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# Application of specific Bacteriophages against prevention of *Staphylococcus aureus* toxin-initiated food spoiling

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**ABSTRACT**

Sample taken from pond water (Padila),river ganga water (Sangam, Allahabad) sewage water (Yamuna drainage) supply water from Municipal Corporation (Daraganj) were found that Ganga water (Sangam) has positive (large, small, medium) plaque formation other pond water, sewage water, supply water from municipal Corporation do not have plaque formation. Since we pressure that Ganga water have high presence of *S. aureus* plaques formation for bacteriophages. Help in therapeutic process, drug target discovery, bacteriophages isolated from environment or Ganga water sample to specifically target pathogenic bacteria and eliminate them from foods. Bacteriophages approved or use in food safety application, genetically engineered phages to specifically target bacterial virulence-associated genes, control multidrug resistant *S. aureus,* implications in biofilm treatment, the progress in the knowledge about phage genomics, immunobiology and experimental therapy in animals and in humans suggest that phages could become the antibacterial drugs of the 21st century.

***Key words*** *: Staphylococcus aureus, Water samples, Bacteriophages, Ganga water, Plague (Phage)*

## Introduction

Bacteriophages are viruses that infect and multiply within bacteria (Mishra, 2013). For many bacteriophages, may release into the environment after replication is accompanied by lysis of the host bacterium (Stone, 2019). This event can be easily observed in test tubes as well as on agar plates (plaque formation), and its exploitation for killing infectious bacteria was suggested almost immediately upon discovery (Hyman, 2019; Joerger, 2003). Bacteriophages are measurable components of the natural microflora in the food production continues from the farm to the retail outlet (Greer, 2005). Phages are extraordinarily stable in these environments and are willing to recover from soil, sewage, water, farm and processing plant effluents, faeces, and retail food products (Moye, 2018). Therefore, purified high-concentration phage and lysates have been use for the species-specific control of bacteria during the pre- and postharvest phases of food production and storage (Greer, 2005). Bacteriophages show an important force in microbial evolution, including their capacity to convert host genes and mediate the acquisition of

novel genetic information (Singh, 2016). The ability of bacteriophages to impart novel biochemical and physiological properties not only provides the host with the opportunity to adapt to new environments (Shruti, 2018), but also in some instances, confers novel virulence properties associated with pathogenesis in human bacterial infections (Tony, 2005). *S.aureus* is significant cause of human as well as animal infections (Nath, 2013). Bacteriophage are a special class of mobile genetic element (MGE) that carry virulence genes and spread them horizontally, including Panton valentine leucocidin (PVL), the immune evasion cluster (IEC) associated with human specificity and enterotoxin A,the major toxin associated with food poisoning (Diana, 2012). *S.aureus* isolates group into major clonal complex (CC) lineages that largely evolve independently due to possession of different restriction modification (RM) system (Alex, 2012). Their use for the treatment of diseases will be probably possible; their development as growth promonants will be more difficult, especially since the bacterial targets for the growth promotants are not fully known (Liliam, 2018). However, bacteriophages as a food additive and use as a control of food borne pathogens and the drawbacks on which more research is needed to further future exploits of these biological entities (García, 2009; Sanna, 2012).

## Materials and Methods

### Selection of food spoiling Microbe

Strains of *S. Aureus* was used for bacteriophage isolation from various water resources including Ganga water. Confirmed by microbial culture technique using blood-agar plate and incubated at 37 °C overnight. One loopful of typical isolated colony was used for smear preparation. Slide processed for gram-staining to observe under microscope. Biochemical observation was done by coagulase test.

### Isolation and propagation of phages against *Staphylococcus aureus*

To isolate bacteriophages, water samples were collected from different sources, these were mainly from four sites Pond water, River Ganga water, Sewage water, Supply water from Municipal Corporation. Phages were generated in the form of plaques using Overlay plaque assay method, table given below (Table 1).

