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The impact of biofilm on the colonization between *Staphylococcus aureus* and *Mycoplasma bovis* under *in-vitro* conditions

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Abstract

Mastitis is a major cause of decrease in milk production in cattle and buffaloes that leads to the economic losses to the dairy industry. *Staphylococcus aureus* and *Mycoplasma bovis* both play an important role in causation of mastitis. Biofilm formation property of these bacteria helped to understand the pathogen city of mastitis in bovine. Twenty milk sample of each sub clinical and clinical mastitis were collected, *Staphylococcus aureu* sand *Mycoplasma bovis* were isolated and their biofilm was developed by cover slip method under *in-vitro* conditions and then they were treated with homologous and heterogonous culture isolates for the cross colonization potential. The homologous biofilm for *S. aureus* and *M. bovis* had resulted into significant increase in growth (CFU) with mean value of 599.8/HPF and 558.8/HPF respectively, while the heterogonous growth of *S. aureus* and *M. bovis* were having mean values of 548.8/HPF and 181.6/HPF respectively. The growth potential of *S. aureus* was equally high irrespective of biofilm source whereas the biofilm of *S. aureus* rarely promoted the growth of *M. bovis*. The overall results proved that the initial *M. bovis* infection may have the potential to promote *S. aureus*as secondary infections with equal intensity. In future more mastitis pathogens may also be studied in similar pattern resolved the pathogen city under heterogonous environment.

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Introduction

Pakistan is an agricultural country and livestock has its major share in the gross domestic product of Pakistan. The value added by livestock in agriculture was 55.91% while in national GDP during the year 2013-2014 its value was 11.8% (Ashfaq *et al.*, 2015). The decrease in milk production and milk quality is the major problem in dairy industry which is associated with mastitis. The major mastitegens are *M. bovis* and *S. aureus* that damage milk quality and quantity (Ibrahim *et al.*, 2013). Mastitis is an expensive disease in the dairy industry that caused a loss of $35 billion on the global scale. The incidence of mastitis in Pakistan is 44% only in Punjab, it not only spoils the milk quality and quantity but also a major public health concern. It accounts for 10% of total economic losses in the dairy industry. The environmental mastitis can be controlled by good manage mental practices, but the contagious mastitis is difficult to control because the pathogens involved in contagious mastitis are resistant to antibiotics and they cause repeated incantations of mastitis (Bradley, 2002).

Staphylococcus aureus is major mastitis pathogen and the colonization of *Staphylococcus aureus* is dependent on its virulence factors, one of which is its ability to form the biofilm. The biofilm formation by *Staphylococcus aureus* makes it resistant towards antibiotics and they use it as a tool to cause chronic infections (Taj *et al.*, 2012). The biofilm formed by *Staphylococcus aureus* is associated with various proteins as surface proteins, BAP, fibrinogen, fibronectin and clumping factors. The adherence of bacteria to living and non-living surface is controlled by BAP. The formation of biofilm by *S. aureus* makes them resistant to antibiotic treatments (Speziale *et al.*, 2014). *Mycoplasma bovis*is a delicate atypical organism and the mastitis associated with *Mycoplasma bovis* can be recognized by an unexpected reduction in milk production, the quality of milk is also compromised. The presence of *Mycoplasma bovis* can be a prime factor for secondary bacterial infections (Pfutzner and Sachse, 1996). The colonization of surface by *Mycoplasma bovis*is because of its biofilm formation property.

In spite the fact that *M. bovis* lack cell wall they form a biofilm for their persistence and survival. The variable surface proteins are involved in biofilm formation by *M. bovis*. The length of Vsa determines the strength of biofilm (Simmons and Dybvig, 2003).

The formation of biofilm helps the bacteria to survive in hostile conditions. When biofilm reaches to a certain extent bacteria start detaching from it and adhere to another site for new biofilm formation (Shivakumar and Chakravortty, 2014). The formation of biofilm by *Staphylococcus aureus* and *Mycoplasma bovis* make them more resistant to antimicrobials that lead to failure of treatments and increased chance of the mastitis to reoccur even in same quarters (Melchior and Vaarkamp, 2006). The missing gaps were identified that there may be a chance that the bacteria promote each-other in the biofilm making a chronic infection that lead to failure of treatment. The study was planned to investigate the interaction of *Staphylococcus aureus* and *Mycoplasma bovis* biofilm in colonization of homologous and heterogonous species.

