coarse suspended particles in the samples were allowed to sediment at room temperature for about 1 h before further processing. 40 ml of each sample was mixed with 5 ml of 5x BHI broth and 5 ml of overnight grown log phase culture (~10^9cfu/ml) of *S. gallinatum* for bacteriophage enrichment. This mixture was incubated with shaking @ 180-200 rpm, 37^{o}C for 12-24 h. The enriched mixture was centrifuged at 10,000 rpm for 10 min and then filter sterilized by passage through a 0.22µm PES membrane filter (Axiva). The bacteria-free enriched filtrate (BFF) can be stored at 4^{o}C for further processing.

Detection of bacteriophage by spot test and double agar-overlay method

The presence of bacteriophage in the BFF was detected by spot test (Mirzaei and Nilsson, 2015). Briefly, 10µl of above BFF was spotted on bacterial lawn culture of *S. gallinatum* and allowed to dry. The Plates were observed for the presence of spot after incubated at 37^{o}C for 6-12 h. A clear zone around the bacterial lawn culture was indicative of the presence of phage in the enriched filtrate. After detection of phage by spot test, double agar overlay assay was performed as per Adams (1959). Serial 10 fold dilutions of the BFF up to 10^{-12} were prepared in sterile SM buffer (5.8 g/L of NaCl, 2.0 g/L of MgSO4, 50ml/L of 1 M Tris, pH 7.5, 5ml/L of pre-sterilized 2% gelatin). 100µl each dilution was mixed with 300µl of overnight grown log phase culture of *S. gallinatum* (~10^9CFU/ml) and incubated for 5 min at 37^{o}C, undisturbed. The above mixture was added to 3ml of molten Luria–Bertani (LB) semi-solids (0.7% agar) at 47^{o}C and mixed thoroughly by rolling the tube in hands. The content of each tube was immediately poured onto LB agar plates. The plates were kept at room temperature to allow the soft agar overlay to solidify. The plates were examined for the presence of plaques after incubation at 37^{o}C for 6-12 h. The plates showing the presence of plaques on the *S. gallinatum* lawn were selected for further propagation of bacteriophage.

Phage propagation and preparation of concentrated phage stock

Phage propagation was done according to Hua *et al.*, (2014). In brief, phage was grown overnight on agar overlay plates containing *S. gallinatum*. Then, a single well-isolated plaque was picked with a sterile loop and crushed completely in 500 µl of SM buffer. Serial 10 fold dilution of the above-crushed plaque up to 10^{-12} was prepared in sterile SM buffer. Using the agar overlay method phage was propagated and individual plaques were observed on plates. This process was repeated three times to obtain homogenous plaques. An isolated plaque from the third purification passage was used to prepare appropriate dilutions that would provide confluent lysis of *S. gallinatum* in a soft-agar overlay plate. To recover phage, 5ml of SM buffer was poured over each agar overlay plate. The plaques were disturbed using a sterile loop and the plates were kept at room temperature for at

least 1h. The elute from each plate was pooled and collected in a sterile tube. The elute was subjected to centrifugation and the supernatant was filtered through a 0.22µm PES membrane filter (Axiva). NaCl and PEG 8000 were added to the supernatant to reach final concentrations of 0.5 *M* and 10% (wt/vol), respectively, and kept at -20^{o}C for 2 h. This mixture was centrifuged at 11,000 × g for 10 min at 4^{o}C, the supernatant was discarded and the tube was kept in an inverted position for 5 min to allow the remaining fluid to drain away. 0.2ml of SM buffer (volume may be increased in case of the large pellet) was slowly and gently poured along the wall of the tube for 50ml of initial suspension taken. A short spin was given and placed in a tilted position for 1 h at room temperature. An equal volume of chloroform was added to the extracted phage particles, gently vortexed for 30 sec, and centrifuged at 3000×g for 15 min at 4^{o}C. The aqueous suspension containing phage particles was taken in a separate tube and phage titer was estimated by double agar overlay method and expressed as pfu/ml. The concentrated phage stock was stored at 4^{o}C.

pfu/ml = No. of plaques × Dilution factor/ Volume of phage used (ml)

Determination of anti-bacterial host range of Bacteriophage

The lytic activity of bacteriophage was assessed against *S. pullorum*, *S. typhimurium*, *S. enteritidis*, *E. coli*, *Klebsiella sp*,, *Pasteurella multocida*, *Streptococcus suis*, and *Staphylococcus aureus*. The host range of bacteriophages was determined by spotting 10 µl of concentrated bacteriophage (~10^{14} pfu/ml) on the lawn of each bacterial isolate. The plates were observed for positive results by observing the appearance of a lytic clear spot after incubation at 37°C for 6-12 h.