### Bacteriophage isolation and propagation technique

After the culture result turned completely clear, then it was treated with chloroform for at least 7 min and centrifuged at 10,000 rpm for 12 min and the supernatant was filtered through 0.45 m filter membrane pore size. The filtered phage lysate was titrated by plaque assay and stored at 5 °C until used. For long-term storage, phage lysate was supplemented with 8% dimethyl sulfoxide and stored at 90 °C. For purification, phage particles were precipitated in the presence of PEG 7000 and NaCl (1 M) on ice cube for at least 1.5 hr. The precipitate was collected by centrifugation at 12,000 rpm for 15 min at 5 °C and suspended in SM buffer (1/ 100 of the volume of the original phage lysate). After adding 0.5 g/ml CsCl, the mixture was layered on top of CsCl step gradients (densities of 1.55, 1.80, and 1.90 g/ml) in ultra-clear centrifugation tubes and centrifuged at 36,000 rpm (Beckman SW41 rotor) for 2.5 hr at 5 °C. The banded phage particles were collected and subjected to a second centrifugation in a CsCl (1.70g/mL) equilibrium gradient at 42,000 rpm in the same rotor for 24 hr at 5 °C. Thus, phages collected were dialyzed against three changes (1,000 vol each) of 0.6 TE buffer, pH 7.0, at 5 °C overnight. When it necessary, the purified phage samples were further concentrated by centrifugation using a Millipore ultra-free-0.6 Biomax-10, as per modified protocol (Hsieh, 2011; Kishor, 2016).

### Application of Bacteriophages to prevent food spoilage

Specific phages will added in food materials and observe CFU of *S. aureus* for successive days till its reached to zero

### Bacteriophage to control *Staphylococcus aureus* contamination

**Bacterial Strains and Growth Conditions**- *S. aureus* strains used for bacteriophage isolation and deter-

**Table 1.** Source of water to isolate *Staphylococcus aureus* Bacteriophages

| S.N | Pond water | River Ganga water | Sewage water | Supply water from Municipal Corporation |
|---|---|---|---|---|
| **1.** | Padila | Sangam Prayagraj | Yamuna Drainage | Daraganj |

mination of host range were previously isolated from milk and other food samples. *S. aureus* isolates from conventional bulk tank and organic milk and other food products (laboratory collection) were also used for phage host range determination. Staphylococcal cells were cultured in 2xYT brothusing basic routine methods (Sambrook, 1989).

**Staphylococcal Strain Typing**- *S.aureus* strains were typed by random amplification of polymorphic DNA (RAPD)-PCR using the oligonucleotide RAPD5 from the RAPD Analysis Primer set amplification conditions were 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 2 min at 31 °C, 2 min at 70 °C, and a final 10-min extension step at 70 °C. The RAPD-PCR band patterns were scanned with the gel doc 2000 gel documentation system equipped with quantity One software Sorensen's similarity coefficient was calculated as a function of the presence or absence of the different bands for each pattern, different patterns being grouped using the un-weighted pair group method with arithmetic averages (Priest, 1995).

**Bacteriophage Enrichment and Isolation-**Bulk tank milk and various food material from different collaborative shop, and traditional cheeses manufactured in different factories were used for bacteriophage screening purposes. Each food product (100 µl) and cheese sample (500 mg) were added to 2 ml of *S. aureus* strains growing in 2xYT containing 10 mg/l CaCl2 and 10 mg/l MgSO4. The cultures were incubated overnight at 37 °C with shaking. Samples were centrifuged at 13,000 × g for 5 min and filtered. The supernatants were subjected to plaque assays using each of the 15 strains as indicators. Plaques were re-isolated, propagated, and stored at "80°C in SM buffer (20 mg/l Tris HCl, 10 mg/l MgSO4, 10 mg/l CaCl2, 100 mg/l NaCl, pH 7.5) containing 50% glycerol (vol/m). Phages were purified by ultracentrifugation (100,000 × g for 90 min) followed by CsCl continuous gradient centrifugation (Kishor, 2016).

