Isolation, characterization and therapeutic potential assessment of bacteriophages virulent to *Staphylococcus aureus* associated with goat mastitis

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Summary

In the present study, the therapeutic potential of bacteriophages virulent to *Staphylococcus aureus* associated with goat mastitis were isolated, identified and assessed. *Staphylococcus aureus* (host or indicator bacterium) was isolated from a goat suffering from clinical mastitis. Based on cultural, morphological, biochemical tests and amplification of *S. aureus* specific thermonuclease gene in PCR, the identity of the organism was confirmed as *S. aureus*. Bacteriophages were isolated from soil and faecal samples (n=42) collected from different parts of the Mathura district in Uttar Pradesh (India), and their identity was confirmed by amplification of the bacteriophage-specific endolysin gene fragment in PCR. The thermal tolerance study revealed that all phage isolates were stable at 30 and 40°C with 100% lytic efficacy and their activities reduced to 62-80% at 50°C declining sharply at 60°C with less than 5% efficacy. Likewise, at pH = 6.5 and 7.5, the survivability of all isolates was 100% which reduced to 70-79% and 84-91% at pH = 5.5 and 8.5, respectively. All isolates were stable up to 3 months at 37°C, and for 16 months at 4°C but the stability of their respective endolysins only lasted for 12-23 days at 37°C and 6 months at 4°C. Three of the bacteriophage isolates, *S. aureus* phage/CIRG/1, *S. aureus* phage/CIRG/4 and *S. aureus* phage/CIRG/5 exhibited lytic activity against over 80% of the staphylococcal isolates. The results of the present study provide insight for the use of lytic bacteriophages for therapeutic interventions against multi-drug-resistant *S. aureus* inducing mastitis in goats.

Key words: Bacteriophage, *Staphylococcus aureus*, Goat, Mastitis, Endolysin

Introduction

Staphylococcus aureus is an important bacterial pathogen in human and animals causing soft tissue abscesses, wound infections, endocarditis, osteomyelitis, mastitis and bacteremia (Mann, 2008). Coagulase positive *S. aureus* is the most common cause of clinical mastitis in dairy goats while coagulase negative *Staphylococci* are the most prevalent in subclinical forms of mastitis (Bergonier *et al*., 2003). Treatment of mastitis is becoming increasingly difficult as many clinical isolates of *S. aureus* have been found to be resistant to β-lactam antibiotics, aminoglycosides, fluoroquinolones and glycopeptides (Bishop and Howden, 2007). Furthermore, mastitis antibiotic therapy causes several other problems including bacterial dysbiosis (Sulakvelidze *et al*., 2001), antibiotic residues in milk and milk products (Sandholm *et al*., 1990), side effects (Yao and Moellerin, 1995), antibiotics inability to eliminate bacteria L-forms (Owens, 1998) as well as adverse effects on milk and milk product exports. Therefore, the search for an alternative treatment for infections caused by multi-drug-resistant (MDR) bacterial agents has become a present day need. Under these challenging circumstances, bacteriophages (phages) have been identified as suitable alternatives to antibiotics, and efforts to explore possibilities of developing phage based therapeutics are gaining popularity all over the world. Phage therapy has been found successful against a variety of bacterial infections such as vancomycin-resistant *Enterococci* (Biswas *et al*., 2002), methicillin-resistant *Staphylococcus aureus* (Matsuzaki *et al*., 2003), *E. coli* (Viscardi *et al*., 2008), *Pseudomonas aeruginosa* (Wright *et al*., 2009), respiratory pathogens (Carmody *et al*., 2010), and several other bacterial infections in clinical cases (Chanishvili, 2009). It also has several advantages over antibiotics including potent *in vitro* and *in vivo* antibacterial activities independent from antibiotic sensitivity patterns, low chance of resistance development, high safety margin and the possibility of easy genetic manipulation (Borysowski *et al*., 2006).

