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Three Bacteriophages SA, SA2 and SNAF can Control Growth of Milk Isolated Staphylococcal Species

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ZA and SUR has designed the study and analyzed the data. AT has performed the experimental work. MS and AT wrote the article. SUR has supervised and reviewed the article.

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ABSTRACT

Staphylococcus aureus along with other coagulase negative Staphylococci are among the major mastitis causing organisms. Consumers of milk and milk products from mastitic animals are at high risk of foodborne infections. Escalating antibiotic resistance strived us to explore the alternate option to control mastitis. In ongoing era, bacteriophages are an alternate possible effective remedy. Therefore, in this study the infection ability of three lytic phages SA, SANF and SA2 was determined against ten isolates of Staphylococci and one *Micrococcus*, isolated from five raw milk samples. Their morphological, biochemical characterization and 16S rRNA sequencing indicated that two strains were *S. aureus*, while all others were different coagulase negative Staphylococci. Their sensitivity against three phages indicated broad host range of phages SANF and SA2, and relatively narrow host range of phage SA. Phage SNAF showed an effective growth reduction of *S. aureus* RP isolate, compared with other bacteriophages. The bacterial challenge test in milk indicated that proliferation of *S. aureus* was successfully ceased until six hours post infection when applied at and MOI of 100.

INTRODUCTION

A vast number of microorganisms, including bacteria, fungi and mycoplasmas are responsible for mastitis in dairy animals, and *Staphylococcus aureus* is the principal causative agent among them, along with other coagulase negative Staphylococci (Buzzola *et al*., 2001; Thorberg *et al*., 2009). These organisms reside and propagate on teat skin of dairy animals, which is coated with keratin that prevents microbes to get access towards teat canals (Robinson, 2005). During milking process, they mix in milk and can pose a serious threat of infection in humans on the consumption of contaminated milk (Oliver *et al*., 2005). *S. aureus* has been reported for many foodborne outbreaks due to consumption of milk and milk products (Asao *et al*., 2003; Johler *et al*., 2015; Ostyn *et al*., 2010). The emergence of multiple drug resistant *S. aureus* necessitated search of alternative methods for their elimination (Morandi *et al*., 2009).

Bacteriophages have an established history as an alternate therapy for bacterial infections. They have been successfully applied to control food contamination due to their host specificity and sparing normal microbial flora in humans (Haq *et al*., 2012; Hermoso *et al*., 2007). Many companies in West have prepared phage based products that are applied on ready to eat foodstuffs at any step from processing to consumption (Garcıa *et al*., 2008; Lu and Breidt, 2015). Listex™P100 and Eco Shield are being used against *Listeria monocytogenes* and *E.coli* O157:H7, respectively in the meat, while Felix-O1 is being used to remove *Salmonella* from chicken (Garcıa *et al*., 2008; Sillankorva *et al*., 2012).

In this study the lytic activity of three bacteriophages have been assessed against mestitic pathogens isolated from milk *in vitro*. Additionally, a preliminary bacterial challenge test in milk has been assessed to determine the effectiveness of phages in milk.

MATERIALS AND METHODS

Isolation and identification of Staphylococci

Five raw milk samples (approximately 15 mL for each) were collected in sterile falcon tubes from different milk shops in Lahore. They were processed for isolation of Staphylococci. The collected samples were serially diluted in buffer peptone water (Oxoid) at 9:1 ratio and 50µL from serial dilutions were plated on mannitol salt agar (MSA) (Oxoid) followed by incubation for 24 h at 37°C. The isolated colonies on MSA were further streaked on Luria Bertani (LB) agar, followed by incubation for 24 h at 37°C to obtain their pure cultures that were used for biochemical testing. The isolates were identified on the basis of their colony morphology on MSA, gram staining, catalase and coagulase tests (Cappuccino and Sherman, 2008; Cheesbrough, 2006). The isolates were directly sent to Macrogen Korea for sequencing the 16SrRNA fragment for their molecular Identification.

Propagation of bacteriophages and their activity against bacterial isolates

All the bacteriophages used in this study were isolated from sewage exhaust of different hospitals located in Lahore, Pakistan. Currently these bacteriophages are partially

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characterized (Hamza *et al*., 2016). The propagation of the bacteriophages (SA, SANF and SA2) and their activity by spot tests against the isolated strains was performed according to already published work (Bibi *et al*., 2016). Sensitive bacterial isolates 3Y, 2P and RP were selected as the host for the enrichment of phages SANF, SA2 and SA, respectively. Phage titer was determined by counting plaques obtained through a serial dilution of the bacteriophages preparation on double layer agar plates (Bibi *et al*., 2016). To calculate phage titer, serial dilutions of phage enrichment were prepared followed by incubation for 45 min. After incubation, the mixture was added in 3 mL soft LB agar, poured on the LB agar plates and further incubated for 24 h at 37°C. The plate with countable plaques (3-300 plaques per plate) was observed to calculate the phage titer using the following formula (Obeso *et al*., 2010).

