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Study on Biofilm Forming Ability of *Staphylococcus* Spp. Isolated from Bovine Mastitis Milk

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Abstract
Mastitis is an inflammation of mammary gland and udder tissue. One of the vital etiological agent *Staphylococcus* sp., mainly *Staphylococcus aureus* is responsible. Biofilm forming ability of such microorganism is considered as one of the vital virulence factors. This study was designed to screen the biofilm forming ability of mastitis-causing pathogens. A total of 48 *Staphylococcus* spp. was investigated which were isolated from mastitis infected milk samples using Baird parker and Mannitol salt differential media having drug resistivity observed from previous study. Tube assay and Microtitre plate assay were conducted for the detection of biofilm for these antibiotic-resistant strains. In tube assay detection method, 19 strong biofilm formers containing all *S. aureus*; 19 moderate formers containing 12 *S. aureus* & 7 *Staphylococcus* spp. and 10 weak biofilm formers containing all *Staphylococcus* spp. were observed at 24 hr. and after additional incubation of 12 hr leads all the isolates to strong biofilm forming category. Similarly, in microtitre plate assay out of 31 Coagulase positive and methicillin-resistant *Staphylococcus aureus*, 9 were moderate and 22 were strong biofilm formers, while out of studied 17 *Staphylococcus* spp., 9 were observed weak and 8 were moderate biofilm formers. No significance difference was observed after more incubation period i.e. 12-24 hr. in microtitre plate assay. Tube assay can be considered as the best method for preliminary detection of biofilm as it can be used to study the growth pattern of microorganism and may help in diagnosis and treatment of such infections caused by pathogens.

Keywords
Biofilm, ELISA, Microtitre, *Staphylococcus* spp.

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INTRODUCTION
Mastitis in bovines known as an inflammation of mammary glands [20]. Many etiological agents are responsible to cause this clinical condition having an ability to form a biofilm. Biofilms are well-defined as the group of bacteria enclosed in a self-synthesized extracellular polymeric matrix (EPM), which attaches to an abiotic or a biotic surface [22].

Biofilm forming ability of a microorganism is considered to be one of the notable virulent factors. *Staphylococcus aureus* and *Staphylococcus epidermidis* are recognised to be vital bacteria for causing the clinical infection [5].

Such infections involving biofilm-forming bacteria are extremely difficult to eliminate because biofilms avenge antibiotic dissemination and it also prevents typical immune responses against infections [3]. The presence of biofilm formation is considered to be an important feature for survival and virulence. It has been identified that resistant nature of *S. aureus* against antibiotics mainly methicillin are due to biofilms. Some studies have shown that the difficulty to treat staphylococcal infection using conventional antibiotics is very difficult [17].

Different pathways and time ranges are observed in the formation of biofilms and sometimes its detection may vary according to the time taken for formation. Time is a dependable parameter for biofilm formation. The time required for biofilm formation is depending upon bacterial strains and it should be taken into consideration for the development of suitable antibiotic rehabilitation measures [7, 14].

Detection of biofilm forming ability of a bacteria at primary level is necessary. Few studies addressed that microtiter plate assay can be used as a rapid and simple method to screen biofilm formation of strains [2, 16].

Here, we studied the biofilm-forming ability of coagulase positive *Staphylococcus aureus* (CoPSA) and coagulase negative *Staphylococcus* spp. (CoNS) of clinical mastitic infection using two different approaches, one is simple tube assay for qualitative and microtitre plate assay to quantitative detection.

MATERIAL AND METHOD

Bacterial strains

We studied biofilm formation assay on 48 strains of *Staphylococcus sp*. Out of which 31 were coagulase positive *Staphylococcus aureus* which was resistant to methicillin and remained 17 *Staphylococcus* spp. were multidrug resistant including methicillin from the previous study. Isolates were confirmed and antibiotic sensitivity assay was performed for all the isolates in a previous study [15].

Tube assay

Identification of biofilm formation qualitatively was performed by tube assay method as described. Briefly, 1.9 ml Luria-Bertani (HiMedia, M1245) broth was inoculated with 100µl of overnight grown culture broth and incubated for 24 hr. 48 hr. and 72 hr. at 37°C with control without culture [11]. The tubes were decanted and washed with Phosphate Buffer Saline (PBS) (Sigma Aldrich, MFCD00131855) at pH 7.3 and tubes were subjected to dry. Staining of dried tubes was done with 0.1 % crystal violet stain (Sigma-Aldrich, C0775). Excess stain was removed by washing the tubes with deionized water (Sigma-Aldrich, 99053). Later, tubes were dried in an inverted position and observed for biofilm formation.

