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Studies on the Efficacy of Phage in Controlling *Staphylococcus aureus* Biofilm Phenotype Associated with Mastitis

Jagnoor Sandhu[1], Mudit Chandra[1], Gurpreet Kaur[1], Deepti Narang[1], A.K. Arora[1]

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ABSTRACT

Background: The study aimed to investigate the effectiveness of phage in controlling *Staphylococcus aureus* (*S. aureus*) biofilm phenotypes associated with mastitis.

Methods: The study was carried out with qualitative assessment and quantitative assessment of biofilm producing ability of the *S. aureus* isolates by using Congo Red Agar Assay (CRA) and Modified Congo Red Agar Assay (MCRA) respectively. The biofilm formed was treated with phage to evaluate its efficacy in removing the biofilm.

Result: A total of 18 isolates of *S. aureus* (S1-S18) were isolated and identified from mastitis cases. Among the 18 isolates of *S. aureus*, many exhibited strong biofilm producing capabilities (22.22% on CRA and 33.33% on MCRA). Biofilm formed was treated with phage to evaluate its efficacy in removing the biofilm and it was observed that exposure to high phage titre (5×10^7 PFU/ml) over 24 h significantly reduced biofilm biomass (83.14%) whereas low phage titre (1.5×10^7 PFU/ml) exhibited significant yet less effectiveness in controlling biofilm. The phage action on biofilm resulted in a decline in bacteria (19.99%) while increasing phage (11.50%). These findings demonstrated the potential of high-concentration phage in treating biofilm phenotype.

Key words: Bacteriophages, Biofilm, Mastitis, *Staphylococcus aureus*.

INTRODUCTION

Mastitis, a multifactorial disease, leads to reduced milk yield along with compromised quality, increased veterinary expenses and impaired animal well-being (Garcia, 2004). Various organisms, including *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli* and *Klebsiella pneumoniae* are involved in causing mastitis. Among these, *S. aureus* plays a significant role. *S. aureus* has the ability to produce biofilm which are cohesive layers of bacteria, surrounded by an organic matrix (Bos *et al*., 1999).

S. aureus infections in bovines have an alarmingly low treatment success rate. Infected bovines often become chronic carriers, sometimes necessitating the culling of infected animals to halt the spread and protect the health of the remaining herd. The culling, while essential, leads to substantial losses for farmers. Moreover, the organism's biofilm producing capability poses significant treatment challenges, as biofilm communities exhibit enhanced nutrient accessibility promoting bacterial growth, retaining water, reducing dehydration and preventing antibiotic penetration, thus leading to treatment failure. The proximity of bacteria within biofilm aids in efficient interactions and genetic material transfer, making them resilient and adaptable to harsh environments (Costerton and Lappin Scott, 1995).

In the era of antimicrobial resistance (AMR), bacteriophages (phage) offer a promising alternative for the treatment of mastitis the most important concern among the dairy farmers. Phage, bacterial viruses with lytic properties, can effectively control biofilms. They demonstrate high specificity for their host bacteria, even at the strain level and are safe for eukaryotic cells (D'Accolti *et al*., 2021).

[1]Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India.

Corresponding Author: Mudit Chandra, Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141 004, Punjab, India. Email: drmuditchandra@rediffmail.com

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Specific phage targeting *S. aureus*, such as phage K, have shown effectiveness in disrupting biofilms and reducing bacterial load. Phage can penetrate biofilms and lyse the bacteria within, offering a targeted approach to infection control (O'Flaherty *et al*., 2005). Their capacity to replicate within host cells and increase in population (up to 1000 times) makes them an excellent option for biocontrol of bovine mastitis (Blowey and Edmondson, 2010). Thus, in the present study, role of phage in controlling biofilm of *S. aureus* was studied that could help in controlling mastitis.

MATERIALS AND METHODS

The research work was conducted at the Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana from December 2022 till December 2023. *S. aureus* isolates were isolated from mastitis milk samples.

All the isolates were screened for their ability to produce biofilm.

Congo red agar assay and Modified congo red agar assay

Congo red agar (congo red dye 0.8 g, sucrose 36 g, brain heart infusion agar 52 g in 1 litre of water) and Modified congo red agar (congo red dye 0.4 g, glucose 10 g, blood agar base 40 g in 1 litre of water) were prepared and sterilized by autoclaving. After autoclaving filter sterilized sugar and dye were added at 50°C and plated. The plates were incubated for 48 h at 37°C for sterility checking. The plates were streaked with the bacteria and checked for colony characters and the colonies were interpreted as strong biofilm formers (black colour), moderate biofilm formers (weak black colour) and non-biofilm formers (red colour) (Mariana *et al*., 2009).