The use of non-characterized phages against bacterial infections is considered to be an important reason for phage therapy failure (Kumari *et al*., 2010). Determining physico-chemical characteristics of phages intended for therapeutic purposes is essential because they are related to the stability and optimum efficacy of phage-products.

Characterizing therapeutically potential bacteriophages in terms of sensitivity to temperature, pH and lipolytic agents like chloroform is essential before subjecting them to further *in vitro* and *in vivo* studies. In the past, phages were isolated from different natural sources, and characterized in terms of optimum conditions for reactivity (McVay *et al*., 2007; Synnott *et al*., 2009; Yang *et al*., 2010; Mishra *et al*., 2011; Mishra *et al*., 2012). The aim of the present study is to isolate phages virulent to *S. aureus* associated with goat mastitis, and to characterize them with respect to their physico-chemical properties, host range and *in vitro* lytic activity of endolysins (lysins).

Materials and Methods

Isolation and identification of the indicator (host) bacterium

Staphylococcus aureus isolated from clinical goat mastitis was used as an indicator organism for isolation, purification, propagation and characterization of the phages. Isolation and identification of the indicator bacterium was carried out as per standard cultural, morphological and biochemical methods. Confirmatory identification was carried out by polymerase chain reaction (PCR) using specific primers; 5′-GCG ATT GAT GGT GAT ACG GTT -3′ (sense primer; Brakstad *et al*., 1992) and 5′-AGC CAA GCC TTG ACG AAC TAA AGC-3′ (antisense primer; Brakstad *et al*., 1992), targeting the *nuc* gene which encodes thermostable nuclease. The PCR mixture consisting of 50 ng template DNA, 20 pmol of each primer and 12.5 µl PCR master-mix (Qiagen, USA) was diluted up to a 25 µl volume with nuclease free water. PCR was performed with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 30 sec, extension at 72°C for 60 sec and a final extension at 72°C for 5 min. To identify gene size, the PCR product was analyzed using 1.3% agarose gel with 100 bp marker (MBI Fermentas, USA).

Isolation, identification, propagation and stability of the phages

Soil/goat-faeces were collected from different areas of the Mathura district, Uttar Pradesh, India. About 10 g of each sample was homogenized in 100 ml of an SM buffer [0.1 M NaCl, 10.0 mM $MgSO_4$. 7 H_2O, 0.05 M TrisHCl (pH = 7.4-7.5), 1% Gelatin (w/v)]. The suspension was centrifuged (7000 × g for 15 min) to remove bacterial cells and debris. The supernatant was then filtered through 0.22 µ syringe filter (Millipore, USA). The filtrate was added to an equal amount of double strength BHI broth supplemented with 0.1% $MgSO_4$. 7 H_2O (HiMedia, India) and inoculated with an amid log phase host culture (*S. aureus*). After incubation at 37°C for 16-18 h, the medium was centrifuged at 8000× g for 10 min. This enrichment procedure was repeated two to three times. The supernatant obtained from the final enrichment step, known as bacteria free filtrate (BFF), was filter sterilized and tested for the presence of phages active against the indicator bacterium. The spot inoculation method of Park *et al*. (2000) was used as an initial test for phage presence by measuring lytic efficacy. A clear zone in the plate resulting from the lysis of host cells indicated phage presence. Phage isolates were purified as per the standard plaque purification method by repeatedly plating and picking individual plaques. For confirmatory identification of the phages, the lysin gene of the phage was amplified by PCR using specific primers: 5′-ATGG CTAAGACTCAAGCAGAA-3′ (sense primer) and 5′-ATTTACCCGTGTGCCAAG-3′ (antisense primer). The oligonucleotide primers used in this study were synthesized by Integrated DNA Technologies (USA). The PCR mixture consisting of 50 ng template DNA, 20 pmol of each primer and 17 µl PCR master-mix (Qiagen, USA) was diluted up to a 25 µl volume with nuclease free water. PCR was performed with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 60 sec, annealing at 51.8°C for 60 sec, extension at 72°C for 60 sec and a final extension at 72°C for 7 min. The PCR product was analyzed using 1% agarose gel with 100 bp marker (MBI Fermentas, USA).

