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Isolation of Phages and Study of their *In vitro* Efficacy on *Staphylococcus aureus* Isolates Originating from Bovine Subclinical Mastitis

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ABSTRACT

Background: India leads the global market in milk production. However, bovine mastitis, which is the mammary gland inflammation in dairy cattle characterized by physical, chemical, bacteriological changes in milk results in commercial losses. *Staphylococcus aureus*, is the major causative agent. The treatment of mastitis caused by this pathogen is mainly by antibiotics. Emphasizing on the one health concept, phage therapy is an appropriate alternative to antibiotic. The present study was aimed to isolate and characterize bacteriophages against *Staphylococcus aureus* associated with bovine mastitis.

Methods: Thirty two isolates of *S. aureus* obtained from the milk of mastitis cows were characterized by phenotypic and genotypic methods. Antibiotic susceptibility of the isolates was carried out by disk diffusion assay. Milk and cow shed wastewater were used for phage isolation. Phages were characterized by host susceptibility and RAPD assay.

Result: Nineteen phages were isolated from the cowshed waste water. All the milk samples showed negative for the presence of phage. The phage 24 (A2) which had the broadest host range, was selected for the CFU drop assay. The phage was able to clear the lawn of *S. aureus* culture when grown on agar at different time points thus indicating that topical application of this phage would be a potential strategy to control *S. aureus* infection leading to mastitis. This study provides a basis to continue the exploration of the potential of PSA2 phage as a candidate for the treatment of Staphylococcal mastitis.

Key words: Bovine mastitis, Phages, *Staphylococcus aureus*.

INTRODUCTION

Milk and dairy products constitute the primary nutrient source for the growt of the microorganisms as it contains all the essential nutrients. Milk acts as a carrier for the transmission of *Staphylococcus* from animal to human and is also frequently associated with subclinical mastitis in dairy animals that may contaminate the udder and milk. Globally, mastitis remains one of the most common economic problems of the dairy industry. It is one of the major infectious diseases of the mammary gland resulting in inflammation and pathophysiological changes in the udder tissue, decreasing the quality and quantity of milk production (Ben Said *et al.,* 2016). *S. aureus* is a milk-borne pathogen causing the severe foodborne intoxication symptoms such as nausea, vomiting, abdominal cramps, diarrhoea, skin diseases, pneumonia, septicaemia, urinary tract infections as well as toxin-mediated syndromes like toxic shock syndrome and food poisoning (Korpysa-Dzirba and Osek 2011). Pasteurization is effective in eradicating the *S. aureus,* but the thermostable nature of enterotoxins makes them hold the biological activity. The incidence of multidrug resistance and the presence of antibiotic residues in the milk and milk products are significant concerns (Madougou *et al.* 2019).

Prevalence of methicillin-resistant S. *aureus* imposes an increasing burden on healthcare resources as well as increased morbidity and mortality (Basdew and Laing 2011). Animals with chronic infections are less likely to be cured by antimicrobial therapy and act as a reservoir in the herd. This understanding has led to a search for alternative treatments. Bacteriophages are the alternative and considered to be one of the effective biological therapy (Wang *et al.* 2019). Bacterial viruses specifically act on bacterium without causing any adverse effect on the surrounding mammary tissues or the environment and can get propagated inside the host. Staphylococcal lytic phages have proved their potential as biocontrol agents of food and phage therapy of human or animal infections

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(Aguayo-Reyes *et al.,* 2018). This brought the phage therapy prominent against mastitis infections associated with *S. aureus* (Ganaie *et al.,* 2018). Hence, the present study is aimed to use phage as a useful tool to combat *S. aureus* causing bovine mastitis.

MATERIALS AND METHODS

Sample collection

Thirty-two *S. aureus* isolate from subclinical cases of mastitis were used and characterized by the phenotypic and genotypic method. Samples from the cowshed wastewater and milk were collected in sterile sample collection tubes in and around Mangaluru belonging to the Dakshina Kannada, District of Karnataka. A total of (32) samples, comprising milk (10) and cowshed wastewater (22) were used for phage isolation.

