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Isolation and characterization of lytic bacteriophages of *Salmonella* Typhimurium and their therapeutic application

LAKSHMI KAVITHA K[1], SAMBASIVA RAO K[2] and SRINIVASA RAO T[3]

Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh 521 102 India

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ABSTRACT

Salmonella Typhimurium is an important bacterial pathogen of gastroenteritis revealing multidrug resistance and has zoonotic implication. In an approach towards alternatives to antibiotics, lytic bacteriophages were isolated against *Salmonella* Typhimurium from sewage effluent using double agar overlay method. The isolated bacteir ohages, viz. ϕST1, ϕST2, ϕST3, ϕST4 and ϕST5 were characterized microbiologically and revealed host range 85–92% individually and 100% collectively within the genus. Biophysical characterization revealed that the phages were stable at 16°, 37°, 42°C and pH 4, 7 and 9 for 3h, supporting their therapeutic application. Electron microscopic examination of the ϕST1 showed icosahedral head (52.5nm), contractile tail (220–250nm) belonging to the family Myoviridae and order Caudovirales. Further, molecular characterization of ϕST1 revealed 38kb nucleic acid and digested by restriction endonucleases i.e., *Eco*RI, *Bam H*I and *Hae* III. The therapeutic application of the isolated phage cocktail was ascertained in Swiss albino mice models by infecting the control and treatment groups with 3×10^8 cfu/ml of the organism intramuscularly and orally. Following challenge the treatment group administered with 3×10^9 pfu/ml of phage mixture showed significant decrease in number of colony forming units of bacteria *in vivo*.

Key words: Bacteriophages, Characterization, *Salmonella* Typhimurium, Therapeutic application

Therapeutic application of bacteriophages need to be addressed in a way to contain *Salmonella* Typhimurium infection in buffalo calves as *Salmonella* spp. are responsible for serious economic losses and also exhibit zoonotic implications. In buffalo calves, it is responsible for severe gastroenteritis with mucus that leads to bloody and fibrinous changes. These organisms are responsible for 70% mortality in water buffalo calves, which is far high compared to bovine calves that were showing 50% mortality (Fagiolo *et al.* 2000, Foster *et al.* 2009). These organisms not only cause severe mortality in terms of disease but are also colonize in older animals shedding heavy loads of bacteria through faeces and are contaminating the environment as well as act as a source of infection to young ones (Borriello *et al.* 2012). In addition to the present scenario, the fast emergence of antibiotic resistance in the pathogens is becoming a major health concern (WHO 2014) due to indiscriminate use of antibiotics in livestock farming. This pressing concern made to search for alternatives to antibiotics. Among such alternatives, bacteriophages are gaining much importance as therapeutic agents and the present study was centred on isolation, characterization and therapeutic application of lytic phages of *Salmonella* Typhimurium.

Present address: [1]Assistant Professor (klkavitha @hotmail.com), JRF(sambasiva.karnati@yahoo.com), Department of Veterinary Microbiology; [3]Assistant Professor (tumaisrinivas2001@gmail.com), Department of Veterinary Public Health and Epidemiology.

MATERIALS AND METHODS

Bacterial strains and prophages: Salmonella Typhimurium isolates (10) were obtained from the collection. To use these organisms as host, presence of prophages was observed using the DNA damaging antimicrobial agent mitomycin (Miller 1998). The host strain bacterial cultures were aliquoted into 1 ml volumes in sterile test tubes and Mitomycin C was added to final concentration of 5µg/ml and incubated for 3h at 37°C. Then 20µl of chloroform was added to controls to lyse bacteria and both drug induced and control tubes were centrifuged. The supernatant was collected and subjected to double agar overlay to observe the exclusion of prophages.

Bacteriophage isolation: For the purpose of isolation of bacteriophages by large scale screening, sewage samples were obtained from the places in and around buffalo farms where there was a possibility of obtaining sewage having more organic matter. The collected sewage samples were centrifuged at 10,000 rpm for 10min, the supernatant was filtered using 0.45µ filters. To this equal volume of SM buffer (100mM NaCl, 8mM $MgSO_4$ and 1M Tris HCl pH7.5) and *Salmonella* Typhimurium (1.5×10^8CFU/ml) was added and incubated in orbital shaker incubator at 37°C

for 24 h. After incubation, the suspension was centrifuged at 10,000 rpm for 10 min and filtered through 0.45µ filters. This filtrate was used to estimate the phage population in sewage water using double agar overlay method using 20% bottom nutrient agar. Then the top agar was prepared using 0.5ml of the filtrate, 1 millilitre of the host culture (0.5×10^8 cfu/ml) and 1.5ml of SM buffer and incubated at 37°C for 20 min. To this suspension, 2.5ml of 20× nutrient agar was added to make 10× agar and layered on bottom agar. After solidification, the plates were incubated at 37°C for 24 h and observed for the formation of clear plaques.

