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THERAPEUTIC EFFICACY OF BACTERIOPHAGE AND ANTIBIOTICS IN STAPHYLOCOCCAL MASTITIS IN CATTLE

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ABSTRACT

Bovine mastitis is one of the most prevalent and economically significant diseases of dairy cattle, causing substantial losses due to reduced milk yield, poor milk quality, increased treatment costs, and milk discards. It is a multifactorial disease caused by a variety of infectious agents, including bacteria, fungi, and algae, along with non-infectious factors. Among these, *Staphylococcus* spp. are the predominant bacterial pathogens due to their persistence as subclinical infections, poor therapeutic response. Although antibiotics remain the primary line of treatment, their prolonged and indiscriminate use has contributed to the emergence of antimicrobial resistant (AMR) strains, necessitating the exploration of alternative therapeutic approaches. The present study evaluated the comparative therapeutic efficacy of intramammary bacteriophage with antibiotics therapy. Twenty four clinical cases of cattle affected with staphylococcal mastitis were randomized into four treatment groups i.e. T1, T2. T3 and T4 for therapeutic trial. Animals in T1 group animals were treated with antibiotic alone, in T2 group with bacteriophage followed by antibiotic eight hours later, in T3 group antibiotic followed by bacteriophage eight hours later, and T4 with simultaneous therapy antibiotic and bacteriophage. Cefoperazone sodium and a *Staphylococcus*-specific bacteriophage cocktail were administered intramammarily. Therapeutic response was assessed based on clinical recovery and analyzed using logistic regression. The highest recovery rate was observed in T2 (83.33%; 5/6; OR=0.038), followed by T4 and T3, whereas T1 showed the lowest efficacy. The results indicate that sequential administration of bacteriophage followed by antibiotic is more effective than monotherapy or other combination regimens. This approach offers a promising alternative strategy for the management of staphylococcal mastitis and may help in reducing antimicrobial resistance in dairy cattle.

KEYWORDS: Bacteriophage therapy, Mastitis, *Staphylococcus aureus*, Cattle, Antimicrobial resistance, Phage-antibiotic synergy.

INTRODUCTION

Mastitis is one of the most prevalent diseases in dairy cows, resulting in significant economic losses due to reduced milk yield, altered milk composition, and decreased processing quality. Mastitis occurs in both clinical and subclinical forms indicated with reduced milk production. The disease is caused by a wide range of pathogens including bacteria, fungi, and algae, with bacterial agents being the most important etiological factors in bovine intramammary infections. Among bacterial pathogens, *staphylococcus* species are the most frequently isolated organisms and are recognized as major contagious mastitis pathogens due to their ability to persist within the mammary gland and establish chronic infections. Conventional treatment of mastitis largely depends on antibiotic therapy. However, indiscriminate use of antimicrobials has contributed to the emergence of antimicrobial resistance (AMR), necessitating the exploration of alternative therapeutic strategies. In this study, bacteriophage therapy has re-emerged as a promising biological approach. Bacteriophages are host-specific viruses capable of lysing bacteria and are widely distributed in the environment. Their high specificity, safety, and eco-friendly nature make them suitable candidates for therapeutic use (Haq *et al*., 2012; Elbreki *et al*., 2014; Golkar *et al*., 2014; Banar *et al*., 2025; Cho *et al*., 2025). Recent studies have demonstrated the potential of bacteriophages in controlling *Staphylococcus aureus* infections, including mastitis, with improved therapeutic outcomes (Mohammed-Ali etbal.,2015; Varela-Ortiz *et al*., 2018; Geng *et al*., 2019; Mohammadian *et al.* 2022; Yadav *et al*., 2024; Ganaie *et al*.,2018). However, limited information is available on their field-level application and comparative efficacy with antibiotics under different treatment regimens. Therefore, the present study was undertaken to evaluate the comparative therapeutic efficacy of bacteriophage therapy and antibiotics, alone and in combination, under field conditions.

MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry (COVSc & A.H), Rewa, Madhya Pradesh. for a period of six months (August 2025–January 2026). The study was approved by Institutional Animal Ethics Committee (IAEC) vide number IAEC/VPP/ COVSc /153/Rewa.

A total of 240 cattle were screened by California Mastitis Test (CMT) from those presented at the Veterinary Clinical Complex (VCC), COVSc & A.H., Rewa, as well as from organized and unorganized farms in and around Rewa district, Madhya Pradesh during the study period. All the milk samples showing positive CMT test results were further cultured for bacterial isolation and those found positive for *Staphylococcus aureus* species were further included in the clinical trail.

CMT was used as cow side test to screen infected quarters in dairy cattle. Milk samples from each quarter (960 quarters) of the 240 animals were collected aseptically and tested. After thorough cleaning of the teats and after discarding the first few streams of the milk from each teat, milk samples were drawn in the CMT paddle and mixed with equal quantities of CMT reagent. The results were scored as given below (Table 01) based on the distinctive characteristics in the milk. Samples found positive for CMT test with score one (01) and above were chosen for clinical trial. The test was performed on day 0 (pre-treatment) and on day 6 (post treatment).

Table 01: Scoring of California mastitis test (CMT)-

Category score	Visible reaction
Negative	Mixture remains normal and there is no evidence of precipitate.
Trace	Slight precipitation is observed, best seen by tipping and it disappears with continued movement.
1	Mixture is slightly thickened but has no tendency toward gel formation.
2	Mixture thickens immediately and moved towards centre.
3	Gel is formed, surface becomes convex and is difficult to shake the mixture.

Sample Collection of mastitis milk for laboratory diagnosis

Milk samples from all the CMT positive cases were collected as per the standard milk sampling procedures in line with those of the NMC (1999) and brought to the laboratory for further study. Approximately 10 ml of milk sample was aseptically collected from all four quarters separately in sterile, disposable tubes of 15 ml capacity and brought to the laboratory in ice-box at 4°C for further testing and analysis. The tubes were labelled with the herd, cattle identification number and the date of previous treatment, if any.

Clinical examination of the udder

The udders of all the animals positive on CMT test results were manually palpated for induration uniformity, atrophy and asymmetry if any, as part of a clinical examination and scored from score 1 to 5 (Table 02). The purpose of the examination was to identify any abnormalities such as fibrosis, inflammatory swelling, pain, etc. Additionally, the milk was checked for discolouration, pus, presence of blood clots or flakes.

Table 02: Udder palpation score

Palpation score	Quarters wise score			
	Front left	Front right	Rear left	Rear right
Soft; fingers easily palpate deep the gland (1)				
Fingers palpate the gland with slight difficulty (2)				
Fingers palpate 3 to 5 cm into the tissue but generally firm tissue (3)				
Fingers cannot palpate into udder tissue (4)				
Hard, fingers cannot palpate into udder tissue (5)				