Material and methods

Source of Sample

Twenty cattle and buffalo milk samples were collected from different dairy and livestock farms around district Faisalabad, Punjab Pakistan. Pre-milking teat dipping was done and surf field mastitis test was performed. The teats positive for mastitis were milked approximately 4ml by stripping the teat. Samples were collected in an aseptic manner and all the samples were collected in sterile containers. Samples were brought to the laboratory of Institute of Microbiology, University of Agriculture Faisalabad, Pakistan.

Isolation and identification of S. aureus

Staph110 media was used to isolate the *S. aureus*. After given time of incubation, the colony morphology was determined and the colonies with circular, flat and undulate margins were selected for further biochemical characterization. For identification of *Staphylococcus aureus* initially Gram staining was performed and morphological characters were studied under the microscope at 10X and 100X.

For confirmatory diagnosis of *Staphylococcus aureus*, hemolysis on blood agar was checked and later on catalase, coagulase and mannitol fermentation tests were performed (Shrihari, 2011).

Isolation and identification of M. bovis

Initially, PPLO broth which was added with 20% horse serum, penicillin and thallium acetate was used for passage. The collected milk samples were centrifuged at 3000rpm for five minutes and the supernatant was discarded. The sediments were poured in PPLO broth and incubated for 24hr. Five such passage were given continuously and then it was streaked on PPLO agar. The incubation of 72hr was given to the culture and it was further resolved under the microscope at 10X. The further confirmation was done by performing glucose fermentation, the activity for phosphatase and Tetrazolium reduction tests (Chima *et al.*, 1995).

Biofilm development

Cover slip method was conducted for the biofilm formation as followed by (Mcauliffe *et al.*, 2006). The Brain Heart Infusion broth supplemented with 0.4% glucose was used as a medium for *Staphylococcus aureus* biofilm. The addition of glucose was done in order to enhance the biofilm formation. Eaton's broth medium was used for *Mycoplasma bovis* biofilm. Eaton's medium was prepared by autoclaving *Mycoplasma* broth base with distilled water and was enriched with 20% horse serum, thallium acetate 50.0mg/L and penicillin 50,000 IU/L.

Mycoplasma bovis and *Staphylococcus aureus* cultures were grown in their respective medium at 37°C in an incubator for 6hr. Petri plates were used for biofilm each having selective medium for *Mycoplasma bovis* and *Staphylococcus aureus* cultures. Six cover slips were placed in each Petri plate, turbidity standard of 0.5 McFarland was maintained by adding 5µl of culture suspension on each glass cover slip submerged in selective medium. Plates were incubated at 37°C and coverslips were observed after 48hrs and 72hrs of incubation.

Biofilm assay

After given time of biofilm development, the cover slips were removed from medium plates, and three cover slips from each set were stained with crystal violet and examined microscopically for bacterial colony forming units. Three cover slips from each set were washed three times with distilled water and stained with 0.1% crystal violet for two minutes at room temperature, excess dye was removed by washing each cover slip three times with 0.85% normal saline, then air dried and observed under the microscope for biofilm examination.

Evaluation of biofilm for homologous and heterogonous growth potential

The cover slips having the biofilm of *Mycoplasma bovis* was placed in Petri plates containing BHI medium supplemented with 0.4% glucose while the cover slips having the biofilm of *Staphylococcus aureus* were placed in Petri plates containing Eaton's medium. Cover slips were placed in each Petri plate and a turbidity standard of 0.5 McFarland was maintained by adding 5µl of culture suspension of *Staphylococcus aureus* on each glass cover slip submerged in BHI medium and 5µl of culture suspension of *Mycoplasma bovis* on each glass cover slip submerged in Eaton's medium. Plates were incubated at 37°C and cover slips were observed after 48hrs and 72hrs.

Statistical analyses

A Tukey test for comparison of multiple means of colony forming units of biofilm with $P > 0.01$was used to analyze the data statistically.

Results

Culture isolation and Identification

Isolated colonies on Staph 110 medium were obtained after the culturing of milk samples, positive for sub clinical and clinical mastitis.