$$\text{Pfu/mL} = \frac{\text{No. of plaques x dilution factor}}{\text{Volume plated}}$$

Bacterial growth reduction assay

To determine the lytic activity of phages (SA, SANF and SA2) on the growth rate of host bacterial strain (RP), 24 h old bacterial culture (100 µL) was added in four flasks containing 50 mL of sterilized LB broth. All flasks were inoculated with 50 µL of correspondingly labeled phages except one, which was used as negative control. During incubation of 24 hours at 37°C, optical density (600 nm) was measured at an interval of two hours by taking sample (1 mL) from each flask.

Bacteriophage antibacterial activity in milk

The effect of phage (SA, SANF and SA2) antibacterial activity on the bacterial growth was tested in commercial pasteurized milk at 25°C and 37°C, at multiplicity of infection (MOI; the ratio of phage concentration/bacterial concentration) of 100. Milk (8.6 mL) in sterile tubes was inoculated with diluted overnight cultures of bacterial strain (10^6 Cfu/mL) and correspondingly labeled phage (10^8 Pfu/mL). Milk inoculated only with the diluted overnight culture of bacterial strain (10^6 Cfu/mL) was used as a control. All tubes were incubated at their correspondingly labeled temperature for six hours and from each tube, (1 mL) sample was taken at an interval of two hours and bacterial load was recorded after growth on Chapman agar. The sterilization of pasteurized milk was tested by direct plating (Obeso *et al*., 2010). The percentage reduction calculated by dividing the decrease in CFU by total number of bacteria in control on a particular time and multiplying by 100.

RESULTS

Majority of milk isolates belong to genus Staphylococcus

Based on cultural and biochemical properties, 11 staphylococci isolates were obtained from 5 raw milk samples. On the basis of 16S rRNA sequencing the isolated strains were identified as different species of the genus *Staphylococcus,* while one strain (2P) which was identified as *Micrococcus caseolyticus* (Supplementary Table I).

Bacteriophages SA2 and SNAF showed broad host specificity

Phage SANF showed complete lytic activity against all isolated strains while phage SA2 was also active against all isolates except 3Y and RI. Phage SA infected the four isolates only (Supplementary Table II).

Tested bacteriophage reduced the host bacterial growth

The growth reduction experiment was performed using the *S. aureus* RP isolates, due to its high susceptibility to all isolated phages. The reduction in growth of *S. aureus* against each individual phage was measured as optical densities (600 nm) during 24 h of incubation. The results of this *in vitro* study indicated that phages reduced the growth of target bacteria. There was a common observation that the bacteriophages sustained the growth reduction during the early 8 h of infection. The bacterial cells started to grow after the 8 hours of infection, but interestingly, the bacterial growth started to decrease again after the 16-18 h post infection (Fig. 1).

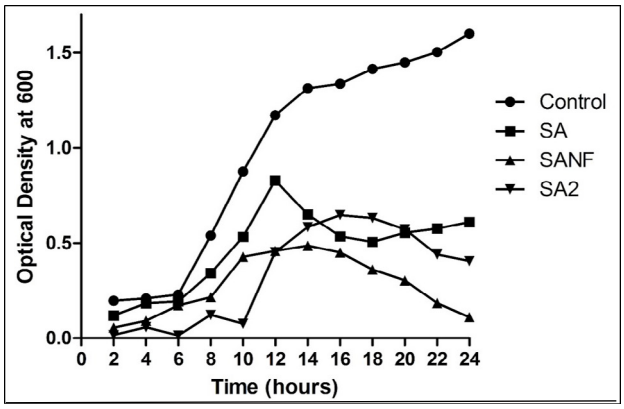

Fig. 1. *In vitro* growth reduction assay of *S.aureus* (RP isolate) by bacteriophages SA, SANF and SA2.

Bacteriophage antibacterial activity in milk

The antibacterial activity of three phages was also tested in the commercial pasteurized milk against *S. aureus* RP isolate. The tested phages showed an increased percentage of bacterial load reduction from 4-6 h, post-infection when incubated at 25°C. The three phages also showed antibacterial activity when incubated at 37°C. The activity at 37°C was however, lower compared to that at 25°C. The maximum bacterial growth control was observed by the bacteriophages SANF and SA2 at 25°C temperature, while bacteriophage SA showed reduction in

bacterial growth from 99 to 60 percent during transition form 4-6 h post infection. The bacteriophage SANF has not shown bacterial growth control ability when incubated at 37°C. However, the phage SA and SA2 showed a growth reduction up to 65 percent from 2-6 h, post infection (Fig. 2).