Culture and identification of microorganisms

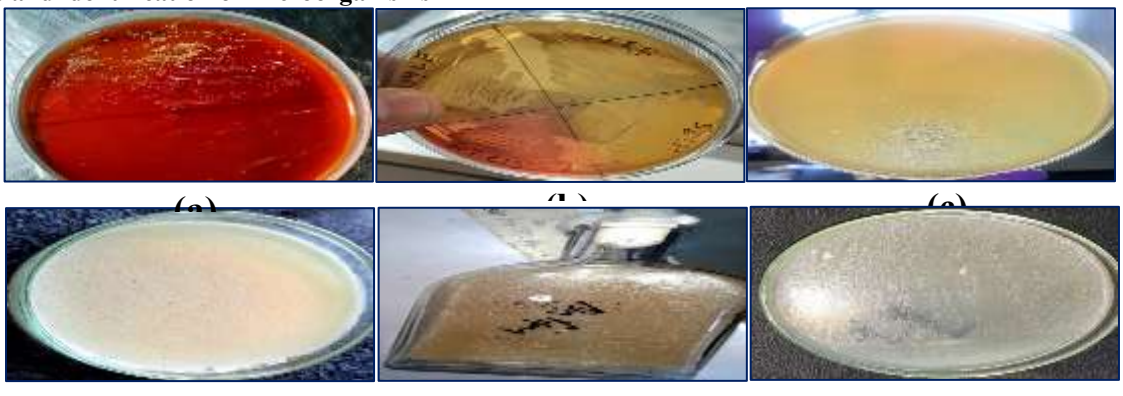


Figure 01: (a) Petri plate showing staphylococcus species growth on MSA agar, (b) Petri plate showing subculture of staphylococcus species on MSA agar, (c) Overlay of Staphylococcal species on MHA plate, (d) Plaque Formation on MHA plate, (e) Roux Bottle showing growth of staphylococcal bacteriophage, (f) Clearence on MHA Plate after Staphylococcal bacteriophage overlay.

Quarter-wise milk samples from all the mastitis positive animals on the basis of CMT test were cultured for isolation of *Staphylococcus* spp. of bacteria using conventional protocols, as described by Quinn (1994).dentification of isolates was done on basis of colony morphology, Grams' staining, catalase test, Oxidase test, Methyl Red test. The colonies confirmed as *Staphylococcus aureus* were further tested for *S. aureus* specific bacteriophage susceptibility Figure 01 (a and (b).

Isolation, Detection and Propagation of Bacteriophages from Water Samples

Bacteriophages were isolated by the method described by Smith and Huggins, 1982 and Szermer *et al*., 2014. The phages were isolated from the wastewater of cleaning udder before milking, sewage water from surrounding drains, milk and from different water bodies, namely the Bichhiya and Beehar rivers (running in the jurisdiction of Rewa municipal corporation). The supernatant of these samples were treated with 1% chloroform solution to remove contaminations, centrifuged for 10 minutes at 4°C at 10,000 rpm and again the supernatant collected after centrifugation.

To check the presence of bacteriophages and their activity, a sterile inoculating loop was used to collect a few bacterial colonies isolated from milk samples, inoculated in normal saline in a micro-centrifuge tube to create a bacterial suspension, thoroughly mixed by vortexing and using a cotton swab sticks, sterile MHA plates were swabbed with these bacterial suspensions. These petri plates were incubated for 2 hours at 37°C to prepare a bacterial lawn and the purified water samples (after treatment with chloroform) were flooded over these lawned bacterial petri plates and incubated overnight at 37°C to monitor bacteriophage activity.

Phages were harvested by pouring 3ml of TMG buffer on the lawned bacterial petri plates to wash the developed phage plaques. The bacteriophages were propagated daily on the lawn on MHA plate**,** incubated at 37°C for 4 hours and the process was continued until the whole plate was cleared and plate became transparent Fgure 01 (c to f).. Serial dilution of the filtered phage lysates was carried out to 12 folds from 10^{-1} to 10^{-12} in the ratio 9:1, in which 1 part of the phage lysates was added to 9 parts of TMG buffer and the activity was measured in order to determine the concentration of the isolated bacteriophage.

Ten microliter of host bacteria (*Staphylococcus species*) was added to each dilution incubated for at least four hours at 37°C, and incubated on agar plates at 37°C for the whole night and on the next day plaques were observed in the decreasing order from 10^{-7} to 10^{-9}. Plaques of similar size were cut and dissolved in SM buffer and used for bulk production. Before using the phages for therapeutic trial, the contaminants from the phage lysate were eliminated by using a 0.2µ membrane filter (Figure 02). The filtered phage lysate was then subjected to membrane dialysis using a semi-permeable dialysis membrane in dialysis buffer (Polyethylene Glycol) and harvesting of phage was done by PBS buffer (Figure 03).

Figure 02: Staphylococcal bacteriophage in dialysis membrane Figure 03: Harvested Staphylococcal bacteriophage after dialysis

Therapeutic trial

For therapeutic trial, twenty-four cattle with mastitis caused by *Staphylococcus species* were randomized into four groups with six animals in each group. *Staphylococcus* specific broad-spectrum bacteriophages and antibiotic cefoperazone (250 mg in 10 ml) were used for therapy as shown in Table 03. Subsequent treatment with above mentioned antibiotics for 4 days was followed in each group.