The culture characteristics were observed at 10X power of the microscope. The colonies of *Staphylococcus aureus* appeared as small and pinhead size with round convex shape, golden yellow in color and 1-4 mm in diameter having sharp edges.

The slide of Gram staining was observed under the microscope at 40X and Gram-positive coccid arranged in the bunch were observed.

The colonies of *Mycoplasma bovis* were found to be atypical and umbonate under the microscope at 10X and 40X. The sizes of colonies were less than 1 mm and were centrally raised with rough edges, by observing under the microscope small projections were seen.

Biochemical characterization of culture isolates

The growth of *S. aureus* on sheep blood agar resulted into the clear zone of hemolysis, *Staphylococcus aureus* showed beta hemolysis on blood agar. They were catalase positive and showed gas bubble formation when a single colony was mixed with hydrogen peroxide.

S. aureus resulted in coagulation of rabbit plasma when incubated for 24hrs at 37°C, it was also positive for sugar fermentation when carried out at mannitol salt agar. Glucose fermentation test was performed for *Mycoplasma bovis*. The color of the indicator (phenol red) slightly changed showing the negative result. Phosphatase activity and Tetrazolium reduction test for *Mycoplasma bovis* were performed the results of both of these tests were positive.

The impact of homologous biofilms of S. aureus and M. bovis

Biofilm of the respective isolates was maintained on the glass cover slip and was observed under the microscope at 40X. The homologous biofilm for *S. aureus* has resulted into significant increase in growth (CFU) as shown in Fig. 1 (a) without the wash and (b) after washing it with double distil water, for observing the biofilm.

The homologous biofilm of *M. bovis* has also resulted into significant increase in growth (CFU) as shown in Fig. 2 (c) without a wash and (d) after washing it with double distil water, for observing cluster forming units of biofilm (CFU).

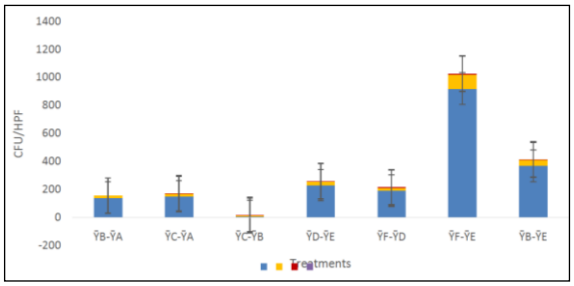

Fig. 1. Mean ± SE for cluster forming unit potential of *S. aureus* and *M. bovis* over homologous and heterogonous biofilms.
(A) Direct *M. bovis* biofilm
(B) *S. aureus*/*M. bovis* biofilm
(C) *M. bovis*/*M. bovis* biofilm
(D) Direct *S. aureus* biofilm
(E) *M. bovis*/*S. aureus* biofilm
(F) *S. aureus*/*S. aureus* biofilm

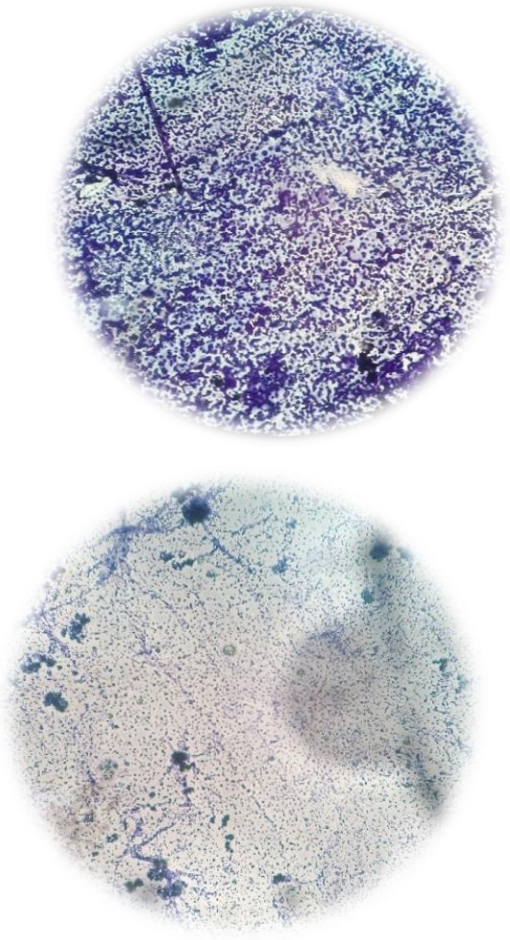

Fig. 1. (a). Photomicrograph of homologous growth of *S. aureus* biofilm on glass cover slip showing active CFU/microscopic field stained with crystal violet after 48h of incubation (b) Photomicrograph of homologous growth of *S. aureus* biofilm on glass

cover slip showing active CFU/microscopic field stained with crystal violet after 48h of incubation and washing with double dist. Water.

