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Lakshmi Kavitha K
Associate Professor, Department of Veterinary Microbiology, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Rajesh K
Assistant Professor, Department of VCC (Veterinary Medicine), Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Sambasiva Rao K
Junior Research Fellow, Department of Veterinary Microbiology, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Corresponding Author
Lakshmi Kavitha K
Associate Professor, Department of Veterinary Microbiology, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Application of lytic bacteriophages in the treatment of environmental buffalo mastitis induced by *Proteus vulgaris*

Lakshmi Kavitha K, Rajesh K and Sambasiva Rao K

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Abstract
Proteus vulgaris accounts for 4.5% of environmental mastitis in bovines. The organism exhibits intrinsic high antibiotic resistance and becomes refractory to treatment. In the present study 185, lytic bacteriophages were screened and based on plaque morphology five were selected as therapeutic agents. The five lytic bacteriophages named ϕPV1, ϕPV2, ϕPV3, ϕPV4 and ϕPV5. These were characterized for host range on the reference strain of *Proteus vulgaris* and on strains isolated from the cases of mastitis and observed 80 to 92.5%. Biophysical characterization of phages was done to ascertain their stability in hostile environments and observed stability at pH 4, 7, 9 and temperature 16 °C, 37 °C and 42 °C. The morphology of the phage PV3 belonged to order *Caudovirales* and the family Siphoviridae. Molecular characterization showed DNA as nucleic acid and phage PV3 was about 28.9Kbp and showed digestion with a restriction endonuclease. Therapeutic activity of phage mixture was evaluated in lactating Swiss albino mice that were divided into control, infected and treatment groups. At the multiplicity of infection 10, lytic bacteriophage mixture eliminated infection (milk bacterial load) and observed a reduction in swelling of the mammary gland.

Keywords: *Proteus vulgaris*, buffalo mastitis, lytic bacteriophages, therapy, lactating mice

Introduction
Proteus vulgaris is an uncommon environmental mastitis pathogen. The occurrence of the disease is due to the presence of virulence factors like fimbriae, flagella, Lipopolysaccharide (LPS), capsule, urease, IgG and IgA protease, haemolysins and siderophores. The incidence of mastitis by *Proteus* spp ranged from 4% (Ngu Ngwa *et al.*, 2020) [13] 1.5% (Kavitha *et al.*, 2016) [9], 11.63% (El-Naker *et al*., 2015) [4], 4.28% (Baloch *et al*. 2011) [1] to 37.5% (Olivares-Pérez *et al.*, 2015) [15]. In general *Proteus vulgaris* typically cause chronic and severe infections that don't respond to antibiotic therapy. Higher antibiotic resistance and non sensitivity to antibiotics under test were observed by Olivares-Pérez *et al.* (2015) [15]. Due to the intrinsic antibiotic resistance exhibited by the organism and the global uproar of the antibiotic resistance an alternative to antibiotics *i.e.*, lytic bacteriophages were applied as therapeutic agents.

Materials and Methods
Bacterial strains and prophages
The *Proteus vulgaris* (ATCC ®33420™) and the isolated organisms from the clinical cases of buffalo mastitis were used for the purpose of the isolation of the lytic bacteriophages. To use these organisms as hosts, the presence of prophages was observed using the DNA damaging antimicrobial agent mitomycin-C as described by Miller (1998) [12].

Bacteriophage isolation
Large scale isolation and screening of lytic bacteriophages was done by using sewage samples that 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 10,000rpm for 10min and then the supernatant was filtered using 0.45µ filters. To this equal volume of SM buffer (Sodium chloride and Magnesium sulphate buffer containing 100mM NaCl, 8mM $MgSO_4$ and 1M TrisHCl pH7.5) and *Proteus vulgaris* ($1.5x10^8$cfu/ml) were added and incubated in an orbital shaker incubator at 37 °C for 24hrs. After incubation, the suspension was centrifuged at 10,000rpm for 10min and filtered through

0.45µ filters. This filtrate was used to estimate the phage population by using the double agar overlay method using bottom nutrient agar containing 2% agar. Then the top agar was prepared using 0.5ml of the filtrate, one millilitre of the host culture ($0.5x10^8$cfu/ml) and 1.5ml of SM buffer and incubated at 37 °C for 20min. To this suspension, 3ml of nutrient agar was added to make agar concentration 1% and layered on the bottom agar. After solidification, the plates were incubated at 37 °C for 24hrs 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 a sterile toothpick, then inoculated into 2ml of nutrient broth having $0.5x10^8$cfu/ml of the host culture and incubated at 37 °C for 24hrs in an 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 in order to obtain a single lytic bacteriophage.