Effect of Heat and pH on the survivability of phage

The purified phage preparation having a titer of ~10^{14} pfu/ml was kept at 25^{o}C, 37^{o}C, 40^{o}C, 50^{o}C, 60^{o}C, and 70^{o}C in a water bath for 30 min to estimate survivability of phage at different temperatures. For pH stability, phage preparation was incubated in BHI broth of different pH ranging from 2 to 11 by adding either 0.1N NaOH or 0.1 N HCl. 100µl of phage suspension was added to 900µl of BHI broth of different pH on microcentrifuge tube and kept at 37^{o}C for 24 hours. Bacteriophage titer was estimated using the double agar overlay method after the treatment.

Invitro efficacy of bacteriophage on the growth of *S. gallinatum*

Briefly, two flasks of fresh BHI broth were inoculated with 500µl of exponentially growing culture of *S. gallinatum* (~10^{9}CFU/ml). In one flask, 100µl of appropriate dilution of bacteriophage was added to get an MOI (Multiplicity of Infection) of 0.01 and the other was mocked with 100 µL of PBS instead of bacteriophage which was kept as untreated control followed by incubation at 37°C overnight in a shaker incubator at 180-200 rpm. The efficacy of bacteriophage was recorded by determination of OD_{600} (optical density) value of both treated and untreated samples.

Results and Discussion

Bacteria identity validation

On Hektoen Enteric Agar (HEA) smooth, transparent, black centered colonies with greenish periphery were observed. On triple sugar iron (TSI) agar slants it produced a K/A reaction with variable production of H_2S and gas. The strain was non-motile, catalase and lysine decarboxylase positive whereas

negative for oxidase, indole and did not utilize citrate. PCR assay for *inv*A, followed by agarose gel electrophoresis revealed specific amplification of 284 bp nucleotide segment indicating the presence of *inv*A gene (Fig. 1).

Multiplex PCR targeting *S. gallinatum* serotype-specific *I137_08605* and ratA ROD genes revealed amplification of 290 bp and 571 bp nucleotide segments indicating the presence of I137_08605 and ratA ROD genes respectively (Fig. 2).

Bacteriophage isolation and purification

Bacteriophage was isolated from sewage samples collected from poultry farms. The isolated bacteriophage exhibited potent lytic activity against *S. gallinatum* which was indicated by the formation of a clear and transparent zone in the spot test (Fig. 3). In a double agar overlay assay, the phage formed clear and round plaques of 2-3mm in diameter with well-defined edges (Fig. 4). The bacteriophage isolated against *S. gallinatum* was designated as SGP-1.

The titer of concentrated phage stock was ~1.9×10^{14} pfu/ml. The concentrated phage stock was stored at 4^{o}C and freeze-dried preparations at -20^{o}C. The titer of preserved phage was monitored at regular intervals during the entire study period. No significant reduction in phage titer was estimated during 1-year storage at 4^{o}C and -20^{o}C.

Host range

The isolated bacteriophage SGP-1also had strong lytic activity against *S. typhimurium* but did not have any lytic effect on *Salmonella pullorum*, *Salmonella enteritidis*, *E. coli*, *Klebsiella sp*, *Pasteurella multocida*, *Streptococcus suis*, and *Staphylococcus aureus*.

Temperature and pH stability

The bacteriophage was found stable up to 60^{o}C for 30 min. Only one and two log reduction was observed in phage titer at 50^{o}C and 60^{o}C respectively in comparison to the original phage titer (~10^{14} pfu/m)**.** No phage was detected at 70^{o}C i.e. the phage was unstable at 70^{o}C when treated for 30 minutes (Table 2 & Fig. 5). The bacteriophage was subjected to various pH(2-11). The phage titer was found similar to the original between pH 3 to 8 and a slight decrease in pH was observed at higher and lower pH, which indicates bacteriophage was stable at a wide range of pH (Table 3 & Fig. 6).

Table.1 Oligonucleotide primer with sequence details

Purpose	Primer sequence (5'-3')	Gene	Product size	Reference
Salmonella sp	F:GTGAAATTATCGCCACGTTCGGGCAA R: TCATCGCACCGTCAAAGGAACC	*inv*A gene	284 bp	Rahn *et al.*, 1992
***S.*Gallinarum serotype**	F: CACTGGA ACTCTGAGGACA R: GGGCAGGGAGTCTTGAGATT	*I137_08605*	290bp	Xiong *et al.*, 2018
	F: ATTGCTCTCGTCCTGGGTAC R: TACCGATACGCCCAACTACC	ratA ROD	571 bp	Xiong *et al.*, 2018