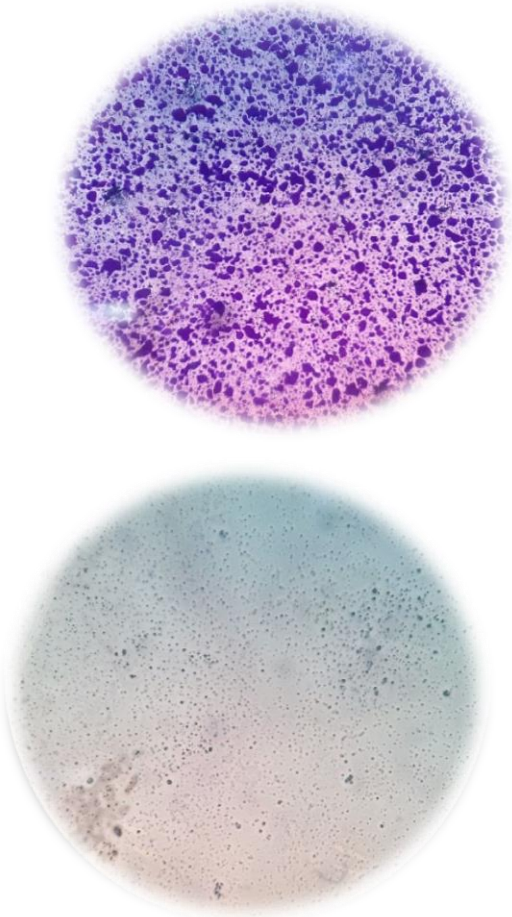

Fig. 2. (c) Photomicrograph of homologous growth of *M. bovis* biofilm on glass cover slip showing active CFU/microscopic field stained with crystal violet after 48h of incubation (d) Photomicrograph of homologous growth of *M. bovis biofilm* on glass cover slip showing active CFU/microscopic field stained with crystal violet after 48h of incubation and washing with double dist. water

The impact of heterogonous biofilms of S. aureus and M. bovis

In the heterogonous biofilm, the growth potential of *S. aureus* was equally high irrespective of biofilm source. When it was allowed to grow over *M. bovis* biofilm the number of cluster forming units (CFU) were observed nearly equal to the homologous growth clusters of *S. aureus* biofilm as shown in Fig. 3 (e) before washing and (f) after washing it with double distil water.

In a heterogonous biofilm, the growth potential of *M. bovis* was not high as it was observed in a homologous biofilm. When it was allowed to grow over *S. aureus* biofilm the number of cluster forming units were observed to be decreased with respect to the homologous growth clusters of *M. bovis* biofilm, the biofilm of *Staph* rarely promoted the growth of *M. bovis* as shown in Fig. 4 (g) before washing and (h) after washing it with double distilled water.