Among the isolated bacteriophages, the host range of five lytic phages was observed using spot assay as described by Santos *et al*. (2011) [18]. Then these bacteriophages were multiplied further, and stocks were prepared.

Biophysical characterization

The obtained bacteriophages at a multiplicity of infection (MOI) one, were subjected to temperatures 16 °C, 37 °C, 42 °C and pH 4, 7, 9 for a period of 4 hours 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 at time intervals of 30min, 1, 2, 3and 4hrs.

***In vivo* lytic activity of the bacteriophages**

Swiss albino mice, aged 40days and under lactation, were selected and grouped into control, infected and treatment groups. Each group has six mice, the infected group received 100µl of $3x10^8$cfu/ml of organisms whereas the treatment group received 100µl of both the organism ($3x10^8$cfu/ml) and endotoxin free (Proteospin endotoxin removal kit, Norgenebiotek) lytic phage cocktail ($3x10^9$pfu/ml) by the intramammary route. During the experiment, the body weights of the mice were recorded, and milk was also collected from three groups of mice. The therapeutic effect of the bacteriophages was estimated by the total microbial count of milk and histopathology of the mammary gland to observe inflammatory changes.

Results

Bacterial strains and prophages

A total of four *Proteus vulgaris* isolates were used for lytic bacteriophages isolation and characterization. Exclusion of temperate phages was done using Mitomycin C induction of prophages.

Isolation of lytic bacteriophages

On average 50 to 100 bacteriophages were obtained on initial isolation from each sewage sample. Among these five lytic bacteriophages showing clear plaque morphology of 1-2 mm were selected (Figure 1). The selected phages were purified, and stock cultures were prepared.

Microbiological characterization

The isolated phage host range was ascertained at a multiplicity of infection 1. The phages revealed a host range of 80 to 92.5% with a collective host range 100% (Figure 2).

Biophysical characterization

The lytic bacteriophages exerted stability at pH 4, 7, 9 and temperatures at 16 °C, 37 °C and 42 °C. At the multiplicity of infection one, the phages were stable and exerted complete lysis. It is observed that there is an increase in titre at pH 7 and temperature 37 °C in comparison to acidic, alkaline and altered temperatures. Further, it is also observed that among the lytic bacteriophages the ϕPV3 exhibited good stability by increasing its titre at different pH and temperatures (Figure 3,4,5)

Morphology

The phage mixture morphology showed that they belonged to Order *Caudovirales* and phage PV3 belonged to family *Siphoviridae* with an icosahedral head of 101nm in diameter and a contractile tail of 343 nm in length (Figure 6).

Molecular characterisation

The phage nucleic acid was observed to be DNA. The phage PV3 nucleic acid is 28.9Kbp in size and showed digestion with *EcoRI* (Figure 7, 8)

***In vivo* lytic activity of the bacteriophages**

Lactating Swiss albino mice were injected with bacterial culture and the treatment group received bacteria along with a bacteriophage cocktail. The infected mice revealed inflammation of the mammary gland with swelling and redness. In comparison to the infected group, the control mice were normal and healthy. When the milk from both the infected and treatment group was subjected to total bacterial count there was a decrease in the number of organisms in the treatment group when compared to the infected group. After 72hrs of post-treatment, there was a complete decline in the total bacterial count. It was evident from histopathology that the neutrophil infiltration was also decreased after three days of post-treatment (Figure 9; Table 1).