**Bacteriophage Host Range**-The host range of phages was determined by the plaque assay: a 0.1-mL volume of stationary-phase host culture (110CFU/ml) was mixed with several dilutions of individual phage suspensions in 4 ml of molten 2xYT top agar (0.8% agar) and the mixture was poured on 2xYT agar plates. Efficiency of plaque formation of selected phages was determined by dividing the phage titer on the test strain by the phage titer on the reference strain *S. aureus*. This strain was selected because it is infected by most of the isolated phages

**Temperate versus Lytic Phage Determination**- To determine if a phage was temperate, putative lysogens (resistant to infection) were isolated from lysis plaques. For each phage, several plaques were scratched and viable cells were colony isolated on 2xYT agar plates. Isolated colonies were challenged by the plaque assay with the corresponding phage to confirm resistance to infection. Exponentially growing cultures were subsequently induced by adding mitomycin C (0.6 µg/ml) to confirm prophage release. After incubation at 37 °C for 5 h with shaking, supernatants were filtered and tested by the plaque assay against all the staphylococcal strains to verify the host range of the phage

**Cross-Immunity Assays**- *S. aureus* was lysogenized by phages ÖH5 or ÖA72 as Ganga water phage. Once it was confirmed that the phage was integrated in the chromosome, cross-immunity was assessed by the spot test. Plates were prepared by adding 100 µl of an over-night culture of the lysogenic strain to 5 ml of molten 2xYT agar (0.6%). A 4 µL volume of the phage stock (110PFU/ml) was spotted onto the surface. Plates were incubated at 37 °C for 17 h and checked forclearing zones.

**Phage Temperature Stability**-Phage stocks were diluted in UHT whole water sample to obtain 105 and 108 PFU/ml. The suspensions were incubated at 4, 24, and 37 °C for 7 h and the phage titer was determined. Similarly, phage suspensions were incubated at 72 °C and samples were removed at 15 s, and 1, 3, 5, and 17 min for phage titration.

**Phage Antimicrobial Activity in food**-The effect of phage infection on *S. aureus* growth was tested in commercial UHT and water sample, and in whole-fat and semi-skimmed raw milks (which were centrifuged at 6,000 × g for 20 min to remove part of the fat). Sample was inoculated with diluted overnight cultures of *S. aureus* (102 CFU/ml) and the phages (104 to 105 PFU/ml). The mixtures were incubated at 37 °C without shaking. In experiments simulating a breakdown in refrigerated storage, Water sample and food sample was inoculated with diluted overnight cultures of *S. aureus* phage (102 CFU/ml) and a mixture of phages (101, 102, and 104 PFU/ml). The mixtures were incubated at 5 °C for 17 h and then shifted to 21 °C for the following 30 h. In all experiments, samples were taken at different time

intervals and scored for *S. aureus* on chapman agar plates and for phages using the plaque assay. The absence of *S.aureus* in water and food sample was verified by direct plating.

## Results and Discussion

### Bacteriophage application to inhibit the effect of *Staphylococcus aureus* food spoilage Morphological, Microscopic and Biochemical observation

Blood Agar is an enriched medium that provided with multiple nutrients and generally comes as a basic media for the preparation of blood agar by supplementation with blood sample, intaking *S. aureus* sample and by applying blood agar medium and done striking and here agar is solidifying agent that provides a stable surface for the organism to grow on (Figure 1), which allows for the observation of colony morphology and enumeration of the organism. On the blood agar medium the *S. aureus* strains were grown as the haemolytic colonies (Figure 2). In the microscopic observation the positive *S. aureus* strains took gram's stain and appeared in the form of irregular clusters (Figure 2). Coagulase positive isolates were used for the isolation of bacteriophage from the water

### Isolation of bacteriophage from different water sources

Processed samples were examined by overlaying the water samples with *S. aureus* culture and the plaque