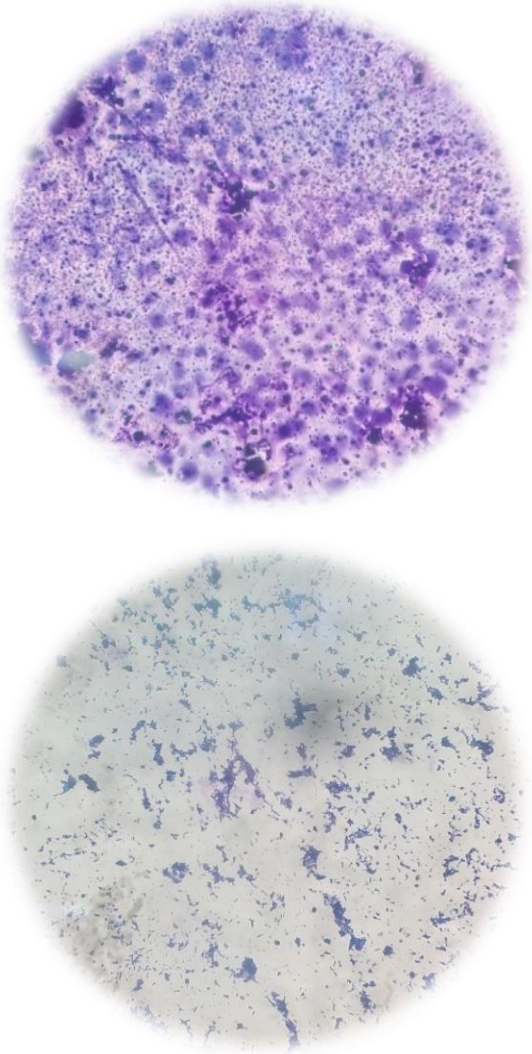

Fig. 3. (e). *S. aureus* growth over biofilm of *M. bovis* stained with crystal violet showing increased growth after 48h of incubation (f) *S. aureus* growth over biofilm of *M. bovis* stained with crystal violet showing increased growth after 48h of incubation and washing with double distilled water.

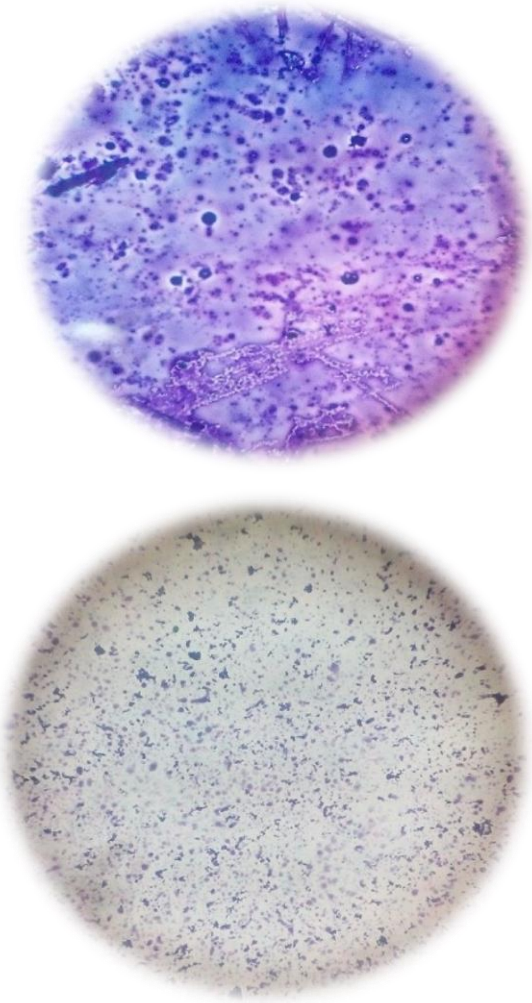

Fig. 4. (g). *M. bovis* growth over biofilm of *S. aureus* stained with crystal violet showing moderate growth after 48h of incubation (h) *M. bovis* growth over biofilm of *S. aureus* stained with crystal violet showing increased growth after 48h of incubation after washing with d. dist. Water.

Tabular Data and Statistical Analysis

The homologous biofilm for *S. aureus* was initially high with the mean value of cluster forming units (CFU) 599.8/HPF when *S. aureus* was allowed to grow over washed biofilm of *S. aureus*. The heterogonous growth of *S. aureus* was also equally high with the mean value of 548.8 CFU/HPF irrespective of biofilm source when allowed to grow over the biofilm of *M. bovis*as shown in table 1. The homologous growth of *M. bovis* resulted into 558.8 CFU/HPF over washed biofilm of *M. bovis*. Whereas the heterogonous growth of *M. bovis* when checked on the biofilm of *S. aureus* it was observed that *S. aureus* did not allow *M. bovis* growth it was having the mean value of 181.6 CFU/HPF as in table 1. The level of significance was checked by applying ANOVA (table 2) and final results were compared by applying the Tukey test for comparison of multiple means, where smaller mean values were taken off from larger mean values (ȲL-ȲS) as in (table 3). The overall results are expressed by in graphical presentation as in graph 1.