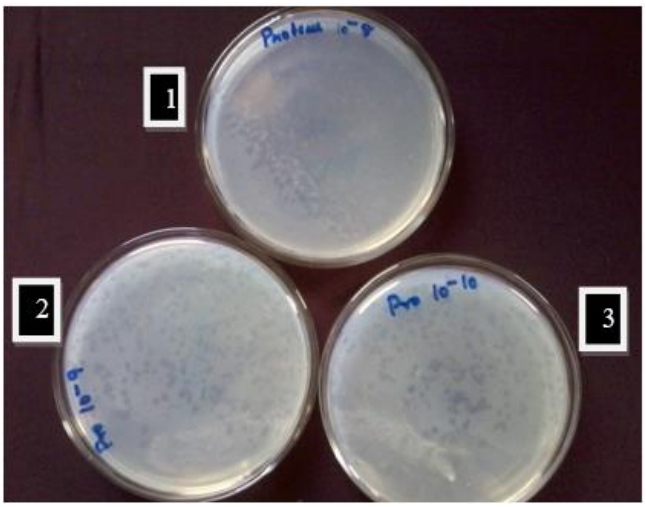

Fig 1: Isolation of lytic bacteriophages of *Proteus vulgaris*. Plate 1 at 10^{-8} concentration- observe 85% lysis of the bacteria. Plate 2 at 10^{-9} concentration - plaque formation Plate 3 at 10^{-10} concentration - clear plaque formation

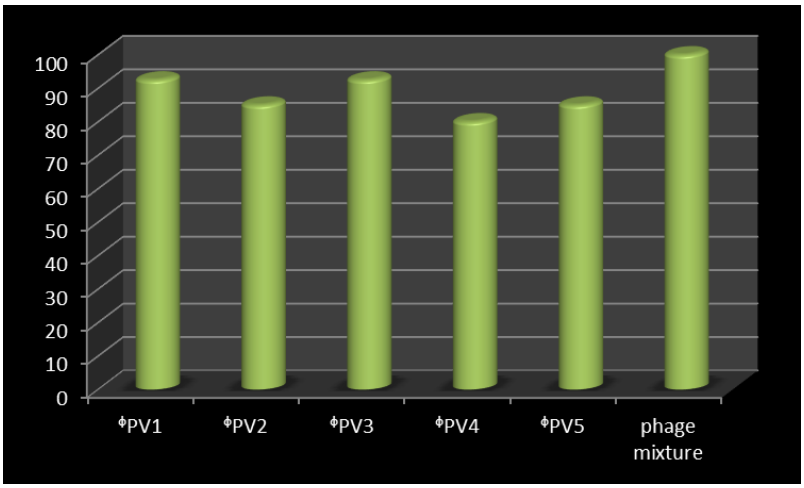

Fig 2: Host range of lytic bacteriophages of *P. vulgaris*

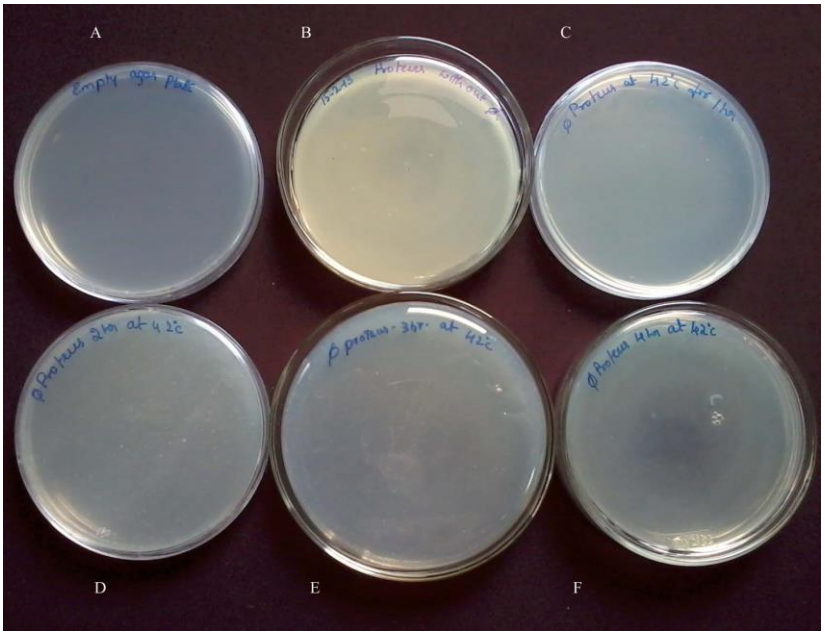

Fig 3: Biophysical characterization of *P. vulgaris* phage mixture. Plate A: Empty agar plate Plate B: Lawn culture of *Proteus vulgaris.* Plate C: 42 °C 1hr – complete lysis of the bacteria Plate D: 42 °C 2hr– complete lysis of bacteria Plate E: 42 °C 3hr – complete lysis of bacteria