Effect of glucose on biofilm

The isolate observed to be a strong biofilm former (indicated by black colonies) in the MCRA assay was cultured overnight in Trypticase Soy Broth (TSB) supplemented with 2% and 4% glucose, respectively. Subsequently, bacterial growth (200 µl) from both culture tubes were inoculated into flat bottom wells of a microtitre plate and incubated for five days at 37°C, with uninoculated TSB serving as the negative control (Stepanović *et al*., 2000).

After incubation the wells were decanted and washed thrice with phosphate buffered saline (PBS) to remove non-adherent cells and adherent cells were fixed with 200 µl of 99% methanol. Staining of biofilm was done using 2% crystal violet for five minutes and excess was removed by rinsing under running tap water. After air drying the stain was resolubilized with 160 µl of 33% Glacial Acetic Acid/ well and the OD was measured at 595 nm using a spectrophotometer (Thermo Scientific, USA) (Chandra *et al*., 2017). The experiment was repeated five times, with twelve replicate wells for each concentration of glucose (2% and 4%) supplemented with TSB. The data was analysed using GraphPad Prism8 version 8.0.2.

Isolation of bacteriophage

Ten sewage samples (50 ml) from dairy farm were collected. These samples were transported in a cold box to the laboratory for isolation of phage. The sewage sample was centrifuged at 5000× g for 10 minutes and supernatant (40 ml) was collected. The supernatant was transferred to 50 ml double strength NZCYM (Nutrient Z-rich Casamino Acids Yeast Extract Medium) (HiMedia, Mumbai) broth along with 10 ml of exponential phase (log phage) broth culture of *S. aureus* grown in TSB (equivalent to 0.5 McFarland) indicating 10^6-10^7 bacteria per ml.

This broth was examined daily by aspirating 10 ml and centrifuging at 8000×g for 15 minutes to collect the supernatant. The supernatant was passed initially through 0.45 µm and later through 0.22 µm PVDF syringe filter (Axiva Sichem Biotech, India) to collect the filtrate. The filtrate was stored at 4°C and indicated as Bacteria Free Filtrate (BFF).

Double agar overlay technique

To check for bacteriophage, 100 µl of exponential phase broth culture of *S. aureus* grown in TSB and 200 µl of BFF were mixed in 5 ml of 0.75% NZCYM agar (maintained at 50-55°C) and spread evenly over a NZCYM agar plate. The soft agar (0.75% NZCYM agar) was allowed to solidify at room temperature and later the plates were incubated at 37°C for 48-72 h to observe for plaques (Chandra *et al*., 2011).

Secondary streaking

Two hundred (200) µl of exponential phase broth culture of *S. aureus* grown in TSB, was added into 5 ml of soft agar and poured over a NZCYM agar plate. After the agar gets solidified a well-isolated plaque was picked from the above plate using a straight wire and streaked horizontally in a straight line across the plate and later streaked vertically at a 90° angle to the horizontal lines. Subsequently, after incubation at 37°C for 24 h, the plates were checked for zones of clearance, indicating a lytic activity by the bacteriophage.

Elution and enumeration of *S. aureus* phage

Sterile SM (Sodium chloride, magnesium sulphate and gelatine) buffer (5.5 ml) was poured over the streak lines showing zone of clearance. The area with clearance was disturbed using a sterile inoculation loop and kept at 4°C for 12 h. Later, SM buffer was collected and centrifuged at 10,000× g for 20 minutes and the supernatant was collected and filtered through a 0.22 µm filter and stored at 4°C.

A serial 10 fold dilution of the phage was prepared up to 10^{-10} in sterile SM buffer. In brief, equal volumes (100 µl) of each phage dilution was prepared and an exponential phase broth culture of *S. aureus* grown in TSB were mixed and plated using double agar overlay technique as described above to observe for plaques. The plaques observed were counted and multiplied by the dilution factor and plaque-forming units (pfu)/ml was calculated.

Biofilm treatment with phage

The bacterial culture grown overnight in TSB supplemented with 2% glucose was inoculated into wells of microtitre plates (200 µl) (in duplicate); one for staining and measuring optical density (OD) and other for enumeration of bacteria and bacteriophage by incubating at 37°C for five days.