Suspensions of the phage isolates were stored over chloroform (5% by volume) at 4°C, and aliquots were frozen at -70°C in an SM buffer containing 25% glycerol. Phage titers were determined as plaque forming units (pfu/ml) using the standard double layer agar plate method. To determine optimal multiplicity of infection (MOI), mid log phase host cells were infected with the phages at 4 different ratios (0.01, 0.1, 1, and 10; pfu/cfu). After incubation for 4 h at 37°C, the phage lysate was centrifuged at 1000 × g for 3 min. The supernatant was filtered (0.22 µ syringe filter) and assayed to determine the phage titer. The MOI resulting in highest phage titer within 4 h was considered as the optimal MOI, and was used for the bulk production of the phages, as well as subsequent experiments. Large-scale production of the phage isolates was done by the conventional liquid culture method.

Resistance expressed by the phages to physical and chemical agents was determined using the method of Chow and Rouf *et al*. (1983). Four temperatures (30, 40, 50 and 60°C) were selected to study the thermal tolerance of the phages in a BHI broth. Results were expressed as the phages' survivability percentage. Likewise, phage stability was assayed at pHs ranging from 6.5 to 8.5 and at chloroform-treatment. For assessing the long term stability of the phages at 4°C and 37°C, 5 ml of each phage-preparation was kept at the mentioned temperatures for 16 months and 3 months, respectively. The lytic activity of each preparation was assessed by the spot inoculation method of Park *et al*. (2000) at a monthly interval.

Isolation and purification of endolysins produced by *S. aureus* phages and the determination of the phages' host range and their endolysins

Endolysins secreted by the phages were precipitated

and separated according to the method described by Sonstein *et al*. (1971). Double precipitation with ammonium sulphate was followed and the precipitated endolysins were dissolved in 0.07 M sodium phosphate buffer and kept at 4°C until further use. To determine the optimum storage condition for endolysins, 100 µl of each lysin preparation was kept at 4°C and 37°C for 1 month and 6 months, respectively. The lytic activity of the each lysin preparation was assessed by spot inoculation method at a weekly interval. *In vitro* lytic activity of the phages and their homologous lysins against 124 isolates of *Staphylococcus* were determined by the spot inoculation method of Park *et al*. (2000).

Results

Circular, convex and golden-brown colored colonies were observed when the milk sample from the clinical goat mastitis case was streak plated on sheep blood agar. The bacterium showed catalase positive, coagulase positive and oxidase negative reactions. Under microscopic examination, it was seen as gram-positive cocci arranged in grape-like clusters. The organism produced jet black colonies on Baird Parker agar (Fig. 1). On the basis of secultural, morphological and biochemical characteristics, the organism was tentatively identified as *Staphylococcus*. PCR amplification of the *nuc* gene resulted in a DNA fragment of approximately 270 bp (Fig. 2).