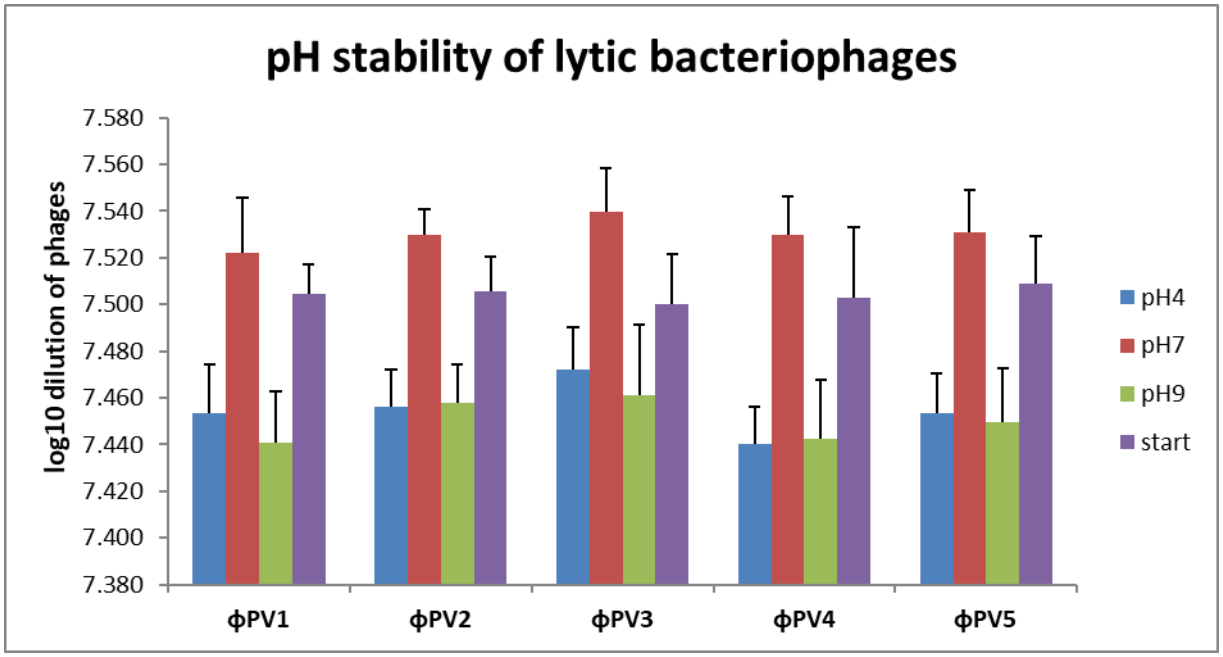

Fig 4: pH stability of lytic bacteriophages of *Proteus vulgaris*

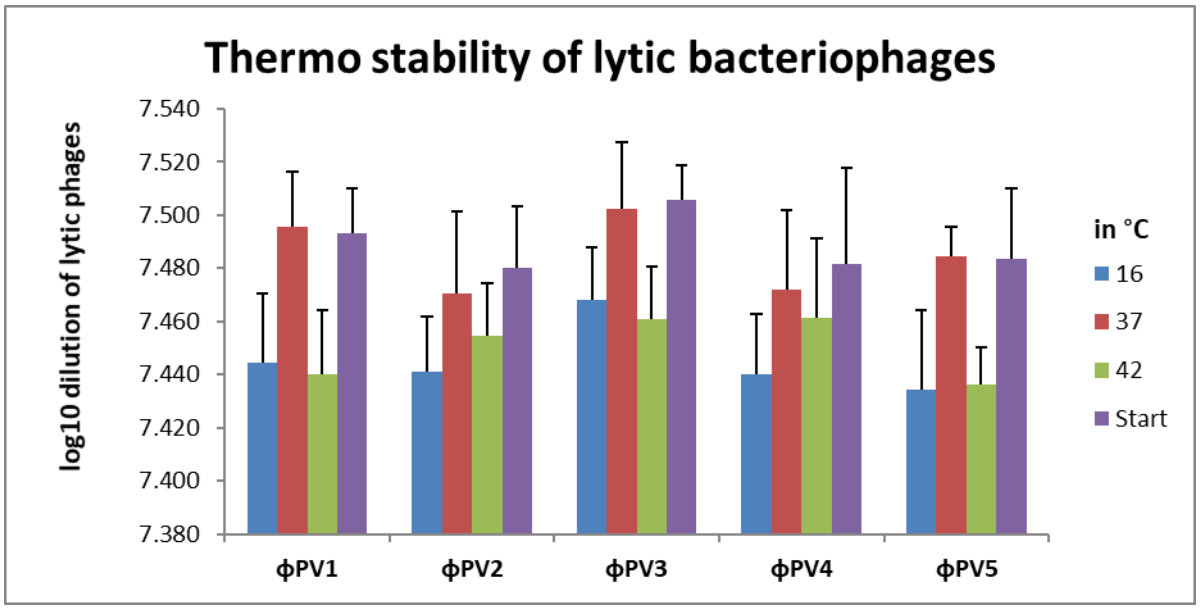