Purification of bacteriophages and microbiological characterization: From the pool of bacteriophages that were obtained on primary isolation, single plaques revealing clear plaque morphology and wide lytic zone were obtained using sterile toothpick, then inoculated into 2 ml of nutrient broth having 0.5×10^8 cfu/ml of host culture and incubated at 37°C for 24 h in orbital shaker incubator. Later it was centrifuged and the supernatant was subjected to double agar overlay as described and the same was repeated thrice sequentially to obtain single lytic bacteriophage.

Among the isolated bacteriophages, 5 lytic phages were obtained. The host range was observed using spot assay as per Santos *et al.* (2011). Then these bacteriophages were multiplied further and stocks were prepared.

Biophysical characterization: The obtained bacteriophages at multiplicity of infection (MOI) one, were subjected to temperatures 16°, 37°, 42°C and pH 3, 7, 9 for 4 h by changing the temperature of incubation and pH of SM buffer respectively and the decrease in bacteriophages count was observed using double agar overlay method.

Nucleic acid isolation and characterization: Bacteriophage stocks were prepared by using MOI one of phage and organisms to yield complete lysis. To the completely lysed plates, 1 millilitre of SM buffer pH 7.5 was added and then incubated for one hour at 4°C, then the supernatant was scrapped with sterile spatula and stored at –20°C for 24 h. Later, it was centrifuged at 10,000 rpm for 10 min and the supernatant was used for the nucleic acid isolation (Santos *et al.* 2011). Later the nucleic acid type was observed by conducting RNase and DNase digestion. Further the ϕST1 nucleic acid was subjected to restriction endonuclease digestion using *Eco*RI, *Bam*HI and *Hae*III enzymes by following manufacturer's instructions.

Morphological characterization: Transmission electron microscopy of ϕST1 was carried out at Central Instrumentation cell, TANUVAS, Chennai.

In vivo lytic activity of the bacteriophages: Swiss Albino mice, aged 40 days, were selected and grouped into control, infected and treatment groups. Each group had six mice, the control group were normal mice, the infected group received 3×10^8 cfu/ml of organisms whereas the treatment group received both the organism (3×10^8 cfu/ml) and endotoxin free (proteospin endotoxin removal kit) lytic phage cocktail (3×10^9 pfu/ml) by oral and intramuscular route. During the experiment the body weights of the mice were collected and the therapeutic effect of the bacteiophages was estimated by enumerating the number of microorganisms in faecal matter.

RESULTS AND DISCUSSION

The *Salmonella* Typhimurium isolates used for cultivation of bacteriophages were initially assessed for the presence of prophages. The presence of prophages was observed using DNA damaging substance Mitmycin C in comparison with chloroform. The bacterial isolates that did not contain prophages were selected for further use. The exclusion of temperate bacteriophages form the host bacterial strains are to avoid the transduction as the prophages are responsible for transfer of genetic material (Merabishivili *et al.* 2009).

The bacteriophage isolation was carried out using sewage water samples obtained from 25 different places using double agar over lay method and obtained a group of 200 lytic phages. From the 200 lytic phages obtained, five (ϕST1, ϕST2, ϕST3, ϕS4 and ϕST5) candidate

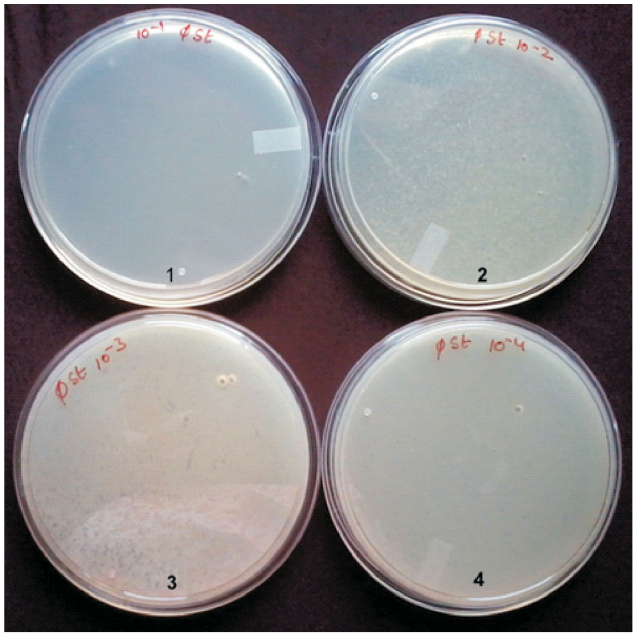

Fig. 1. Isolation of lytic bacteriophages of *Salmonella* Typhimurium.