Table 03: Study groups with dose of treatment

Groups	Treatment	First day treatment	Subsequent treatment

(T1) (n=6)	Control	250 mg Antibiotic* intramammary	250 mg Antibiotic intramammary OD for 4 days
(T2) (n=6)	Bacteriophage cocktail followed by antibiotic therapy.	3 ml (10^5 PFU/ml) of bacteriophage cocktail followed by 250 mg antibiotic intramammary after 8 hrs.	250 mg Antibiotic intramammary OD for 4 days
(T3) (n=6)	Antibiotic followed by Bacteriophage cocktail then continued with antibiotic.	250 mg antibiotic followed by 3 ml (10^5 PFU/ml) bacteriophage cocktail intramammary after 8 hrs.	250 mg Antibiotic intramammary OD for 4 days
(T4) (n=6)	Antibiotic + Bacteriophage cocktail simultaneously.	250 mg antibiotic +3ml (10^5 PFU/ml) bacteriophage cocktail Intramammary.	250 mg Antibiotic intramammary OD for 4 days

Intramammary *Cefoperazone sodium antibiotic (Mammicef, Intas Pharmaceuticals Ltd.) was used, Symptomatic therapy with anti-inflammatory drug isoflupredone acetate@10mg I/M (Sefticort, Virbac India Health Private Ltd.) were given to all the animals. Animals which did not respond to the above therapy in five days were continued with other sensitive antibiotic therapy until cure.Treatment efficacy was assessed based on clinical recovery and changes in udder palpation score, CMT score and milk pH, with observations recorded on day 0 (pre-treatment) and day 6 (post-treatment). To evaluate the therapeutic efficacy of the intramammary preparations, odd ratio at 95% confidence intervals (CI) was calculated to know whether the treatment groups were statistically significant or not.

RESULTS AND DISCUSSION: -

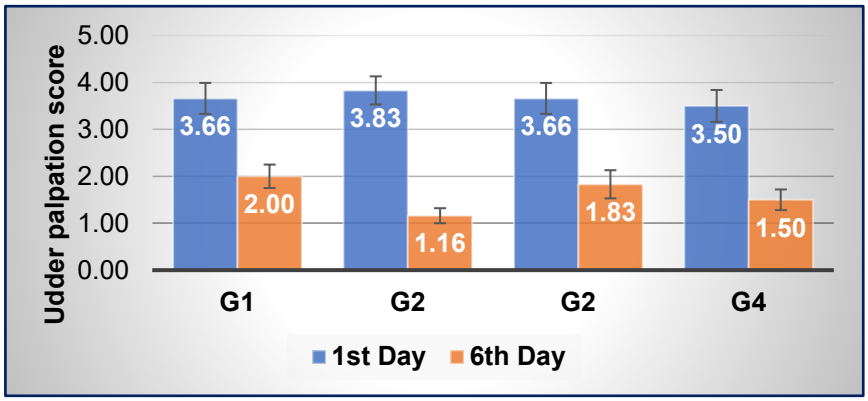
Antibiotic Cefoperazone sodium @250 mg in 10 ml per teat and broad-spectrum *Staphylococcus species* specific bacteriophage cocktail suspension @ 3 ml ($3X10^5$ PFU/ml) were used for comparative therapeutic study. Animals in T1 group were treated with antibiotic Cefoperazone sodium and were used as control for statistical analysis. Animals in T2 group were first treated with bacteriophage cocktail followed by intramammary antibiotic infusion after 8 hrs, those in T3 group were first treated with intramammary antibiotic infusion followed by bacteriophage cocktail after 8 hrs. whereas animals in T4 group were treated with combination of antibiotic and bacteriophage cocktail simultaneously. Subsequent treatment with above mentioned antibiotics for 4 days was followed in each group.