For observing effect of phage on the biofilm, microtitre plate wells were decanted and washed thrice with PBS without disturbing the biofilm and 200 µl of phage (1) 1.5×10^7 (PFU A) and 5×10^7 (PFU B) PFU/ml was suspended for 4 h, 8 h, 12 h and 24 h. The pfu/ml was estimated using the supernatant at each time point as per the method described above. To quantify bacteria, microplate was rinsed twice with PBS and adherent cells were collected by scrapping and suspended in 100 µl of PBS for calculating CFU/ml using serial dilution in PBS.

RESULTS AND DISCUSSION

Isolation of *S. aureus* from mastitic animals

In the present study, a total of 50 mastitis milk samples from dairy animals were collected in and around Ludhiana. Out of these 50 mastitic milk samples a total of 18 *S. aureus* (S1-S18) were isolated and confirmed using biochemical tests.

Congo red agar assay

The ability of the *S. aureus* isolates to produce biofilm was evaluated using the congo red agar assay. Among the 18 isolates, 22.22% of the isolates were strong biofilm producers (produced black colonies), 38.88% of the isolates were moderate biofilm producers (produced weak black colonies) and remaining 38.88% were non biofilm producers (produced red colonies) (Table 1).

Modified congo red agar assay

The ability of the *S. aureus* isolates to produce biofilm was also evaluated using the modified congo red agar assay. Among the 18 isolates tested, 33.33% of the isolates were strong biofilm producers (produced black colonies), 44.44% were moderate biofilm producers (produced weak black colonies) and 22.22% were non-biofilm producers (produced red colonies) (Fig 1, 2 and 3).

Effect of glucose on biofilm

Following staining of the microtitre plate, the OD obtained at 595 nm was plotted using GraphPad Prism8 version 8.0.2 (Fig 4) and paired t-test was utilised to check the relation between effects of different concentrations of glucose on biofilm formation. The analysis revealed no significant difference in the OD values obtained when using different glucose concentrations (2% and 4%), indicating that the concentration of glucose did not influenced the biofilm formation.

The biofilm formed was interpreted by calculating the cut-off OD (ODc). The cut-off optical density (ODc) was determined by calculating three standard deviations above the average optical density (OD) of the negative control. In the present study an ODc value of 0.396 was obtained that indicated moderate to strong biofilm formation in the microtitre plate wells.

Isolation of bacteriophage

From a total of 10 sewage samples collected from the dairy farm, two bacteriophages (Phage 1 and Phage 2) were isolated and later confirmed using secondary streaking. Upon culturing on NZCYM agar plates using the double agar overlay technique, distinct circular plaques (Fig 5) measuring 1-2 mm in diameter with clear zones of lysis were observed. Subsequently, secondary streaking of the plaques revealed distinct zones of clearance alongboth horizontal and vertical streak lines (Fig 6). These isolated phage, demonstrated lytic action against



Fig 1: Black colonies of *S. aureus.*

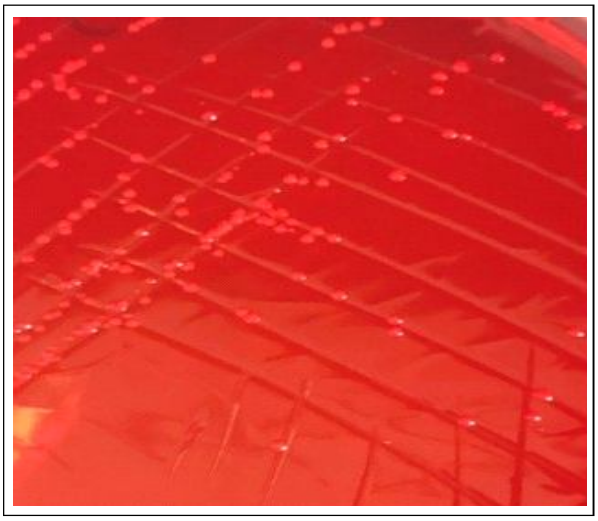


Fig 2: Red colonies of *S. aureus.*

Table 1: Assessment of *S. aureus* isolates (S1-S18) for their biofilm producing ability.