Fig 5: Thermal stability of lytic bacteriophages of *Proteus vulgaris*.

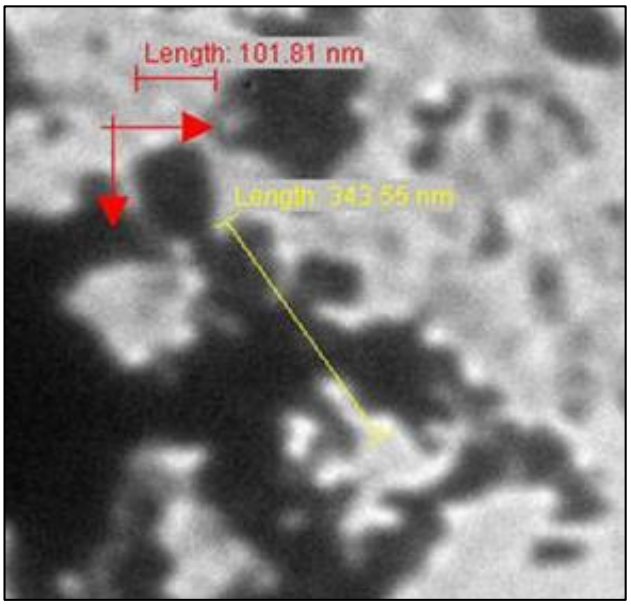

Fig 6: Morphology of lytic bacteriophage PV3 (Family *Siphoviridae*)