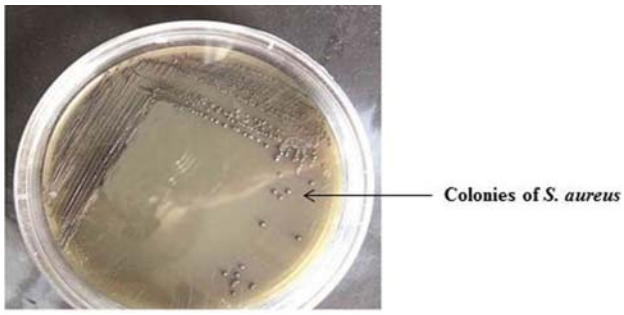

Fig. 1: *Staphylococcus aureus* on Baird Parker Agar

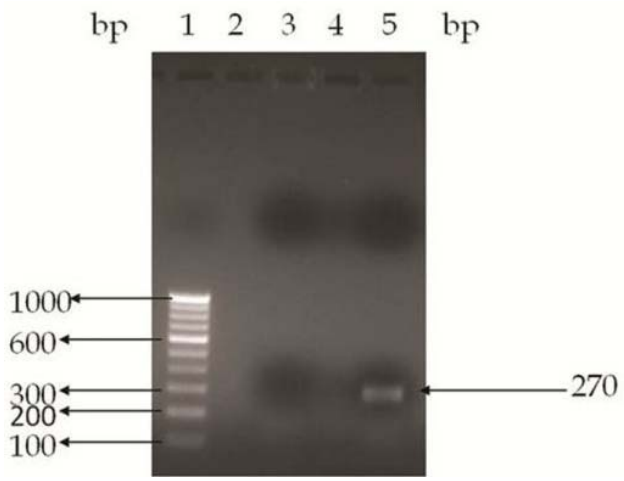

Fig. 2: PCR amplification of the *nuc* gene of *S. aureus*. Lane 1: 100 bp DNA ladder. Lane 5: Amplified PCR product

From the 42 faecal and soil samples, phage presence was detected in only six samples (Figs. 3, 4). All six isolates of *S. aureus* phages were tentatively named as *S. aureus* phage/CIRG/1, 2, 3, 4, 5 and 6, respectively. Each phage isolate was purified, and the stocks of phage isolates were prepared with titers 1.5×10^{11}, 1.2×10^{8}, 2.7×10^{8}, 1.8×10^{9}, 1.3×10^{9} and 2.4×10^{10} pfu/ml, respectively. The endolysin gene of the *S. aureus* phage was successfully amplified using lysin specific primers with a single PCR product of 802 bp (Fig. 5). The optimal MOI for the each isolate was found to be 0.01.

The thermal tolerance study revealed that all phage isolates were stable at 30 and 40°C with 100% lytic efficacy (Table 1). The activity reduced to 62-80% at 50°C and declined sharply at 60°C with less than 5% efficacy. Likewise, at pH= 6.5 and 7.5, the survivability of all six isolates was 100% but reduced to 70-79% and 84-91% at pH = 5.5 and 8.5, respectively (Table 2). The phages were found highly stable on chloroform treatment with an activity ranging between 90-95% (Table 3). In the present study, all phage isolates were stable up to 3 months at 37°C, and for more than 16 months at 4°C.

Endolysin produced by each phage isolate was purified by the precipitation of fresh phage-bacteria culture lysate with ammonium sulphate, followed by high speed centrifugation and dialysis. The activity of all six endolysins at 37°C storage lasted between 12 to 23 days (Table 4), while all showed stability even after 6

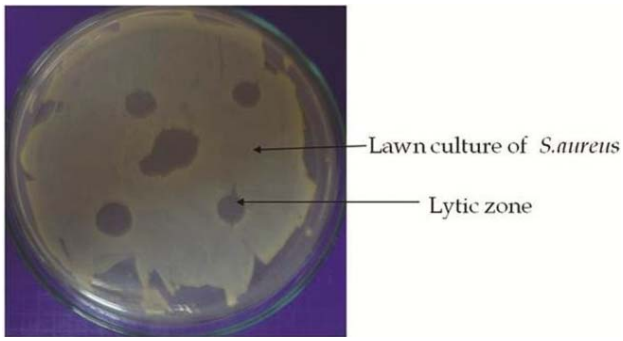

Fig. 3: Lytic zones produced by the phages. The zones are formed due to the lysis of bacterial host cells by phages