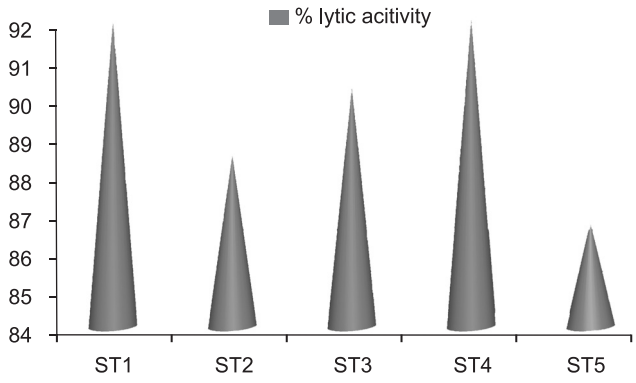

Fig. 2. Lytic activity of *Salmonella* Typhimurium bacteriophages.

bacteriophages were selected for further studies based on the degree of lytic activity (estimated on the basis of the visual clarity and diameter of plaques). The diameter of the clear plaques was found to be 1 to 2 mm (Fig. 1). According to the plaque size diameter the bacteriophages belong to Family Myoviridae as the phages of this family produce plaques of 1mm diameter (Kesik-Szeloch *et al.* 2013). Moreover the lytic activity of the phages belong to Myoviridae is higher compared to other two families of the order Caudovirales. Further, the host range of lytic phages yielded 85–92% individually (Fig. 2) and 100% collectively. The collective use of lytic bacteriophages for therapeutic application was suggested (Smith and Huggins 1983, Smith *et al.* 1987, Atterburry *et al.* 2007, Merabishvili *et al.* 2009).

In industrialized countries, bacteriophage therapy is undergoing a renaissance (Anonym.us 2004). Several studies proved the use of lytic bacteriophages in therapeutics (Miedzybrodzki *et al*. 2007, Vinodkumar 2008, Merabishivili *et al.* 2009, Vinodkumar 2010, Santos *et al.* 2011). The candidate strains for therapeutic application should also be selected using biophysical characterization. In this perspective, the 5 bacteriophages subjected to

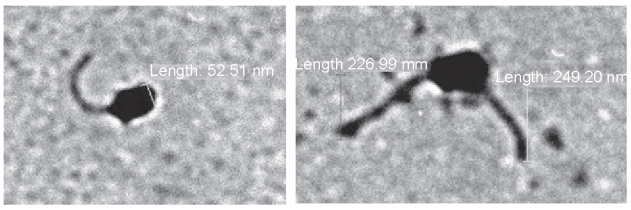

Fig. 3. Electron micrograph of ϕST1.

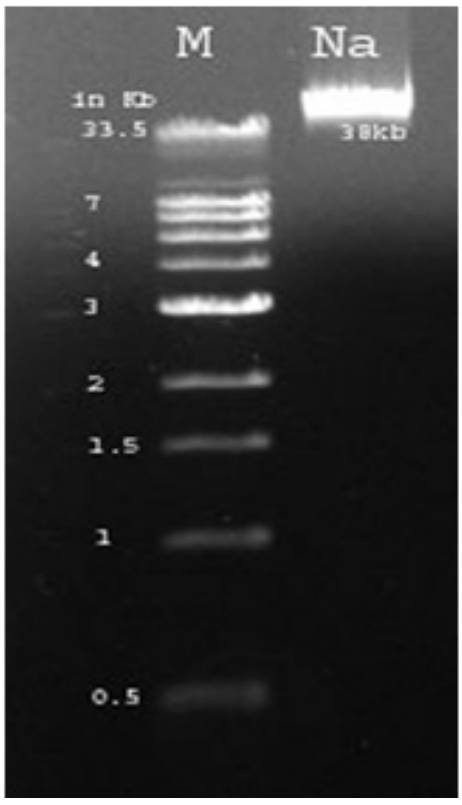

Fig. 4. Nucleic acid of ϕST1.