Table 1. Direct and Cross Colonization impact of *S. aureus* and *M. bovis* biofilm.

Treatments	Cultures	No. of clusters					Mean values
A	Direct growth of *M. bovis* biofilm	456	392	412	384	403	2047/5=409.5
B	Growth of *S. aureus* over *M. bovis* biofilm	592	548	564	524	516	2744/5=548.8
C	Growth of *M. bovis* over *M. bovis* biofilm	564	567	553	561	549	2794/5=558.8
D	Direct growth of *S. aureus* biofilm	436	398	424	407	387	2052/5=410.4
E	Growth of *M. bovis* over *S. aureus* biofilm	192	176	180	173	187	908/5=181.6
F	Growth of *S. aureus* over *S. aureus* biofilm	608	594	597	611	589	2999/5=599.8

Table 2. Analysis of Variance of mean cluster forming units obtained through homologous and heterogonous biofilm of *S. aureus* and *M. bovis*.

S.O.V	D.F	Sum. Sq.	Mean sq.	F cal.	F tab.	Conclusion
Treatment	5	596405	119281	306.7	2.64	Significant
Error	24	9334	389	-	-	-
Total	29	605739	119670	-	-	-

Table 3. Comparative cluster forming units appeared on homologous and heterogonous biofilm of *S. aureus* and *M. bovis* of mean values.

Comparison	ȲL-ȲS	q cal.	q tab.	Conclusions
ȲB-ȲA	139.3	15.79	5.37.	Significant
ȲC-ȲA	149	16.89	5.37	Significant
ȲC-ȲB	9.7	1.099	5.37	Non-Significant
ȲD-ȲE	228.8	25.94	5.37	Significant
ȲF-ȲD	189.4	21.476	5.37	Significant
ȲF-ȲE	918.2	104.11	5.37	Significant
ȲB-ȲE	367.2	41.637	5.37	Significant

Discussion

The aim of the study was to isolate and characterize mastitis causing *Staphylococcus* and *Mycoplasma* species and to study the impact of biofilm across the colonization of homologous and heterogonous species. *Staphylococcus aureus* and *Mycoplasma bovis* grow and develop their biofilm on coverslips, initially, the cover slips were washed and stained with crystal violet to observe their growth and biofilm. Later the coverslips were submerged in reverse media to find out the heterogonous growth potential of both bacteria. For this purpose, *Staphylococcus aureus* and *Mycoplasma bovis* were isolated from the sub clinical and clinical mastitis milk samples of both cattle and buffalo. Staph 110 media was used for the isolation of *Staphylococcus sp. as Staphylococcus aureus* while PPLO media was used for *Mycoplasma bovis*. Colonies of *Staphylococcus aureus* were the pinhead and of golden yellow color while center raised with rough edge colonies were of *Mycoplasma bovis*. The colonies of *Staphylococcus aureus* were of 1-4 mm while colonies of *Mycoplasma bovis* were less than 1 mm. Gram staining, hemolysis on blood agar, catalase, coagulase and mannitol fermentation tests were positive for *Staphylococcus aureus* as they contain thick peptidoglycan, catalase enzyme. On another hand, glucose fermentation was negative while phosphatase activity and Tetrazolium reduction were positive for *Mycoplasma bovis*.

The formation of biofilm by *S. aureu* sand *M. bovis*is a distinct property that made them enable to colonize a surface for the longer duration. Their colonization and persistence lead to enhanced infectivity where some of the bacteria show a synergistic effect while others show an inhibitory effect. The formation of biofilm boosts the non-biofilm forming bacteria to cause infection (Fox *et al.*, 2005).