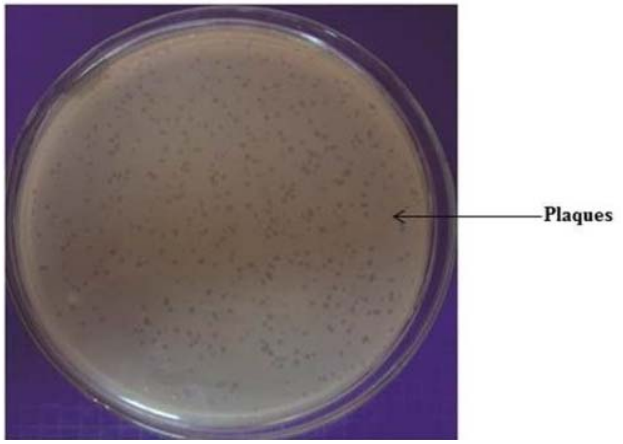

Fig. 4: Plaques produced by phages against indicator *S. aureus*. Plaque-formation indicates presence of phages

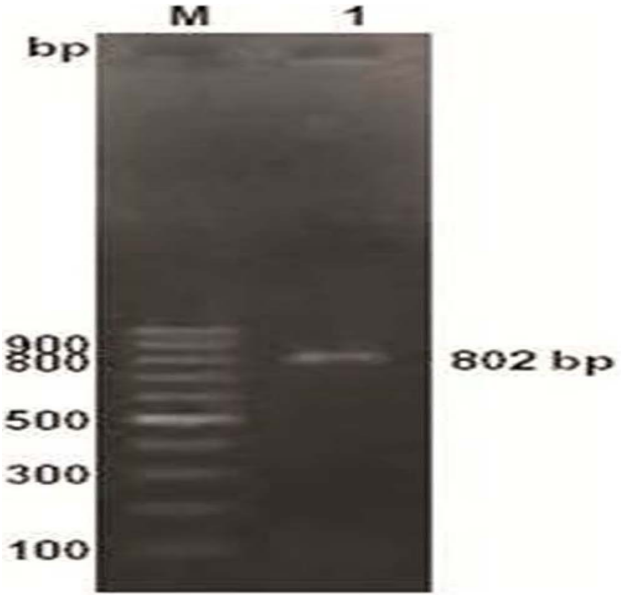

Fig. 5: Amplification of the lysin gene of *S. aureus* phages. Lane M: 100 bp plus DNA ladder. Lane 1: Amplified PCR product

months of storage at 4°C. The lytic potential of the phages and their respective lysins was determined against 124 isolates of *Staphylococcus*. The sensitivity of the target organism against phages was observed by the formation of clear circular lytic zones. Lytic efficacy against more than 80% of the bacterial isolates was demonstrated by *S. aureus* phage/CIRG/1, *S. aureus* phage/CIRG/4 and *S. aureus* phage/CIRG/5 (Table 5). Likewise, the host ranges of the endolysins were determined and found to be slightly higher than those of their homologous phages (Table 6).

Discussion

The use of antibiotics is popular in our daily lives. The indiscriminate and improper use of these remarkable drugs is the most common cause of the emergence of drug resistant bacterial strains. This has created interest among scientists to develop alternatives for antibiotic therapy. Bacteriophage therapy is re-emerging as a possible alternative for the treatment of bacterial infections (Lorch, 1999).

Table 3: Stability of the *Staphylococcus aureus* phages to chloroform treatment

Phage isolate	% Survivability of the phages
1	90
2	95
3	90
4	92
5	91
6	95

Table 4: Stability of *Staphylococcus aureus* phage-lysins at 37°C storage

Endolysin	Duration of *in vitro* activity (in days)
LysCIRG 1	23
LysCIRG 2	12
LysCIRG 3	21
LysCIRG 4	17
LysCIRG 5	19
LysCIRG 6	15

Table 5: *In vitro* lytic efficacy of the *Staphylococcus aureus* phages against different isolates of *Staphylococcus*

Phage-isolate	Lytic efficacy against % isolates
1	82.26
2	76.61
3	70.97
4	86.29
5	84.68
6	73.39

Table 6: *In vitro* lytic efficacy of the endolysins produced by different *S. aureus* phages against the various isolates of *Staphylococcus*