In the present study, five animals out of six in group T2 showed recovery, in group T3 two animals out of six and in group T4, three animals out of six while in group T1 one out of six animals showed clinical recovery. Using T1 as the reference group, binary logistic regression analysis revealed a marked reduction in the odds of infection in T2, with an odds ratio (OR) of 0.038 (95% CI: 0.002–0.833; P = 0.038), demonstrating a statistically significant protective effect at the 5% level (Table 04). On the contrary, although T3 (OR = 0.512; 95% CI: 0.026–6.176) and T4 (OR = 0.239; 95% CI: 0.014–2.911) showed lower odds of infection compared to T1, these reductions were non-significant, as evidenced by confidence intervals having unity and non-significant P values.

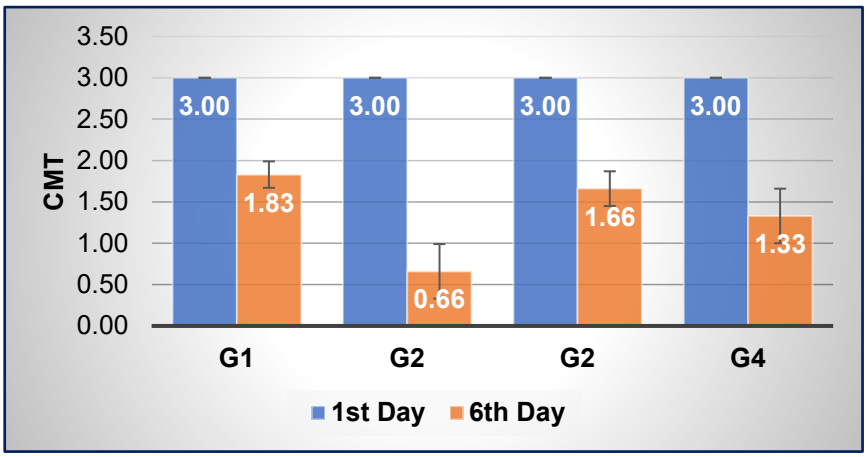
Table 04: Odds ratio of treatment groups (Reference group: T1)

Group	Non-infected	Infected	Odds Ratio (OR)	95% CI	P Value
T1	1	5	1.00 (Reference)	-	-
T2	5	1	.038	0.002-0.833	0.038*
T3	2	4	.512	0.026-6.176	0.512
T4	3	3	.239	0.014-2.911	0.239

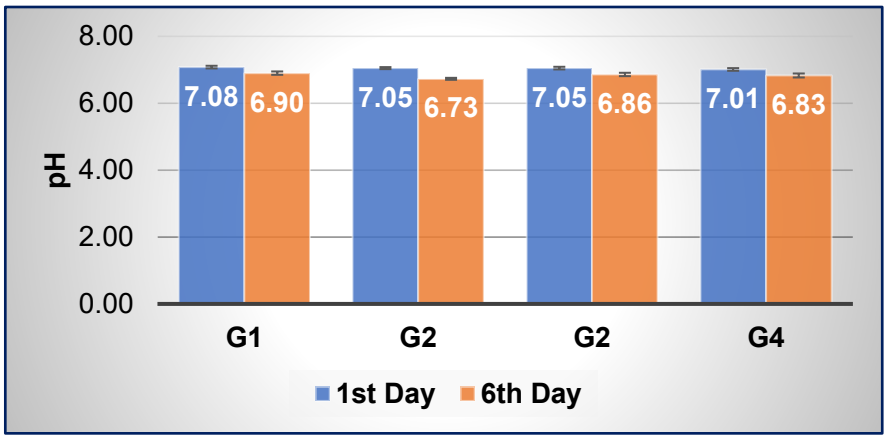
Based on the scores and faster resolution of the clinical signs, on the basis of udder palpation score, CMT score pH (Figure 04) ,and values of odd ratio (Table 04), T2 group proved to be the best treatment group out of all.the treatment groups. Positive results of phage therapy have also been reported in the treatment of udder diseases in dairy cattle, both clinical and subclinical, by several workers.