Isolation and characterization of phages

Samples were centrifuged and filtered using a 0.22 µm syringe filter and used as phage source. The agar overlay assay was used for the examination of phage in the filtrate. Spot assay was used for the detection of phages by the enrichment technique by placing five µL of the supernatant on a lawn of *S. aureus* culture. Plates were incubated at 37°C overnight and observed for plaques. Plaques were purified by single plaque purification and titre of phages was determined by soft agar overlay technique. The isolated bacteriophages were further propagated on their respective host. Tested bacteria were considered as either sensitive or resistant to the phage depending on the appearance of plaque. Bacteriophage DNA was extracted by zinc chloride precipitation followed by phenol extraction as described by Su *et al.* (1998).

Molecular typing of bacteriophage was performed using Random Amplified Polymorphic DNA (RAPD) analysis with RAPD5 primer (Gutiérrez *et al.,* 2011). The reaction mixture (30 µl) consisted of nuclease-free water (21.5 µl), 10X Buffer with 2.5 mM $MgCl_2$ (3 µl), 10 mM/µl dNTP (1 µl), 10 pmol of primer and 2 µl DNA template. The program used was as follows: 3 min at 94°C followed by 35 cycles of 50 sec at 94°C, 36 sec at 36°C and 30 min at 72°C followed by a final extension at 72°C for 5 min. PCR products were then electrophoresed on 1% agarose gel and visualized by ethidium bromide staining.

In vitro assessment of the lytic activity of bacteriophages: CFU reduction assay

Bacterial culture was inoculated to 250 ml broth and incubated till the OD 600 reached. 0.5. Spread plating on nutrient agar was performed to determine the initial cell density. The culture was distributed into conical flasks labelled as control, 10 Multiplicity of infection (MOI), 1 MOI, 0.1 MOI and 0.01 MOI in duplicates. To each flask different quantity of phages were added to attain the respective MOI. At every 0, 4, 8, 12 and 16 h sampling was done and the spread plate was performed to determine viable cell count. Plates were incubated at 37°C overnight and following day colonies were enumerated.

Antibiotic susceptibility test

Antibiotic susceptibility of *S. aureus* isolates was tested using the protocol described by Bauer *et al.* (1966) on Muller-Hinton agar and the isolates were classified either as resistant or sensitive according to the guidelinse recommended by the Clinical Laboratory Standards Institute. Total of fifteen antibiotics were used for the study which comprised of methicillin (5 mcg) levofloxacin (5 mcg), rifampin (5 mcg), ampicillin (10 mcg), imipenem (10 mcg), meropenem (10 mcg), erythromycin (15mcg), azithromycin (15 mcg), cephalothin (30 mcg), vancomycin (30 mcg), kanamycin (30 mcg), streptomycin (10 mcg), amikacin (30 mcg), piperacillin/tazobactam (100/10 mcg), cefotaxime (30 mcg).

RESULTS AND DISCUSSION

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium, causing a broad range of infections among humans and animals. *S. aureus* causes a wide variety of diseases which are ranged based on severity, from slight skin infection to highly severe pneumonia and septicaemia (Ben Said *et al.,* 2016). Also, it is regarded as the most frequent etiological agent of bovine mastitis with the ability to contaminate and reduce the quality of the milk (Sahebekhtiari *et al.*, 2011). It is challenging to eradicate endemic disease like bovine mastitis. Due to the development of antibiotic resistance, interest has shifted from conventional antibiotic therapies to the field of biological control of the disease (Ben Said *et al.*, 2016; Magro *et al.,* 2017).

Bacteriophages have received renewed attention in recent years as a possible antibiotic alternative to eliminate or control harmful bacterial infections. The re-awakening of interest in the use of phages to control bacterial infections is also finding immense application in human and veterinary medicine to agricultural settings and the food industry (McDougal *et al.,* 2010; (Breyne *et al.,* 2017; Ganaie *et al.*, 2018).