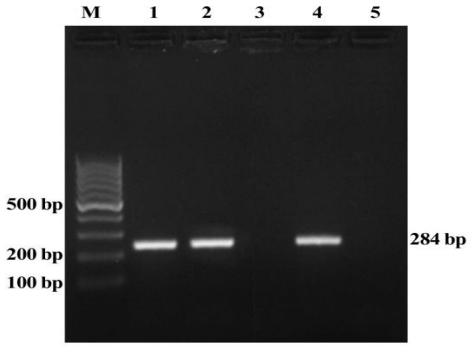
Table.2 Stability of bacteriophage SGP-1 at different temperature

Temperature	Phage titre after exposure (log_{10}pfu/ml)
4°C	14.27
25°C	14.14
30°C	14
40°C	13.17
50°C	13.27
60°C	12
70°C	0

Table.3 Stability of Bacteriophage SGP-1 at pH

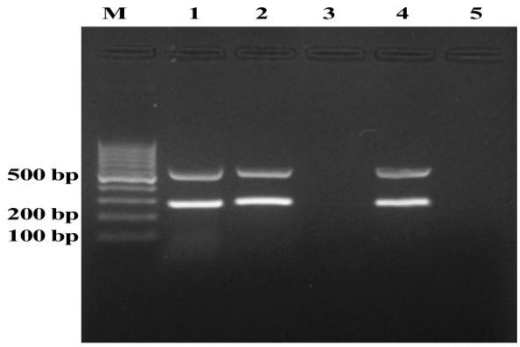
pH of the medium used	Phage titer after exposure (log_{10}pfu/ml)
2	12.4
3	12.9
4	13.6
5	13.9
6	14
7	14.27
8	13.8
9	12.9
10	12.6
11	11.6

Fig.1 PCR amplification of *inv*A gene (genus specific) for confirmation of *Salmonella gallinarum* isolate



Lane M = 100 bp plus DNA ladder
Lane 1 = *Salmonella gallinarum*
Lane 2 = *Salmonella typhimurium*
Lane 3 = *E. coli*
Lane 4 = Positive Control
Lane 5 = Non Template Control

Fig.2 PCR amplification of *I137_08605* and ratAROD genes (serotype specific) for confirmation of *Salmonella gallinarum* isolate



Lane M = 100 bp plus DNA ladder
Lane 1 = *Salmonella* Gallinarum
Lane 2 = *Salmonella* Gallinarum
Lane 3 = *Salmonella* Typhimurium
Lane 4 = Positive Control
Lane 5 = Non Template Control