Colony colour	Congo red assay			Modified congo red assay		
	Black	Weak Black	Red	Black	Weak Black	Red
Strains positive	S2, S4, S7, S12	S1, S3, S6, S8, S11,S13, S16	S5, S9, S10, S14, S15, S17, S18	S2, S4, S7, S11, S12, S13	S1, S3, S5, S6, S8, S9, S10, S14	S15, S16, S17, S18
Percentage	22.22	38.88	38.88	33.33	44.44	22.22

the targeted *S. aureus* strains and no lytic activity for other tested bacterial species; *Salmonella* Typhimurium and *E. coli.* Phage (1) had bigger plaque size and was selected for treatment studies. *S. aureus* strain (S4) exhibited strong biofilm producing ability and was selected for treatment studies.

Biofilm treatment with phage

Biofilm was produced in microtitre plate (in duplicate), one for enumeration of the CFU and PFU and the other for measuring the optical density. The developed biofilm was treated with two different phage concentrations; PFU A (1.5×10^7) and PFU B (5×10^7) in triplicate and the activity of phage on biofilm was noted at different time intervals by measuring OD_{595}, enumerating bacteria (CFU) and phage (PFU). The mean of biofilm biomass OD_{595} of experiments at 4 h, 8 h, 12 h and 24 h interval was calculated.

After exposure for 24 h the total biofilm biomass decreased significantly and there was a substantial reduction in the OD values after exposure with PFU A (0.502±0.0472) and PFU B (0.229±0.0024) as compared with the OD of the control (1.510±0.0155). It was also observed that PFU B exhibited a more extensive reduction in the OD value after an exposure of 24 h compared to PFU A (Table 2).

It was also observed that both the phage (1) concentrations PFU A and PFU B led to a major fall in the OD value (0.948±0.0339 and 0.693±0.0601 respectively) after an exposure of 8h when compared with 4 h treatment (1.243±0.0583 and 1.241±0.0349) respectively. The PFU values also increased after each time interval while a decrease in the CFU after 24 h of treatment with PFU A and PFU B was recorded (Table 3).

Qualitative assessment of the biofilm production by congo red agar assay (CRA) was described by Freeman

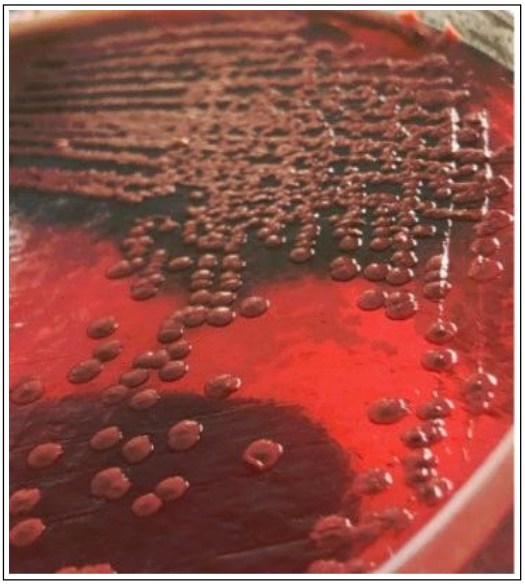


Fig 3: Weak black colonies of *S. aureus.*

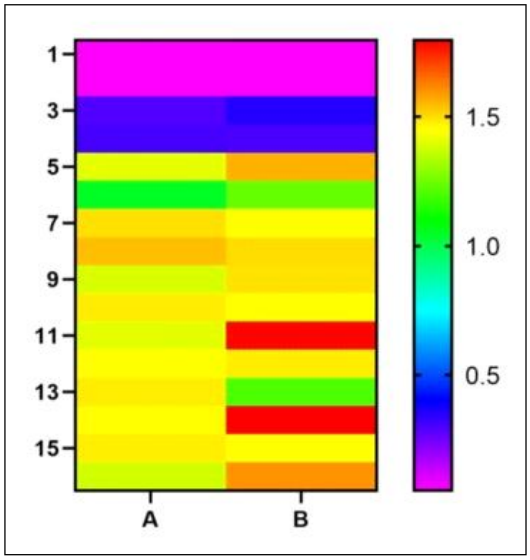


Fig 4: Heat map of biofilm developed (A- TSB +2% Glucose, B- TSB+4% Glucose).

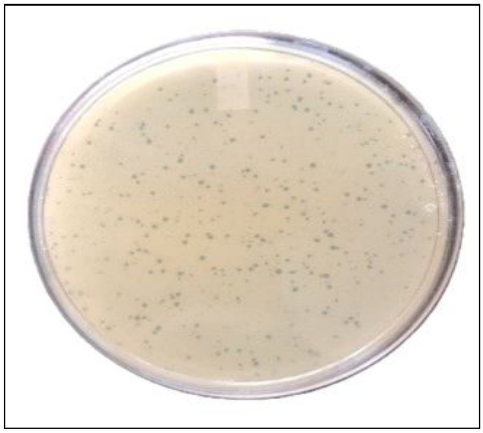


Fig 5: Plaques of phage on NZCYM agar.