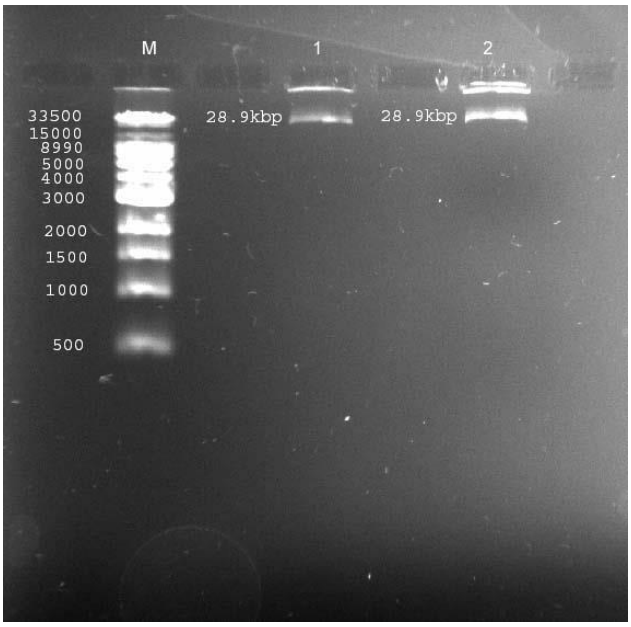

Fig 7: Nucleic acid of phage PV3

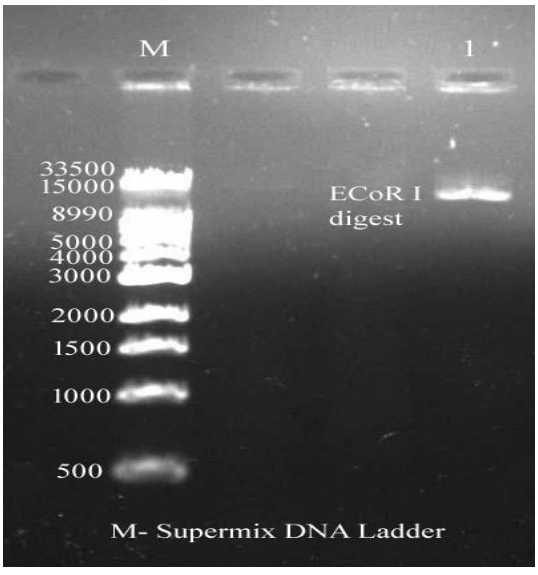

Fig 8: *ECoR I* restriction endonuclease digestion of phage PV3

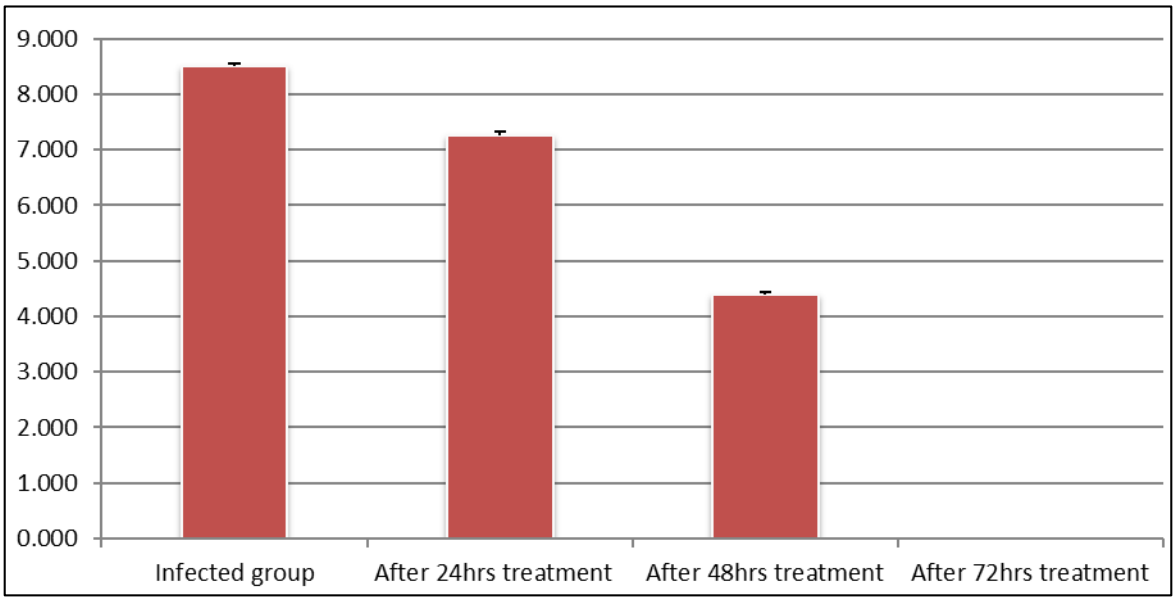

Fig 9: Average total microbial count of milk

Table 1: The results of the *invivo* lytic activity of the bacteriophage mixture in mice.

Conditions	Control group	Infected group after 72h	Treatment group		
			24h	48h	72h
Swelling and discoloration of mammary gland	-	+++	++	+	-
Dull and depression	-	+++	++	-	-
Average bacterial count as org/ml	-	$3.3x10^8$	$1.8x10^7$	$2.5x10^4$	-
Histopathological changes	-	+++	After 72hrs reduced neutrophils		

Discussion

Bacterial pathogens are accounting for a major role in the pathogenesis of mastitis and the extensive use of antibiotics properly or improperly the organisms are attaining higher antibiotic resistance (Fischetti, 2008) [5]. In this regard research towards alternatives to antibiotics has gained the most importance and one such alternative is the application of lytic bacteriophages. To isolate lytic bacteriophages as therapeutic agents; necessitates the use of host bacteria. The source bacteria were logically determined based on the target of phage therapy *i.e., Proteus vulgaris*. A total of three *Proteus vulgaris* isolates with one ATCC culture was narrowed down for the purpose of the isolation and characterization of the lytic bacteriophages. The host bacteria under Mitomycin C excluded the prophages. The exclusion of prophages is essential to avoid the transfer of virulence as well as antibiotic resistance genes (Merabishivili *et al*., 2009, Pei and Lamas-Samanamud. 2014) [11, 6].