Endolysin	Lytic efficacy against % isolates
LysCIRG 1	83.87
LysCIRG 2	78.23
LysCIRG 3	72.58
LysCIRG 4	88.71
LysCIRG 5	86.29
LysCIRG 6	76.61

Table 1: Stability of the *Staphylococcus aureus* phages at various temperatures

Temperature (°C)	% Survivability of the phages					
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
30	100	100	100	100	100	100
40	100	100	100	100	100	100
50	70	80	62	66	63	72
60	3	0	0	5	0	0

Table 2: Stability of the *Staphylococcus aureus* phages at different pH

pH	% Survivability of the phages					
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
5.5	74	79	70	75	71	72
6.5	100	100	100	100	100	100
7.5	100	100	100	100	100	100
8.5	91	85	84	87	84	80

PCR can be used for the confirmatory diagnosis of bacteria or bacterial products in clinical materials (Valentine *et al*., 1991). PCR amplification of the *S. aureus nuc* gene resulted in a DNA fragment of approximately 270 bp (Fig. 2). This confirmed the organism to be *S. aureus*, and to be subsequently used as an indicator/host bacterium for isolation, purification, propagation and characterization of the phages in this work.

In the present study, we identified six isolates of *S. aureus* phages from soil/faecal samples. and gene encoding bacteriophage-endolysin was amplified for the confirmatory identification of *S. aureus* phages. Characterizing new phages is very important for assessing their therapeutic potential (Watnick and Kotler, 2000) and the use of non-characterized phages against bacterial infections is considered to be an important reason for the failure of phage therapy (Kumari *et al*., 2010). Characterizing phages with respect to their physico-chemical properties is a prerequisite to their therapeutic trials (Hanlon, 2007). Determining the physico-chemical characters of the phages intended for therapeutic purposes is essential because they are related to the stability and optimum efficacy of products. Therefore, all six isolates of the phages were characterized and their *in vitro* lytic activities were assessed.

All six isolates showed stability to a wide range of temperatures which indicates that the phages could withstand the hot climatic conditions of India. Similarly, the phages were able to withstand the pH of mastitic milk which varied from 6.9 to 7.4 because of their stability over a wide range of pH (Hussain *et al*., 2012). The phages also showed considerable survivability rates at chloroform treatments. Chloroform treatment of a phage suspension is necessary because it removes cells infected with lysogenic phages, eliminating any possibility of transfer of toxic/resistance genes (Carlton, 1999). All isolates demonstrated considerable long-term stabilities at 37°C and 4°C. Long-term stabilities are the essential requirement for any phage-preparation to be used therapeutically. The shelf life should be enough for the time needed for quality control, packaging and labelling, distribution to clinical sites and treatment. All the phage isolates qualified on these physio-chemical and stability parameters.

Phage enzymes (lysins) are known by their ability to break covalent bonds of peptidoglycan/murein layers of the host cell wall, leading to the release of newly assembled progenies of the phage (Fischetti, 2005). Endolysin has a unique ability to lyse the murein layer of the cell wall of the host cell in a species-specific manner. Therefore, endolysins could be used as a novel class of therapeutic agents without any side or toxic effects. In our study, we isolated and purified the lysin secreted by all six phage isolates. Double precipitation with $(NH_4)_2SO_4$ was carried out to attain good quality lysin-preparation.

The first and mandatory step for developing a universal phage based therapeutic system is to search for the broad host range lytic phages isolated from an epidemiological area (Barrow, 2001). Therefore, we assessed the lytic potential of the phages and their respective lysins against 124 *Staphylococcus* isolates. Three phage isolates and their homologous lysins showed very wide host ranges and could hence,be used as therapeutic agents against *S. aureus* associated with goat mastitis. The results of the present study provide insight for using lytic bacteriophages for therapeutic interventions against multi-drug-resistant *S. aureus* induced infections including goat mastitis.

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