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 higher 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*.

**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 MgSO4 and 1M Tris HCl pH7.5) and *Salmonella Typhimurium* (1.5×10⁶CFU/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 EcoRI, BamHI and HaeIII 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 bacteriophages 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 Mitomycin 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
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, Atterbury 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, Merabishvili 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 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 3×10^9 pfu/ml concentration for future use.

The morphological study of ɸST1 revealed that it has icosohedral head of 52.5nm diameter and 220–250nm long contractile tail (Fig. 3). The electron microscopic analysis revealed ɸST1 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 ɸST1 was found to have 38kb in size (Fig. 4) and digested by EcoRI, BamHI 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 ɸST1 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 Typhimurium 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 3×10^9 pfu/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 \( \phi 10 \) decreased in > 2.19log\(^{10}\) 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 \( \phi 1 \) 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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REFERENCES