Staphylococcus aureus (MRSA) denotes a group of *S. aureus* isolates generally resistant to methicillin as well as erythromycin, levofloxacin, tetracycline, clindamycin, mupirocin, gentamicin, trimethoprim, or doxycycline but is normally susceptible to vancomycin. It is difficult to treat MRSA associated infection using common antibiotics (Shukla and Hirpurkar 2011). All 32 isolates were resistant to at least one of the antibiotics tested. Among the different antibiotics used, maximum resistance was observed to kanamycin, streptomycin, rifampicin, methicillin nalidixic acid and tetracycline (71% followed by nitrofurantoin (50%) and ampicillin (43%) (Fig 1). Out of 19 phages isolated for this study, 5 phages showed lytic activity against methicillin-resistant *S. aureus* strains.

For the exploration of indigenous bacteriophages, milk and cowshed wastewater samples were collected from different areas located in and around Mangalore. In this study, 19 phage's were isolated from the cowshed waste water and none of the phages was isolated from milk samples. All the nineteen *S. aureus* phages gave clear zones

of lysis for all the tested isolates. This might be due to the fact that cattle wastewater is rich in bacterial contaminants (Kwiatek *et al.,* 2012). All milk samples were found to be negative for the presence of bacteriophages it may be suggested that Staphylococcal phages were not present freely in bovine mastitis affected cow milk, always linked to some particles (Labrie *et al.,* 2010). For the isolation of bacteriophages, overlay and enrichment technique was followed. The lysate with the highest titre value was used for the spot assay. Isolated phages were differentiated based on their plaque morphology (Fig 2) and Random Amplified Polymorphic DNA (RAPD) (Fig 3) analysis. RAPD relies on amplification of random sections of DNA wherein phage mechanisms do not inhibit this process. Phage 46A3b and P2G4b showed a similar pattern in RAPD using RAPD5 primer and P2G4a and P2G4b showed a similar pattern in RAPD using OPL5 primer.

All the thirty-two isolates of *S. aureus* were used to studying the host range of the nineteen phages (Fig 4). Phage 24 (A2) showed broad host range of 63% (19) tested against total *S. aureus* isolates (n=30). Due to its broad host range, phage 24(A2) for further studies.

The multiplicity of infection, which was initially done using phage taken from its own isolates for 16 hrs, did not give satisfactory results in liquid phase assay. Hence, the solid-phase assay was performed for 16 hrs. The phage showed lysis only till 4 hrs (Fig 5a and Fig 5b) Since the liquid phase showed good results only for 10 MOI, solid-phase was used as an alternative to check the activity of the phage for 16 hrs. The phage appeared to be active only in the solid phase activity in the liquid phase may require augmentation.

When phage 24A2 was tested for 16 hrs at MOIs ranging from 10 to 0.0 1MOI in the liquid phase, a mild decrease in cell counts compared to control was observed only at 10 MOI. However, the reduction was not statistically significant. On agar medium, the phage was found to be active on cultures that were less than 4 hrs old when tested by the routine test dilution method. In a liquid culture, using phages directly from enrichment, the maximum attainable MOI is 10 MOI, but when the same step was carried out in the solid phase, approximately 1000 MOI can be achieved. A possibility of failure of the phage to reduce cell counts in the liquid phase may be due to the requirement of a high MOI by this phage. The activity of this phage on a lawn of bacteria is promising as a topical application. Thus, the isolated phage will be effectively used for prophylaxis of mastitis caused by *S. aureus* infection.