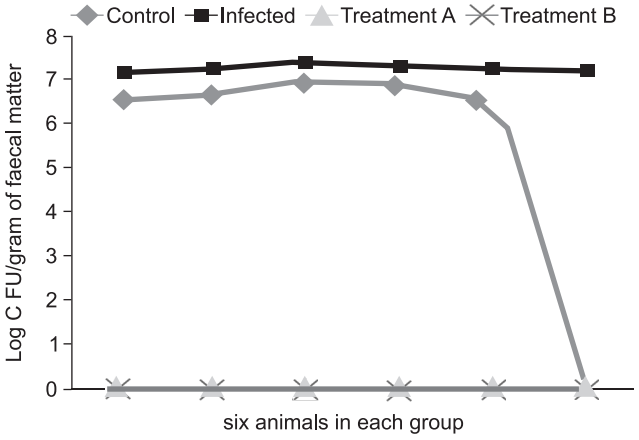

Fig. 5. Bacterial burden in control, infected and treatment groups.

different temperatures 16°C, 37°C and 42°C and pH 4, 7 and 9 for 3h. Over these detrimental pH and temperatures the lytic phages were stable and revealed complete lysis at MOI one. This study supported the therapeutic use of lytic bacteriophages and phage cocktail was prepared a 3×10^9pfu/ml concentration for future use.

The morphological study of ϕST1 revealed that it has icosohedral head of 52.5nm diameter and 220–250nm long contractile tail (Fig. 3). The electron microscopic analysis revealed ϕST1 belong to family Myoviridae and order Caudovirales. Atterbury *et al.* (2007) also observed that the phages of family Myoviridae can produce more number of phage particles in caecum than that of family Siphoviridae.

The nucleic acid of ϕST1 was found to have 38kb in size (Fig. 4) and digested by *EcoR* I, *BamH* I and *Hae* III. However, the number of bands obtained is less which indicated that the bacteriophages are resistant to the endonucleases released by the bacteria, which is in agreement with Kêsik-Szeloch *et al.* (2013). Furthermore, the molecular characters of ϕST1 supported that the phage belong to family Myoviridae and also thrown light over the quest for endolysins.

In water buffalo, salmonellosis is a wide spread disease with a prevalence of 25% (Borriello *et al.* 2012), 11% (Adlaka and Sharma 1992) and 0.8% (Amrousi *et al.* 1971). Furthermore, there is an increased association of *Salmonella* Tyhimurium in water buffalo and also considered to be a potential pathogen of gastroenteritis and subsequent septicaemia (Borriello *et al*. 2012). Moreover, these organisms also exhibit zoonotic potential and multiple antibiotic resistance in many countries over the past two decades (Barrow *et al.* 2010). The increased antibiotic resistance in microorganisms is viewed seriously by WHO and many other health agencies. In an approach towards alternatives to antibiotics, the therapeutic effect of the *Salmonella* Typhimurium phage cocktail was studied on mice. In this study, the treatment group mice received 3×10^9pfu/ml of phage cocktail after challenging with 3×10^8 cfu/ml of bacteria and found a significant decrease in the number of *Salmonella* from infected and control groups

(Fig. 5). In the treatment group complete loss of the *Salmonella* was found, which indicated a promising choice to control over disease, whereas the infected mice died after 2 days. In similar studies to control *Salmonella* Typhimurium in broiler chicken a single phage ϕ10 decreased in > $2.19\log^{10}$ cfu within 24 h (Atterbury *et al.* 2007). The use of a cocktail of 5 lytic bacteriophages against *Salmonella* Typhimurium is advantageous and could be able to reduce the complete number of *Salmonella enterica* Typhimurium. Further in a study to reduce *Salmonella enterica* serovar Virchow in mice by using a ϕ1 which resulted a significant reduction in the number of *Salmonella* in intestine and liver and it was also noticed that the bacteria developed phage resistance at O antigen and could not able to survive for long duration (Capparelli *et al.* 2010).

These results invite an objective assessment of phage prospects as antimicrobials. The appreciation of use of bacteriophages as therapeutic agents can be assessed by observing the use of *Salmonella* specific phage preparation to reduce contamination level of live poultry before processing by U.S. Food safety and inspection service (Capparelli *et al.* 2010).

Further the phage cocktail can also be applied over meat and meat products to reduce contamination. Though a bit neglected in the past research, in the present era of antibiotic resistance, bacteriophages are the promising choices as antimicrobials and bio-preservatives.

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