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Original Article

Molecular Characterization of *Salmonella* Bacteriophages Isolated from Natural Environment and its Potential Role in Phage Therapy

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Bacteriophages or phages are the viruses that infect bacteria. Phages like all viruses are made up of a nucleic acid core which is surrounded by a protein coat. The majority of phages are known to contain DNA but some contain RNA also. In this study, four salmonella phages were isolated and were subjected to mollecular characterization. All the four *Salmonella* phages were found to contain DNA as their genetic material. Comparison of protein profiles of phages by SDS-PAGE revealed near identical protein profiles for phages SaP2 and SaP4. Study of storage stability of phage indicated that the titer of the *Salmonella* phage SaP1 almost remains unaltered during the 14 days storage at different temperatures ranging from - 20ºC, 0ºC and 4ºC. This study shows that the isolated phage have good storage stability. The isolated phages also show narrow host range.

Keywords: **stability, host range, SDS-PAGE, titer, strains**

Introduction

Salmonella phages are believed to play a critical role in *Salmonella* evolution and to mediate horizontal transfer of virulence genes among *Salmonella* strains (Baumler, 1997; Porwollik and McClelland, 2003; Rabsch *et al*., 2002). *Salmonella* bacteriophages occur naturally in manure and can be isolated for future characterization and potential use as typing reagents, indicators and biocontrol agents. Studies of *Salmonella* bacteriophages particularly those associated with lysogenic strains, appears to have been undertaken by Burnet (Burnet, 1930). He described four main sub divisions which he designated as phage group A, B, C and D. Later Boyd published his first paper on the temperate bacteriophage isolated from lysogenic strains of *S.* Typhimurium in which he described two main groups of bacteriophages designated as subgroup A and subgroup B (Boyd, 1950).

Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infection could be prevented by the administration of bacteriophage **(Sulakvelidze *et al.*, 2001).** Twort d'Herelle had suggested for the first time that phages could be used for treating bacterial infections. Report of treating bacterial diseases by applying phages came from France in 1921 for the first time. There have been several reports on phage therapy, but many of these were not scientifically well-demonstrated and later the discovery of antibiotics as chemical agent led to the replacement of phage therapy (Barrow and Soothil, 1997; Alisky *et al.*, 1998). The increase in problem of multidrug resistant bacteria made researchers to reconsider use of phages to treat against experimental *E. coli* infections in mice, calves, lambs and piglets (Smith and Huggins, 1982; Smith *et al*., 1987).

With this background, the objectives of the study were Molecular characterization of *Salmonella* bacteriophages, Storage stability studies of *Salmonella* bacteriophages and Study of susceptibility of non-*Salmonella* species to phages isolated in this study.

Materials and Methods

Analysis of bacteriophage nucleic acid

For extraction of nucleic acid from phage particles, method described by Su *et al.* (1998) was followed. The phage was precipitated from filtered growth medium with sterile 2mol/ $ZnCl_2$ at the rate of 1:50 for 5 minutes at 37 ºC and pelleted at 4000 rpm for 5 minutes. The pellet was suspended in TENS buffer (50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl and 0.3% SDS) and proteinase k (100 mg /ml final concentrations) and incubated at 65 ºC for 10 minutes. Proteins were removed by two extractions with phenol/chloroform/ isoamyl alcohol and DNA was precipitated with isopropanol. After washing in 70% ethanol, pellets were resuspended in TE (10 mM Tris pH 8.0, 1m M/EDTA) and analyzed using 0.8% agarose gel electrophoresis.

Electrophoresis

0.8 g of agarose (Hi media, Mumbai) was added to 100ml of 1.0 x TAE buffer and melted. After cooling to 50 ºC, Ethidium bromide was added to the agarose solution at the rate of 0.5 mg/ml. The agarose solution was mixed well and poured on a horizontal agarose gel base and allowed to solidify after keeping the comb at one side of the gel base. After solidification the comb was slowly removed. The gel was then placed in electrophoresis tank. DNA preparation was then loaded to the wells along with the molecular weight marker and electrophoresis was performed at voltage of 100 millivolt.

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DNA Molecular weight markers (Bangalore Genei, Bangalore)

Lambda DNA digested with EcoR1 and Hind III yielding 13 fragments 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125 base pairs was used as molecular weight marker.

Protein profiles of phage structural proteins

The protein profiles of phage structural proteins were analysed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electorphoresis (SDS-PAGE) as described by Laemmli (1970). High titer phage suspension (100 ml) obtained from confluent lysis plates was taken. After the addition of 5% b-mercaptoethanol, samples were boiled for 5 min with equal volume of sample buffer, electrophoresed on 15 % discontinuous gels (15 % acrylamide, 0.5 % bis-acrylamide) by using the vertical slab gel electrophoresis system (Bangalore Genei, Bangalore) and stained with 0.1 % coomassie blue. Standard molecular weight markers (Bangalore Genei, Bangalore) were run along with the samples to determine molecular weights of sample bands.