Endolysins are peptidoglycan hydrolases which are encoded with double-stranded DNA bacteriophage and are produced in bacterial cells that are infected with phage

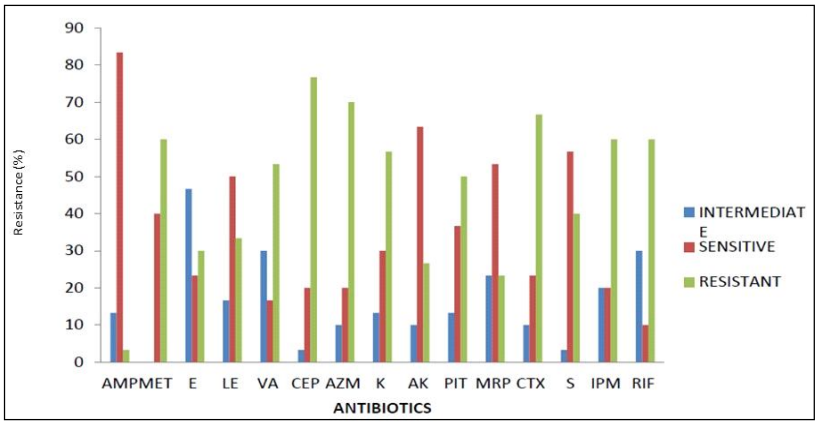

Fig 1: Antibiotic resistance pattern of all isolates against fifteen different antibiotics.

(AMP: Ampicillin, MET: Methicillin, E: Erythromycin, LE: Levoflaxacin, VA: Vancomycin, CEP: Cephalothin, AZM: Azithromycin, K: Kanamycin, AK: Amikacin, PIT: Piperacillin/Tazobactan, MRP: Meropenem, CTX: Cotrimoxazole, S: Streptomycin, IPM: Imipenem, RF: Rifampin).

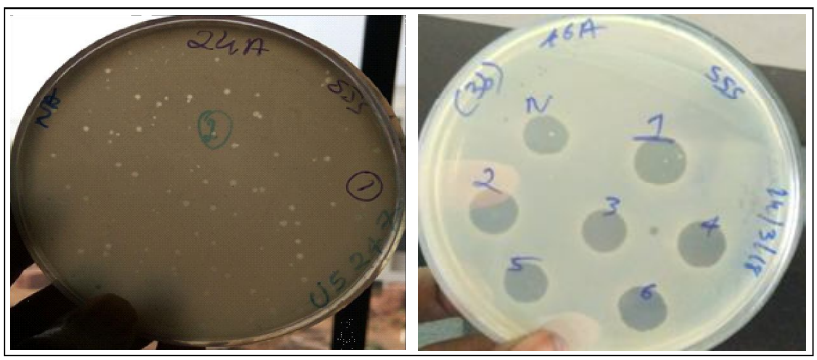

Fig 2: Plates showing clear zone on bacterial lawn.

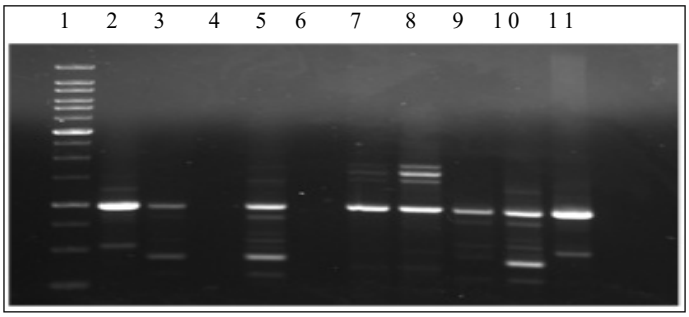

Fig 3: Amplification of phage DNA using RAPDL5 primer.
Lane 1: High range marker, Lane 2: 24A2, Lane 3: 89A, Lane 4: 46A4a, Lane 5: 89B, Lane 6: P2g 4a, Lane 7: 46A3b, Lane 8:P2g4b, Lane 9: 14, Lane 10: 89C, Lane 11: 24A1a.