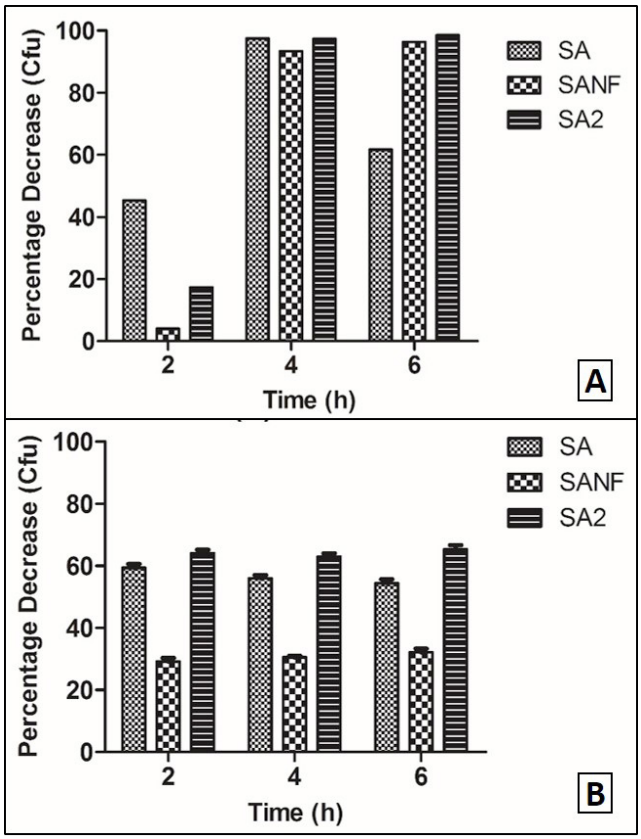

Fig. 2. Inhibitory activity (percentage reduction in bacterial colony count) of phage SANF, SA2 and SA against *S. aureus* in comparison with uninfected control in milk at MOI 100: **A,** at 25°C; **B,** at 37°C.

DISCUSSION

In the current study, the susceptibility of milk isolated bacterial strains against three phages (SA, SANF and SA2) indicated broad host range of SANF and phage SA2 while SA indicated relatively narrow host range than others. El Haddad *et al*. (2014) reported the broad host range of three polyvalent phages Team1, phi812 and K. He found 52 out of 57 raw milk isolated strains of of *S.aureus* isolated from raw milk samples, susceptible to all of three polyvalent phages (El Haddad *et al*., 2014). The individual phage SANF showed efficient antibacterial activity against Staphylococci isolated from milk as Jia *et al*. (2015) reported the effective lytic activity of a newly isolated phage JS01 with broad host range against *S.aureus*. Broad host range of phage vB_SauM_JS25 phage against *S.aureus* strains was also observed by Zhang *et al*. (2015).

The three tested phages in this study showed above 90 percent decrease in the growth of target bacteria when incubated at room temperature, while the percentage reduction was reduced to 30-60%, when incubated at physiological temperature. Bacteriophage SA2 showed a considerable potential for growth reduction of target organism in the milk environment. The decrease in the lytic ability of the bacteriophages when applied in the milk environment compared with their activity in broth is similar to previous studies of O'Flaherty *et al*. (2005) and Gill *et al*. (2006) who found inactivity of phage K in milk against *S. aureus*. The presence of milk proteins is mainly responsible for the inactivity of phages in milk. The milk proteins after being adhered to the bacterial cell surfaces change the receptors' morphology of *S. aureus* and make it tough for phages to access the host receptors. *S. aureus* can bind to the broad range of proteins such as immunoglobulin, collagen and fibrinogen (Gill *et al*., 2006). The presence of IgG and some other unknown components in the lacteal secretion of dairy animals cause aggregation of *S. aureus* cells in milk. These aggregates also inhibit the absorbance of phages on the bacterial cell receptors (Tanji *et al*., 2015). O'Flaherty *et al*. (2005) studied that the immunoglobulins, adhered to the bacterial cells associate them with the fat globules and the coating of fat globules around *S. aureus* aggregates also hinders the access of phages to *S. aureus* receptors. The bacterial growth reduction by the tested bacteriophages can be employed for controlling Staphylococcal contamination in raw milk. In future, more detailed studies of bacterial control by bacteriophages might give confidence to dairy industry for their routine use.

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Conflict of interest statement

We declare that we have no conflict of interest.

Supplementary Material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2017.49.2.493.496

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