Preparation of SDS-PAGE apparatus (Sambrook et al., 1989)

Glass plates and glass wares were washed in chromic acid and rinsed in de-ionized water before drying. The spacers were placed in position and edges sealed with 0.5 % agarose solution. The 16.5 cm wide and 20 cm high rectangular glass plate was assembled together with the notched plate and the spacers were placed such that the vertical spacers were in contact with the horizontal one at the bottom.

Preparation of resolving gel

Resolving gel mixture was prepared in a small beaker with a magnetic stirrer by mixing the components for a desired concentration of acrylamide according to the following chart (Harlow and Lane, 1998).

Immediately upon addition of APS followed by TEMED, the solution was poured into the assembled plates and overlaid with iso-butanol to prevent diffusion of oxygen into the gel and for obtaining a uniform margin of the gel. It was allowed to polymerize for about 30 min.

Preparation of stacking gel

In a similar manner, stacking gel mixture with 4 % acrylamide mix was prepared by mixing the components as below (Harlow and Lane, 1988).

Storage stability studies of Salmonella bacteriophages

The effect of different temperatures on the viability of *Salmonella* phages during storage was studied. The phage was prepared in large scale using soft agar overlay method and harvested with phage buffer at 10^{12} pfu/ml concentration. 100ml of phage was distributed to each storage bottle containing 10ppm chloroform and stored at –20 ^{o}C, 4 ^{o}C, and 37 ^{o}C for 14 days. Periodically the titer was measured.

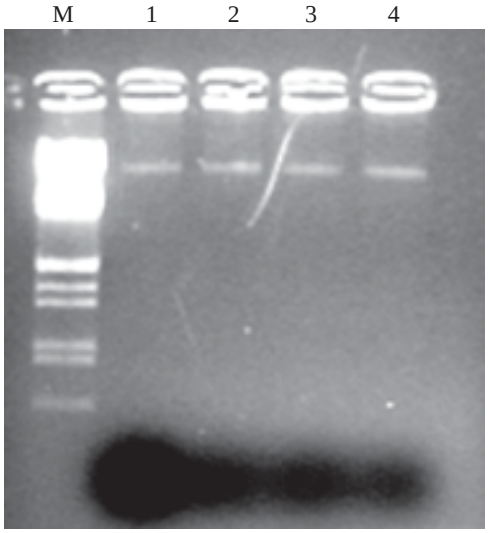
Study of susceptibility of non-Salmonella species to phages isolated in this study

To know the susceptibility of salmonella phages to other non-*Salmonella* species, susceptibility study of non-*Salmonella* species to phages isolated in this study was conducted. The host range of phages outside *Salmonella* group was studied using *E. coli*, *S. aureus*, and *V. harveyi*.

Results

Analysis of phage nucleic acid

The DNA of phages were isolated and analyzed by agarose gel electrophoresis. DNA bands corresponding to about 20 kb (Fig. 1) were visible after electrophoresis indicating that the phages contained DNA as their genetic material.

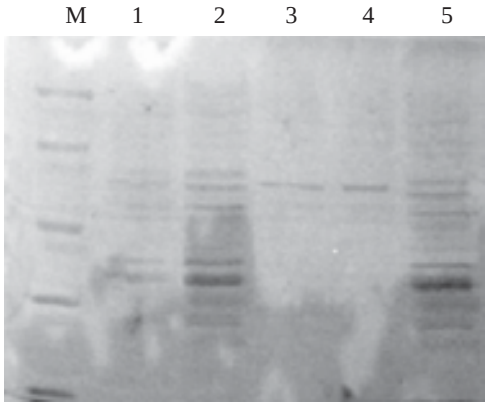


Lane M: Marker- DNA Eco RI HindIII
Lane 1 : SaP1 phage
Lane 2 : SaP2 phage
Lane 3 : SaP3 phage
Lane 4 : SaP4 phage

Figure 1. *Agarose electrophoresis of DNA extracted from different Salmonella phages.*

Protein profile of the phages

All the four *Salmonella* phages which were distinguishable by morphology were further characterized by SDS-PAGE analysis of structural proteins. All the four isolated *Salmonella* phages show different banding pattern with some main structural proteins as well as several minor proteins (Fig. 2). In SaP1 phage, major 36 kDa and 33 kDa structural proteins and 56 kDa, 53 kDa, 48 kDa, 45 kDa, 40 kDa and 27 kDa minor proteins were detected. In SaP2 phage, major 56 kDa, 52 kDa, 48 kDa, 36 kDa and 33 kDa structural proteins and 54 kDa, 39 kDa, 30 kDa and 27 kDa minor proteins were detected. In SaP3 phage, major 52 kDa structural proteins and 48 kDa, 45 kDa, 37 kDa and 28 kDa minor proteins were detected. In SaP4 phage, major 54 kDa, 50 kDa, 43 kDa, 36 kDa, 32 kDa structural proteins and 68 kDa, 61 kDa, 46 kDa, 29 kDa, 27 kDa and 24 kDa minor



Lane M: Protein molecular weight marker (PMW M, Bangalore Genei)
Lane 1 : SaP1 phage
Lane 2 : SaP2 phage
Lane 3 : SaP3 phage
Lane 4 : SaP4 phage

Fig. 2. *SDS-PAGE of bacteriophage structural protein*

proteins were detected. The molecular weights of phage structural proteins are given in Table 1.