Fig.3 Spot test of isolated bacteriophage SGP-1

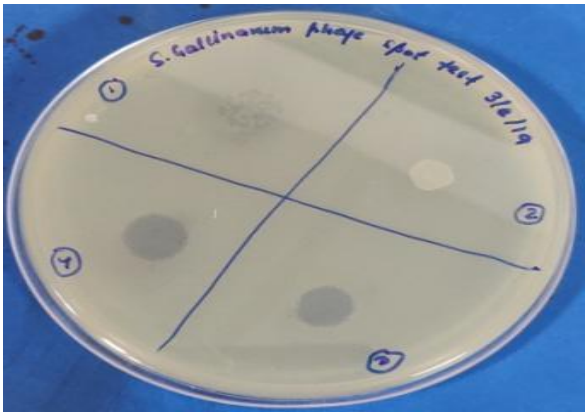


Fig.4 Plaque morphology of isolated bacteriophage SGP-1 on agar overlay plate

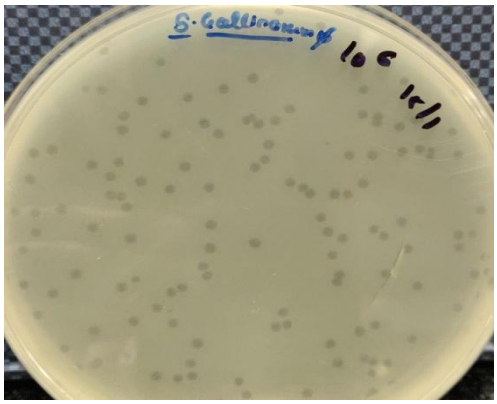


Fig.5 Effect of temperature on the stability of bacteriophage SGP-1

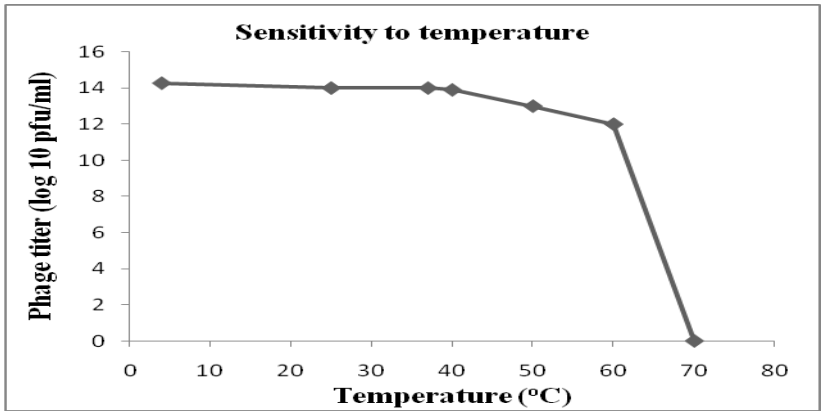
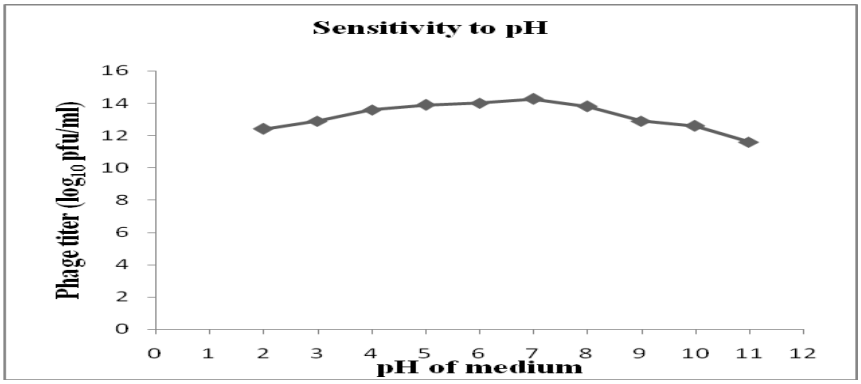


Fig.6 Effect of pH on the stability of bacteriophage SGP-1



In vitro efficacy of bacteriophage on the growth of *S. gallinatum*

In vitro, the efficacy of SGP-1 bacteriophage was also evaluated. In phage treated flask OD_{600} value of an exponentially growing culture of *S. gallinatum* was decreased to 0.75 in comparison to the control one (OD_{600} = 1.35) which showed the lysis of bacteria by the phage.

Isolation and characterization of lytic phages against target organism is the first and foremost step for developing phage-based therapeutics. In the present study, bacteriophage against *S. gallinatum* was isolated from sewage effluent collected from a poultry farm. Bacteriophages are widely distributed in the environment and can be isolated from seawater, soil, freshwater, and sewage ecosystem (Jensen *et al.*, 1998). Bacteriophages against *Salmonella* have been isolated from sewage water and poultry litter (Berchieri *et al.*, 1991; Sklar and Joerger, 2001), which indicates their natural occurrence in the environment. It has been observed that for almost all the bacteria that exist in the environment, a phage corresponding to that bacterium is also present there and so phages offer the potential for targeted biological control of bacterial pathogens in human, animal, and plant diseases (Lederberg, 1996; Schuch *et al.*, 2002). Isolation of phages from the environment in which a suspected bacterium resides has been a common finding against various bacteria (Xie *et al.*, 2005).

Bacteriophage isolated against *S. gallinatum* also had a lytic effect on *S. typhimurium*. Bacteriophages are very host-specific and this factor limits their use as a therapeutic agent in other bacterial infections (Bielke *et al.,* 2007). *Salmonella* bacteriophages are generally host-specific and often infect only one bacterial species or only one serotype within species (Welkos *et al.,* 1974). The isolated bacteriophage can be used to control *S. typhimurium* along with *S. gallinatum*.

The isolated bacteriophage was able to survive between temperature 4^{o}C to 60^{o}C and only 1 to 2 log reduction in phage titer was observed at a higher temperature. No phage was detected after exposure to 70^{o}C for 30 min. The response of phages to the varying temperature is considered as a key model for understanding the ability of the organism under the question to adapt to the novel environment (Johnston and Bennett, 1996). The bacteriophage SGP-1 was found stable at a wide range of pH between 2 to 11. Their stability in a wide range of pH can be explained by the presence of biotic and abiotic factors in sewage where the pH can oscillate from acidic to basic. This oscillation in pH might have induced the phage to adapt itself to survive a wide range of pH (Sridhar *et al.,* 2013).

In phage treated flask OD_{600} value of an exponentially growing culture of *S. gallinatum* was decreased which showed the lysis of bacteria by the phage. Wong *et al.,* (2014) analyzed the interactions and dynamics of phage-host populations at different MOI and a decline of 6.6 $\log_{10}$cfu/ml of Salmonella at 0.1 MOI in 8 h were recorded. Their results support the findings of this research. The bacteriophage isolated in this study may have good potential to be used as a therapeutic and prophylactic agent for controlling the bacterial infections of chicken caused by *Salmonella* Gallinarum.

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