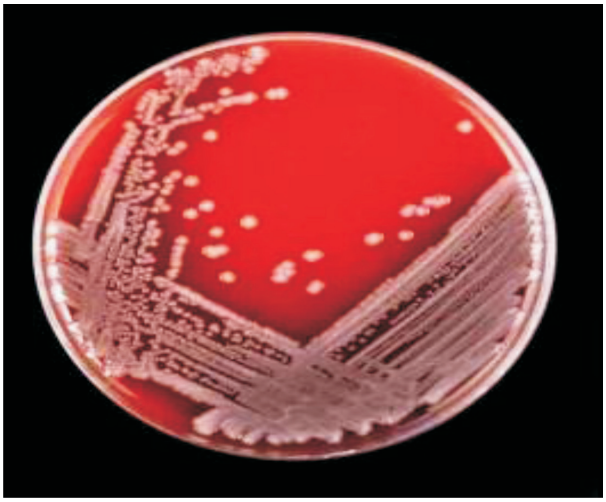

**Fig. 1.** A typical growth of *S. aureus* on blood agar medium

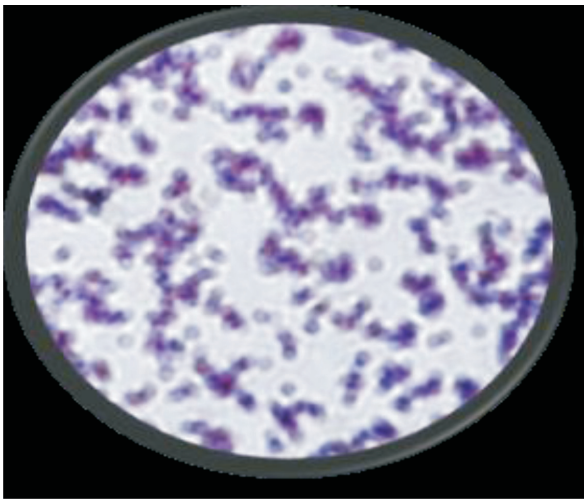

**Fig. 2.** Bunch like appearance of gram-positive bacteria *S. aureus* on microscopic observation.

formation resulted by all the water samples of Pond water, River Ganga water, Sewage water, Supply water from Municipal Corporation (Table 2).

The plaques appeared on the plates with different morphology. In the plate, three types of plaque morphology appeared:

(1) Some plaques were irregular in size,
(2) Some were medium
(3) Some plaques were smaller in size, in Fig. 2.

### Application of bacteriophages to prevent food spoilage

The isolated phages at $10^6$PFU would use to prevent *S. aureus-initiated* spoilage of food material by addition of quantities, amount in the food material. *S.aureus*. CFU reached zero after 72 hours of addition of specific phages with host. Although, there is a need to devise methods of enhancing the size and also visibility of the formed tiny plaques of *S.aureus* phages in order to increase their chances of isolation, detection, and better evaluation (Figure 3). Presence of plaques over the lawn of *S. aureus*, confirm the result is positive for particular experiments of phages from different source of water specially in Ganga Water (Figure 3)

## Conclusion

Application of bacteriophages in *S.aureus* food poisoning can able to inhibit the effect of food toxicity and can overcome effect of food poisoning by *S.aureus*, so bacteriophages act as a role therapeutic

**Table 2.** Water samples that form plaques on *Staphylococcus aureus* lawn culture

| S.N | Water samples | Plaque formation |
|---|---|---|
| 1 | Padila | Not observed |
| 2 | Ganga (Sangam) | Positive (Large, small and medium) |
| 3 | Yamuna Drainage | Not observed |
| 4 | Daraganj Municipal corporation Supply | Not observed |

**Fig. 3.** Presence of plaques over the lawn of *Staphylococcus aureus*.

target for further use. After experiment we are providing evidence that phages can be successfully used to control *S.aureus* in food and food products. There are phage-based diagnostic tools available to detect *S. aureus*, which includes antibiotic resistant (MRSA) and susceptible (MSSA) strains, more the potential of phages is being evaluated mostly due to the increasing emergence of multiresistant strains. Future studies should also focus on improving the general understanding of the mechanisms of phage resistance acquired by the hosts and the rate of elimination from the animal body. Phage therapy is able to reduce foodborne pathogen levels in animals and consequently control the pathogen load on entry at the slaughter houses, the strategies applied for phage bio-control of pathogens in foods reduce significantly the levels in a variety of products and seem to be a promising alternative to traditional food safety and preservation measures, phage use in agricultural settings is as efficient or more than the conventionally used agents to control the growth of plantbased bacterial pathogens

## Future Prospects

Bacteriophages used for spoiled food samples so it will decrease the effect of *S. aureus* in food samples and increase the self-life and also prevent the spoilage of food and further bioinformatics tools and techniques will be applicable for diagnosis and drug discovery. Incorporation of *S. aureus* bacteriophages is helpful in various irrigations, help in saving food items and prevent spoilage of food and help in food sanitation techniques.

**Conflicts of Interest:** All authors declare that there are no conflicts of interest.

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