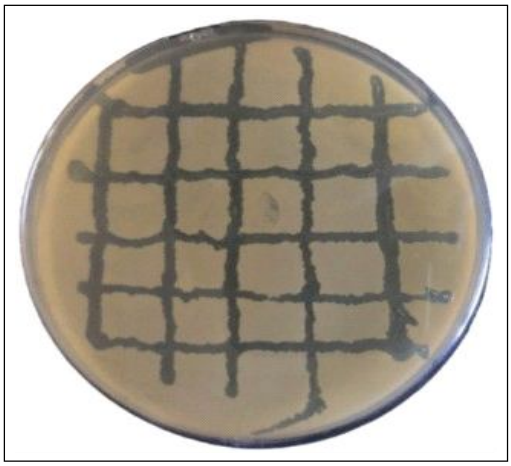


Fig 6: Secondary streaking of phage on NZCYM agar.

et al. (1989) as a diffusion of black pigment in the agar with growth of black-pigmented colonies. Among the 70 strains Milanov *et al*. (2010) tested they observed 11.42% biofilm-producing isolates with black colonies having a dry crystalline consistency.

In another study CRA was used by Krukowski *et al*. (2008) and Szweda *et al*. (2012) in which the biofilm producers were observed to be 42% and 57% respectively. In the present study it was observed that 22.22% of *S. aureus* isolates isolated from clinical mastitis cases were strong biofilm producers, which was higher than what was observed by Milanov *et al*. (2010) in their study but lower when compared with the studies of Krukowski *et al*. (2008) and Szweda *et al*. (2012) in which biofilm producers were 42% and 57% respectively. The regional variation among the isolates studied could be one of the factors leading to variation in biofilm producers. However, further studies are required to identify what leads to variation in the biofilm producing abilities of clinical isolates. The ability of the isolates to produce biofilm was further evaluated using modified congo red agar assay which too revealed that 33.33% isolates had strong biofilm producing ability. Further, it was observed that all the isolates which were strong biofilm producers on CRA were also producing strong biofilm on MCRA but not vice versa indicating that MCRA was able to detect those biofilm producing isolates that CRA was not able to detect qualitatively. In a study Mariana *et al*. (2009) reported that a lower percentage of agar in MCRA resulted in the growth of permanent black-pigmented colonies which too was similar to the findings of the present study. On the contrary, Leshem *et al*. (2022) demonstrated the effectiveness of CRA in identifying biofilm producers in *S. aureus* clinical isolates.

For quantitative analysis of biofilm, crystal violet staining method was used for staining as this stain is a basic protein dye capable of staining negatively charged surface molecules and extracellular matrix composed of polysaccharides. However, it does not provide information on the functioning aspects of the biofilm (Xu *et al*., 2016). Similarly, it was observed by Stepanović *et al*. (2000) that correlation between the CRA assays and spectrophotometric tests was 96% and almost every strain that the spectrophotometric assay identified as biofilm-producing was also positive in the CRA assay which too is aligning with the observations in the present study. In this study biofilm formed was interpreted by calculating the cut-off using crystal violet staining and it was possible to determine a cut off value using optical density to identify moderate to strong biofilm producers which is similar to findings of various earlier studies.

Glucose concentrations (2% and 4%) was supplemented in TSB and tested for their role in rendering biofilm formation. Both the concentrations helped and enhanced the biofilm producing ability of the bacteria but it was observed in the current study that no significant difference in the OD values obtained when two different concentrations of glucose supplementation were used, indicating that the concentration of glucose did not influence the biofilm formation. The role of glucose in biofilm-producing ability aligns with the study conducted by Stepanović *et al*. (2000), in which the supplementation of glucose significantly enhanced the adherence capability of the isolates but this effect was noted to be independent of the concentration of glucose used. Also, in a study by Grinholc *et al*. (2007) importance of nutrient composition, particularly glucose, in biofilm formation revealed that varying glucose concentrations did not significantly altered biofilm formation which is in tandem with the findings of the present study. Lade *et al*. (2019) found that TSB supplemented with glucose effectively enhanced robust biofilm production, enabling consistent quantification of *S. aureus* biofilm formation *in vitro*. However, the additional NaCl resulted in considerable variability in biofilm measurement. However, further studies are required to fully understand the role of nutrients in formation of biofilm *in vitro*.