For detection of biofilm different methods are there but here biofilm was developed for *Staphylococcus aureus* and *Mycoplasma bovis* in a cover slip based technique. Coverslips were used for the development and quantification of biofilms as described by (Mcauliffe *et al.*, 2006). In the biofilm formation by Mcauliffe, cover slips were placed on multi well glass plates while in this study the cover slips were placed in Petri plates. Cover slip based technique is the reliable and readily available technique to study and quantify biofilms as compared to Congo red agar method, microtiter plate method, testtube biofilms and flow chamber biofilms. Congo red agar (CRA) method categorizes the bacteria on the basis of their colony colors. Strong biofilm producing bacteria show black color colonies while moderate biofilm producing bacteria show pink colored colonies and white color colony indicates weak biofilm producing bacteria. The color of colonies is not a good indicative of biofilm production as color identification may vary from person to person. Likewise, CRA is also not a reliable method as results are not repeatable (Mathur *et al.*, 2006). The test tube biofilms also depend on visual aids, here the categorization biofilm producing bacteria is done on the basis of the thickness of material attached to the test tubes. In the flow chamber biofilms continuous provision of media is needed along with specified materials and instruments. This is not an appropriate method for bacteria producing static biofilms like *Staphylococcus aureus*. In our conditions, the microtiter plate assay was not suitable because it was difficult to observe the homologous and heterogonous growth potentials in it. A coverslip based biofilm method does not have all these drawbacks and it was the easy and dependable method for biofilm quantification so this method was used in this research.

Biofilm of *Staphylococcus aureus* and *Mycoplasma bovis* was developed for 72 hours and maximum biofilm formation was observed after the incubation of 48 hours. Biofilm forming ability of both bacteria was observed, their homologous and heterogonous growth potential were verified. The colony forming units of homologous biofilms of both *Staphylococcus aureus* and *Mycoplasma bovis* were found increased. The biofilm formed by *M.bovis* supported the growth of *S. aureus* as a number of colonies forming units were elevated. *Mycoplasma bovis* alone has the capability to grow and produce disease in dairy animals and cause the major increase in somatic cell count, infection due to *Mycoplasma bovis* cause the suppression of immune system hence makes udder more liable to other pathogens (Ghadersohi *et al.,* 1999). The growth of *Staphylococcus aureus* was significantly increased when on *Mycoplasma bovis* biofilm. The supportive effective of *M. bovis*to wards other pathogens was studied and the positive relation was observed for *S. aureus*, the colonization of *M. bovis*was followed by other pathogens that were not before associated in infection (Radaelli *et al*., 2011).

The colony forming units of *M. bovis* were counted over *S. aureus* biofilm but the growth of *M. bovis* was not as much. There was no supportive effect of *Staphylococcus aureus* for *Mycoplasma bovis*. *S. aureus* is major mastitis pathogen the infection of *S. aureus* was observed with coagulase negative bacteria and it was reported that if CNS infect the udder they make it resistant towards *Staphylococcus aureus* infection (Pyorala and Taponen, 2009). The infection of *Mycoplasma bovis*is followed by other contagious pathogens because the ability of *M. bovis* to evade the immune system and make animal immune compromised to allow the colonization of other bacterial species to cause infection.

Conclusions

Our studies clearly reveal that the homologous biofilm for *S. aureus* and *M. bovis* had resulted into significant increase in growth (CFU). The growth potential of *S. aureus* was equally high irrespective of biofilm source whereas the biofilm of *Staph* rarely promoted the growth of *M. bovis*.

The overall results proved that the initial *M. bovis* infection has the potential to promote *Staph. aureus* infections with equal intensity. The biofilm formation by *Mycoplasma bovis* makes the udder more vulnerable to invasion and colonization by *Staphylococcus aureus* species. The initial *M. bovis* infection has the potential to promote *Staph. Aureus* infections with equal intensity. The animal suffering from pneumonia with *M. bovis* may suffer from mastitis in the later stage of the disease so it must be treated for mastitis as well. More mastitis pathogens may also be studied in similar pattern resolved the pathogenicity under heterogonous environment.

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Conflict of interest

All co-authors have no conflict of interest regarding these research findings and its publication.

In this graph blue area indicates the difference between highest and lowest mean value of colony forming units while orange color is showing the calculated value of cluster forming unit. Here the minute red shows the tabulated value obtained from the tables which help in comparison with the graphical values. The line over error bar showed the lowest value from below than the line above lowest value indicates 1st quartile than comes 2nd quartile after that comes mean value than 3rd quartile and above all the highest value. The difference between the highest value and mean value indicates the standard error. In this obtained graph the distance between 3rd quartile and highest value shows the highest value of cluster forming unit e.g. in C-A bar and F-E bar the two lines almost merge with each other showing that number of cluster forming units fall under this area.

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