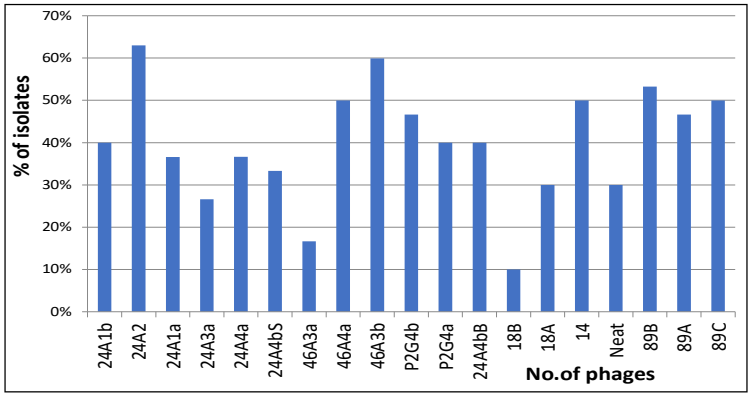

Fig 4: Graph showing the per cent of lysis activity of phages against the 32 isolates.

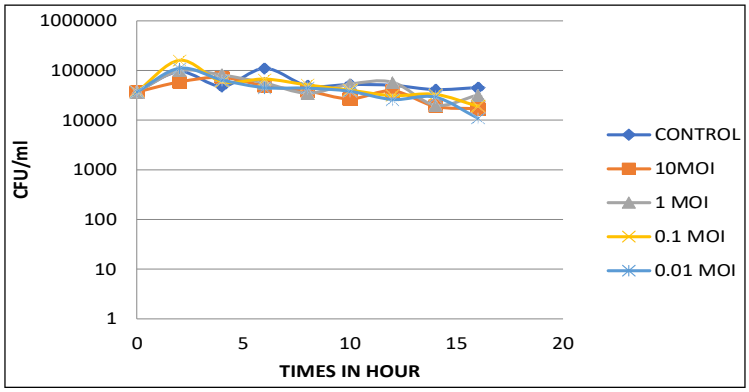

Fig 5a: Graph showing the reduction of *S. aureus* counts at various time intervals in presence of different quantity of phage 24(A2).

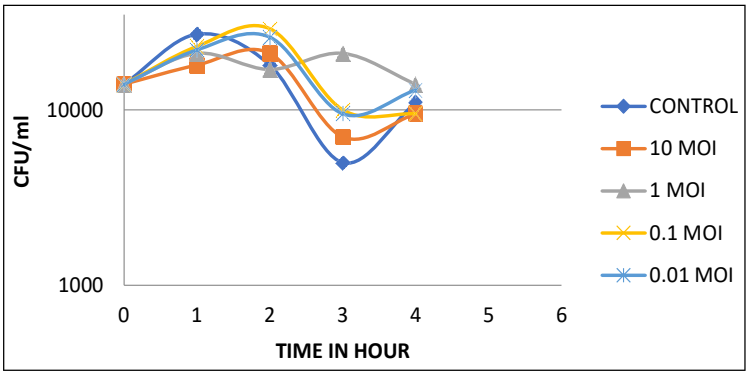

Fig 5b: Graph showing the reduction of *S. aureus* counts at various time intervals in presence of different quantity of phage 24(A2).

toward the end of the lytic cycle. They induce lysis of the bacterial cell enabling the release of progenies of virus apart from this; they are also capable of degrading peptidoglycan when applied externally to the bacterial cell wall resulting in its rapid lysis. Due to this feature, they have received considerable attention as possible antimicrobial agents against Gram-positive bacteria and have been applied to a variety of pathogens, such as *Bacillus anthracis* (Ganaie *et al.*, 2018).

CONCLUSION

Phage isolated in this study were able to lyse antibiotic-resistant *S. aureus*. Combining the phage with enzymes like lysozyme or peptidoglycan hydrolase may increase activity in the liquid phase as well. Phage therapy will compensate for unavoidable complications of chemotherapy, such as the appearance of multidrug resistance or substituted microbes. Earlier phage therapy for bovine mastitis has limited potential the developments made so far and the pertaining of antibiotic resistance, has reopened the doorway for phage therapy.

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Conflict of interest

There is no conflict of interest towards the study.

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