The selected host bacterium was used for the isolation of lytic bacteriophages from sewage influents as it yields a wide diversity of phages (Synnott *et al*., 2009) [19]. An average of 50-100 lytic bacteriophages were obtained from each source and five lytic bacteriophages (ϕPV1, ϕPV2, ϕPV3, ϕPV4 and ϕPV5) were selected. The plaque morphology was studied and the phages showing a diameter of 1mm to 2mm with clear plaque morphology were selected. The phages with clear plaque morphology of 1-2mm and above belong to the family *Siphoviridae* (Jurczak-Kurek *et al*., 2016) [6]. The phages of the family *Siphoviridae* have a short eclipse phase of 15 min and a burst size of approximately 50–60 phage particles per bacterium. This feature enhances the removal of bacterium quickly compared with the phages of family *Myoviridae* which had higher lytic activity compared to the other two families (Kesik-Szeloch *et al.*, 2013) [10]. Further, the isolated phages were tested on the lawn of different isolates of *Proteus vulgaris* to assess the host range. The host range of five lytic bacteriophages ranged between 80 to 92.5%. The collective host range is 100%. In order to overcome the host specificity of lytic bacteriophages, the collective use of phages as a cocktail was also suggested by many workers (Merabishvili *et al*., 2009; Wall *et al*., 2010; Raya *et al*., 2011; Yen *et al*., 2017) [11, 20, 17, 21].

Further, the phages on biophysical characterization revealed stability through acidic, alkaline pH as well as temperature variations. However, there is a decrease in log concentrations of the phages at pH 4,9 and temperatures 16 °C and 42 °C. But this decrease didn't affect the lytic activity of phage through different pH and temperatures. Similar results were observed by Santos *et al*. (2011) [18]. Moreover, the increase in the phage concentration over initial concentration may be due to the effective multiplication of the host bacterium at pH7 and temperature 37 °C which was in concurrence with O' Flynn *et al*. (2004) [14].

The morphology and electron microscopic analysis of ϕPV3 showed that it belonged to the family *Siphoviridae* and order *Caudovirales*. Wide spectrum lytic activity of *Siphoviridae* phages against *Proteus vulgaris* was also observed by Zhilenkov *et al*. (2006) [22]. The therapeutic application of family *Siphoviridae* phages was also supported by Kesik-Szeloch *et al.* (2013) [10] and Cao *et al.* (2015) [2].

The nucleic acid of ϕPV3 was found to have 28.9kbp in size and was digested by *Eco*RI, *Bam*HI and *Hae*III. However, the number of bands obtained was less, which indicated that the bacteriophages were resistant to the endonucleases released by the bacteria, which agrees with Kesik-Szeloch*et al*. (2013) [10]. Furthermore, the molecular characters of ϕPV3 supported that the phage belonged to the family *Siphoviridae* and threw light over the quest for endolysins.

Prior to the application of phage cocktails as therapeutic agents, endotoxins must be removed from the phage mixtures which are the common components that were released into the solution because of bacterial lysis due to phage lytic activity. As the endotoxins were proinflammatory the endotoxin removal in the bacteriophage cocktail was also supported by Merabishvili *et al*. (2009) [11].

The phage mixture at a multiplicity of infection 10 was used for the therapeutic application in lactating mice. Under *in vivo* studies in comparison to the infected group, the treatment group revealed a reduction in swelling and discolouration of the mammary gland, improved health condition, reduced pain. In addition to the improvement in the clinical condition, the total bacterial count of milk after treatment was reduced. Moreover, the infiltration of neutrophils was also reduced in the treatment group due to a reduction in the inflammatory process. Similar experiments in the application of lytic bacteriophages were also applied in the treatment of *Pseudomonas aeruginosa* mastitis (Kavitha *et al*., 2018) [8], *Klebsiella pneumoniae* induced mastitis in Swiss albino mice (Kavitha *et al*., 2017) [7] and *Staphylococcus aureus* induced mastitis in mice models by Chilamban *et al*. (2004) [3].

In conclusion, the study demonstrated that lytic bacteriophages can be used significantly to reduce mastitis by *Proteus vulgaris* in mice models. The results of the study were promising, although further work needs to be undertaken to determine real-life settings. The practice of bacteriophage therapy is promising in the context of the rise of antimicrobial resistance (AMR).

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