From a total of 10 sewage samples collected from dairy farm, two bacteriophages (Phage 1 and Phage 2) were isolated and later confirmed using secondary streaking. Both the *S. aureus* bacteriophages isolated were narrow spectrum and didn't show heterogeneous lytic ability. Similar observations were recorded by Song *et al*. (2021) in which narrow spectrum *S. aureus* specific phage was isolated in their study. In another study performed by Mahadevan *et al*. (2009) and Vahedi *et al*. (2018), the phage isolated by them were also narrow spectrum and had no effect on other bacteria similar to the findings of the present study.

Further, the lytic ability of the phages was tested to treat biofilm at two varied concentrations of PFU A (1.5×10^7) and PFU B (5×10^7) and it was observed that higher titre of phage reduced the biofilm more effectively. The results were almost similar to the findings of Alves *et al*. (2014) where they studied interrelation of biofilm removing capacity of three strong biofilm producing isolates of *S. aureus* by treatment with different multiplicity of infection (MOI) of phage and it was observed that the phage mixtures with a higher MOIs (10 compared with 1) gave a more rapid reduction in biofilm mass.

Table 2: Optical density (OD) value after phage treatment at different time.

Treatment	4 h	8 h	12 h	24 h
Positive control	1.294±0.0235	1.339±0.05	1.497±0.0335	1.510±0.0155
PFU A	1.243±0.0583	0.948±0.0339	0.705±0.0523	0.502±0.0472
PFU B	1.241±0.0349	0.693±0.0601	0.453±0.0193	0.229±0.0024

Table 3: PFU/ml and CFU/ml after phage treatment of biofilm.

Groups	After 4 h		After 8 h		After 12 h		After 24 h	
	PFU	CFU	PFU	CFU	PFU	CFU	PFU	CFU
Control	0	9.28935±0.0218	0	9.3818±0.0032	0	9.61265±0.00775	0	9.81165±0.07856
PFU A (1.5×10^7)	7.21725±0.0136	9.2899±0.00825	7.40975±0.0671	9.1376±0.05829	7.7396±0.0917	9.03135±0.04405	8.07305±0.0731	8.52705±0.12926
PFU B (5×10^7)	7.8440±0.0312	9.22885±0.01828	8.073±0.073	8.97225±0.06912	8.4358±0.0556	8.4603±0.2300	8.69005±0.155	7.8495±0.15023

A reduction in biofilm biomass, rise in PFU and a reduction in CFU was observed in the present study. A reduction in OD after phage treatment was observed by Song *et al*. (2021) in which the OD decreased to 0.25 after exposing the staphylococcal biofilm to phage for 24h similar to the findings of the present study where after exposure for 24 h the total biofilm biomass decreased significantly, 0.502 ±0.0472 and 0.229±0.0024 with PFU A and PFU B respectively as compared with the control (1.510±0.0155). These observations provided an insight that the phage isolated in the study was effective in reducing the biofilm of the *S. aureus*.

The OD values obtained at each time interval were indicative of a considerable biofilm clearing action. Our findings supported the observations recorded in a study by Vandersteegen *et al*. (2013) in which no substantial effect of phage treatment was found on the biofilm of *S. aureus* during 2 h to 6 h. However further studies are required to know precisely the timeline which is needed for effective cleansing of biofilm by phage.

CONCLUSION

A total of 18 *S. aureus* were isolated and confirmed using biochemical tests from mastitic animals in the study. All the isolates were tested for their biofilm producing ability and it revealed that 22.22% of the *S. aureus* isolates produced biofilm when tested on congo red agar assay and 33.33% on modified congo red agar assay. Also, two phages Phage 1 and 2 were isolated against *S. aureus* from ten sewage samples in the study. Two different concentrations of Phage 1 (PFU A and PFU B) was used for treating biofilm produced by *S. aureus* (S 4) isolate that was strong biofilm producer. The treatment with PFU A (1.5×10^7) decreased biofilm by 2.20% at 4 h, 31.74% at 8 h, 51.84% at 12 h and 67.10% at 24 h and PFU B (5×10^7) reduced biofilm by 5.84% at 4 h, 50.10% at 8 h, 70.41% at 12 h and 83.14% at 24 h. From the study it can be concluded that phage with high concentration was better in treating biofilm.

Conflict of interest

The authors do not have any conflict of interest in the research presented in this article.

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