Microtitre plate assay

The isolates were screened for their capability to form biofilm by microtitre plate method with modification with culture dilutions and incubation time [7, 13, 21]. Isolates from fresh nutrient agar plates were inoculated in LB broth supplemented with 1% glucose and incubated for 18 hours at 37°C in a stagnant condition. Sterile flat bottom 96 wells plate (HiMedia, DM3) was filled with 0.2 ml aliquots of the diluted overnight cultures (Dilution Factor 10^{-1}). The control well contained only LB broth without inoculation. The plates were incubated for 24 hr and 48 hr at 37°C to observe biofilm.

After the respective incubation period, the content of each well was blandly removed by lightly tapping / instant inverting the plates. The wells were washed twice with phosphate buffer saline pH 7.3 to remove free-floating planktonic bacteria. The plates were then stained with 0.1% crystal violet solution and washed. Excess stain was washed off completely with 70% ethanol and plates were kept for drying. In order to quantify the adhered cells, 220 µl of decolouring solution (ethanol/acetone, 80:20%) was added to each well for 15 min. The absorption of the eluted stain was measured with a micro ELISA auto reader at a wavelength of 630 nm. These values were considered as an evidence of attachment to surface and forming biofilms. The experiment was performed in triplicates with proper hygiene conditions and the mean optical density values were considered.

Statistical Analysis

Biofilm forming ability was evaluated for coagulase-positive *S. aureus*(CoPSA) and coagulase negative *Staphylococcus* sp. (CoNS) using Friedman's test (SPSS 20) including 24 hr. and 48 hr. data of microtitre plate assay [23].

RESULTS AND DISCUSSION

Formation of biofilm was confirmed with the presence of attachment on the wall and bottom of the tube as shown in figure 1 [7].

Figure 1. Biofilm attached to the bottom of the tube

As a result of tube assay, positive biofilm formation was considered when a visible stained film was observed on the wall and bottom of the tube. Ring formation at the liquid interface was not considered an indication of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as negative (-), weak (+), moderate (+++) and strong (+++++).

Tube assay was performed on 48 isolates, out of which 19 *S. aureus* showed attachment of biofilm at the bottom and wall of the tube when stained with crystal violet after 24 hr. and remaining 29 isolates showed attachment after 24-36 hr (Table 1).

Table 1. Biofilm formation by Tube assay after 24 hr. & 24-36 hr. incubation

Biofilm Formation	Isolates showing biofilm after 24 hr. incubation	Isolates showing biofilm after 24-36 hr. incubation
Negative (-)	Control	Control
Weak formation (+)	10 (*Staphylococcus* spp.)	-
Moderate formation (+++)	19 (12 *S. aureus*; 07 *Staphylococcus* spp.)	-
Maximum formation (+++++)	19 (*S. aureus*)	29 (12 *S. aureus*, 17 *Staphylococcus* spp.)

Microtitre plate assay gave promising and comparatively similar results to a preliminary evaluation of tube assay. Microtitre plate assay is an updated method of tube assay in a smaller scale with the larger number of samples. In this method categorization of biofilm was done based on absorbance value. On the basis of microtitre plate assay, biofilm formation ability was weak for 9 *Staphylococcus* spp. isolate (OD <0.120), moderate for 17 isolates (9 MRSA, 8 *Staphylococcus* spp. where OD range was between 0.120-0.240 and high for 22 (*S. aureus*) isolates having absorbance above 0.240. The results at the point justifies tube staining outcomes (Table 2; Figure 1). After 12 hr. to 24 hr. of absorbance measurement; negligible change in OD value was observed.

Table 2. Biofilm Categorization on the basis of microtitre plate assay

Absorbance at 630 nm	Biofilm Formation
<0.120	Non/Weak
0.120 – 0.240	Moderate
>0.240	High