(a)



(b)



(c)

Figure 04: (a) Udder palpation score of the staphylococcal mastitis positive animals before and after treatment (Mean ± SE), (b) Milk CMT score of the staphylococcal mastitis positive animals before and after treatment (Mean ± SE), (c) Milk pH score of the staphylococcal mastitis positive animals before and after treatment (Mean ± SE)

Bacteriophages were used as alternative and adjunct therapy to antibiotics in the present study. Bacteriophages, as ubiquitous bacterial viruses in various natural ecosystems, play an important role in maintaining the homeostasis of the natural microbiota. They have positive effects on eukaryotic cells, especially in the course of bacterial infections, which are extremely important in treatment exploiting phages as alternative to antibiotics.

Staphylococcus species of bacteria have ability to produce biofilms rendering action of antibiotics ineffective on them (Teng *et al*., 2022). Bacteriophages have the capability of breaking these biofilms. They also have affinity for mucus which activates their ability to penetrate cells and tissues, usually by endocytosis.

Phages can migrate through layers of epithelial cells and then enter the bloodstream activating immune responses (Van Belleghem *et al*., 2018). This is the result of interactions between phages and eukaryotic cell membranes via transmembrane mucins and specific and nonspecific receptors, which enable signal transmission in epithelial cells.

The mechanisms by which bacteriophages activate cellular immune response i.e. B and T lymphocytes are not yet fully understood. Earlier research by Sausset *et al.,* (2020) however, indicates that activation of B lymphocytes as a result of

contact between dendritic cells and phages induce the production and release of anti-phage antibodies. Bacteriophages have also been shown to influence the production of cytokines, such as IFN-γ and IL-6, due to activation of T cells in lymph nodes. Bacteriophages can also exhibit anti-inflammatory activity, taking part in controlling inflammatory responses by- (i) inducing an increase in the expression of and stimulating the production and release of certain interleukins, (ii) blocking the expression of pro inflammatory cytokines, (iii) inhibiting the activity of certain cells and macrophages (Lusiak-Szelachowska *et al*., 2017). (iv) inhibiting the development of oxidative stress (Souza *et al*., 2023); (v) Inhibiting migration of neutrophils and granulocyte macrophage colony-stimulating factor to the infection site (Van Belleghem *et al*., 2017; Popescu et el., 2022).

Some bacteriophages used in therapy may exhibit both pro and anti-inflammatory activity at the same time, as confirmed in research by Zhang *et al*., (2018) in their study. They reported that intramammary application of phages specific to *S. aureus* resulted in a reduction in the levels of cytokines Tumor necrosis factor-α, Interleukins (IL)-1β, IL-6 and IL-8 in mammary epithelial cells (mammary alveolar cells -Ts) stimulated with lipopolysaccharide. It also resulted in a reduction in the levels of inflammatory mediators in the absence of LPS.

Nale *et al*., (2023) reported that in treatments application of a suspension of bacteriophages specific to various pathogens responsible for udder diseases in dairy cattle reduced inflammation and clinical symptoms. Our present study on therapeutic trial with bacteriophages on clinical mastitis associated with *S. aureus* also proved to be a success in the path of alternative therapy to combat antimicrobial resistance.

CONCLUSION

The study demonstrates that bacteriophage therapy, particularly when used in combination with antibiotics, is highly effective in the treatment of staphylococcal mastitis in cattle. This approach offers a promising alternative to conventional antibiotic therapy and can play a crucial role in combating antimicrobial resistance. The odds of infection in T2, with an odds ratio of 0.038 (95% CI), demonstrated a statistically significant protective effect at the 5% level. Thus, T2 group showed the best results in treating the infection. These findings highlight the potential of bacteriophages as alternative treatments for antibiotic-resistant Staphylococcus infections while also underscoring the need for further research to optimize phage formulations for use in complex dairy environments.

ACKNOWLEDGEMENT

The authors expresses sincere gratitude to the College of Veterinary Science and A.H., Rewa, Madhya Pradesh, India and Department of Microbiology, Institute of Medical Science, BHU, Varanasi, U.P. for providing necessary facilities and support.

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