Table 1: *Molecular weight of phage structural proteins*

Phage isolate	Mol. Weight (kDa) of major proteins	Mol. Weight (kDa) of minor proteins
SaP1	36 and 33	56, 53, 48, 45, 40 and 27
SaP2	56, 52, 48, 36 and 33	54, 39, 30 and 27
SaP3	52	48, 45, 37 and 28
SaP4	54, 50, 43, 36 and 32	68, 61, 46, 29, 27 and 24

Storage stability of Salmonella phage

The storage stability of phages was tested by storing phage preparations at 37ºC, 4ºC, and –20ºC and estimating the titer values at 1, 7 and 14 days. The results of storage stability studies are given in Table 2. A test for lytic activity of SaP1 phage revealed that SaP1 phage retained its lytic activity at all temperature of storage even after 14 days. The titer of SaP1 phage was almost unaltered during 14 days of storage at 37ºC, 4ºC, and –20ºC.

Table 2: *Titer values of SaP1 phage at different storage temperatures*

Length of storage in days	pfu/ml of phage at different temperature -20 ºC	4 ºC	37 ºC
1	$2.8´10^{12}$	$2.7´10^{12}$	$2.7´10^{11}$
7	$2.6´10^{12}$	$2.5´10^{12}$	$2.5´10^{11}$
14	$2.6´10^{12}$	$2.5´10^{12}$	$2.4´10^{11}$

Study of susceptibility of non-Salmonella species to phages isolated in this study

The host range of phages outside *Salmonella* group was studied using *E. coli*, *S. aureus*, and *V. harveyi*. All the four *Salmonella* phages isolated in this study did not lyse any of the non-*Salmonella* strains used demonstrating that the phages are highly specific to *Salmonella* spp (Table 3).

Table 3: *Susceptibility of non-Salmonella species to Salmonella Phages*

Bacterial species tested	No. of strains tested	No. susceptible to phages SaP1	SaP2	SaP3	SaP4
V. harveyi	3	0	0	0	0
S. aureus	3	0	0	0	0
E. coli	1	0	0	0	0

Discussion

The emergence of multidrug resistant bacteria has opened a window of opportunity for phage therapy. Modern innovations combined with careful scientific methodology can enhance the ability of mankind to make it work. The potential unique advantage of phage treatment over chemotherapy is the narrow host range of phages and it does not harm the normal intestinal microflora (Carlton, 1999). The DNA of phages were isolated and analyzed by agarose gel electrophoresis. DNA bands corresponding to about 20 kb (Fig. 1) were visible after electrophoresis indicating that the phages contained DNA as their genetic material. There were reports of various *Salmonella* phages having DNA as their genetic material. *Salmonella* bacteriophage P22 is an unassigned species of temperate bacteriophage in the family *Podoviridae* that infects *Salmonella* species. The genome consists of double–stranded DNA, terminally redundant, and circularly permuted (Schmeiger, 1982).

The structural proteins of all the four isolated *Salmonella* phages were analyzed by SDS-PAGE. Both major structural proteins and several minor proteins were found in the isolated phage. The *Salmonella* phages SaP2 and SaP4 were showing slightly similar banding pattern. The sizes of major protein bands in all the four *Salmonella* phages were as follows; In SaP1 phage 36 kDa and 33 kDa major proteins were detected. In SaP2 phage 56 kDa, 52 kDa, 48 kDa, 36 kDa and 33 kDa major proteins were detected. In SaP3 phage, only one major protein was detected i.e. 52 kDa and in SaP4 phage 54 kDa, 50 kDa, 43 kDa , 36 kDa and 32 kDa major protein were detected. The observed difference in the protein profile could be responsible for difference in host range and plaque morphology of the phages. Similar results have been reported previously using T even phages of *Escherichia coli* (Hantke, 1978). Even though the phage belongs to the same family, the structural proteins are unique to each phage and depend on their morphotype. It was suggested that the protein pattern could be used for phage morphotype, characterization and differentiation (Barangou *et al*., 2002).

When the phages are produced in large quantity for its application, it is necessary to store them in optimum condition

such as temperature, pH and salt concentration. In this study, the titer of the *Salmonella* phage SaP1 almost remains unaltered during the 14 days storage at different temperatures ranging from - 20ºC, 0ºC and 4ºC. This study showed the stability of SaP1 phage while storing at different temperature. Rossi and Arageno, (1999) had earlier reported that some phages might be inactivated at higher temperature. All the four isolated *Salmonella* phages isolated in this study did not lyse any of the non-*Salmonella* strains used demonstrating that the phages are highly specific to *Salmonella* spp (Table 3). This study also supported the fact that the host range of phages is narrow, i.e., they do not cross their generic boundaries (Ackermann *et al.*, 1987). This has been documented from studies of more than 4000 bacteriophage isolates described at that time.

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