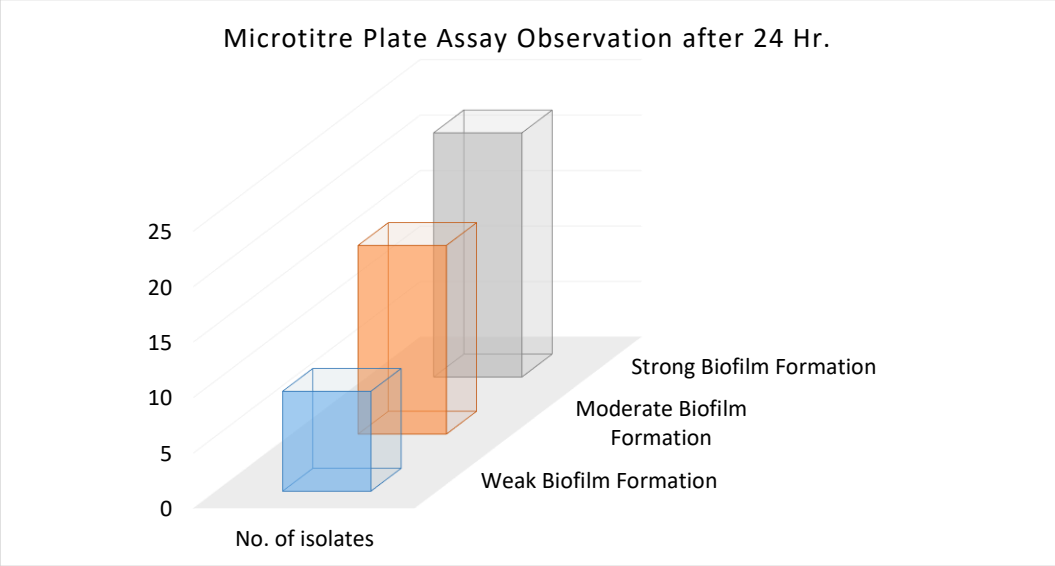

Figure 2: Numbers of isolates showed biofilms in category

According to the Friedman's test, no significant difference was observed between groups (CoPSA and CoNS for time intervals) in terms of biofilm-forming ability at each time point (24 hr: p = 0.027; 48 hr: p = 0.040) and verified no difference in outputs after 24 hr supplementary incubation.

When free whirling microbial cells encounter any surface, normally they switch themselves from a planktonic life type to the slimy communities which are known as biofilms that are enclosed by a dense layer of the self-produced slippery matrix material [12].

The ability of a pathogenic organism to form biofilm is a trait that is diligently associated with bacteria's virulence and persistence. Thus, many persistent and chronic bacterial infections like mastitis are nowadays believed to be linked with the formation of biofilm by pathogens [4, 9].

In this study, we analysed the ability of multi drug resistant *Staphylococcus* spp. to form a biofilm. *S. aureus* is a pliable opportunistic pathogen whose ability to persist and multiply in a variety of environments causes a wide spectrum of diseases in animals. This may lead to an increase in the risk of animal health. Biofilm forming ability of *Staphylococcus* sp. from mastitis cases is nowadays a major concern pertaining to animal health and milk quality.

A study from microtitre plate assay showed all the 31 *S. aureus* strains were found biofilm producers, classified as strong (70.97% = 22/31), moderate (29.03% = 09/31), and in *Staphylococcus* spp. 52.94% (09/17) were weak and 47.04% (08/17) were moderate biofilm producers. These data are in accordance with the tube assay outputs where all the isolates were found biofilm producers.

It is very notable observation that all the 48 (100%) *Staphylococcus* spp. were biofilm producer as well as antibiotics resistant. This virulent factor assurances the pathogenicity of *Staphylococcus* spp. and its connexion and conservation in the mammary gland tissue of udder. The results of this study are in accordance with a study from China, USA, Taiwan and India [4, 10, 12, 13, 21].

For detection of biofilm Congo Red Agar method is also used for primary detection but according to a study by T. Mathur *et al.*, it suggests that tube assay method for detection of biofilm forming ability of an organism is accurate and reproducible for screening [13].

Prevalence of biofilm producing ability of *Staphylococcus* spp. in different colonizing conditions may defer. It is highly depending upon the in-vitro conditions. In many cases, simple bacteria are unable to produce such biofilm until and unless it is provided with the susceptible environment to enhance toxins within and that is directly affecting the strain health. It has been previously demonstrated that phenotypic expression of biofilm formation is highly susceptible to in vitro conditions and hence can be detected variably by different methods [6, 7, 23].

This study was designed on single species biofilms and since beginning single-species biofilms have been extensively investigated. However, the biofilms in nature comprises mostly multiple species whether from same genera or different, where interspecies interactions help in shaping development, structure

and molecular functions from biofilm populations *S. aureus* [1, 8, 18, 19].

CONCLUSION

Detection of biofilm by the mainstream producers *Staphylococcus aureus* and *Staphylococcus* spp. can be detected in 24-36 hr. cultures. Tube assay and microtitre plate assays are the best approaches for preliminary screening qualitatively and quantitatively. The understanding of time progression of biofilm expression may subsidize the microbiologist to improve the treatment strategies with the use of antimicrobials for Staphylococcal infected mastitis therapy because it's an important virulent factor of microorganism.

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ETHICAL APPROVAL

No need for ethical approval.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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