

EVALUATION OF DIFFERENT DETECTION METHODS OF BIOFILM FORMATION IN STAPHYLOCOCCUS AUREUS ISOLATES FROM BOVINE MASTITIS

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ABSTRACT

Biofilms are self produced extra polymeric matrices that comprises of sessile microbial community where cells are characterized by their attachment to either biotic or abiotic surfaces. Biofilm associated diseases pose serious health challenges, resulting in high economic losses. *Staphylococcus aureus* is the most frequent and dreadful etiological agent for biofilm formation in bovine mastitis associated cases. The present study was undertaken to evaluate three in vitro techniques of biofilm formation in *Staphylococcus aureus* isolates obtained from Bovine mastitis cases. 150 bovine mastitis samples were collected from veterinary clinics F.V.Sc & A.H. Shuhama and local villages to assay biofilm production in vitro. The ability of the isolates to form biofilm was examined using tube assay, liquid interface coverslip assay and congo red assay. Of the 150 samples of acute and subacute mastitis 80(53.33%) isolates of *Staphylococcus aureus* were obtained. Of the 80 isolates analyzed, 65% showed biofilm formation by Tube assay, 45% by Liquid interface coverslip assay and 25% by Congo red assay indicating that tube assay is the most reliable method of assaying in-vitro *S. aureus* biofilm formation. The results indicate a high potential for pathogenicity among *S. aureus* isolated from bovine mastitis samples due to their ability of biofilm formation.

Keywords: *Biofilm, Liquid interface, Mastitis, S. aureus, Tube assay.*

I. INTRODUCTION

Bovine mastitis is the most infectious disease of dairy animals affecting quality and quantity of milk (1). Mastitis of early lactation results in long term production losses in dairy industry and is the common cause of death in adult dairy cows causing an estimated loss of billion dollars worldwide (2). A biofilm is a structured community of bacterial cells that are enclosed in a self-produced, polymeric matrix that adheres to an inert or

living surface and constitutes a protected mode of growth that allows survival in a hostile environment (3). Biofilms are inherently tolerant to host defenses and antibiotic therapies and is the root cause of many persistent and chronic bacterial infections (3), including bovine mastitis (4). *Staphylococcus aureus* is one of the most important pathogenic bacteria causing clinical/sub-clinical mastitis. Bacteria attach to mammary gland epithelia, form colonies surrounded by extracellular matrix thus forming biofilms and promotes chronicity of infection (5). *Staphylococcus aureus* biofilm formation is regulated by expression of polysaccharide intracellular adhesion (PIA), which mediates cell-to-cell association. Main constituent of *Staphylococcal* biofilms responsible for intercellular interactions is exo-polysacchride poly-N-acetyl-B- 1, 6-glucosamine (PNAG) synthesized by enzymes encoded in *ica ADBC* operon (6). The present study was undertaken with the aim to screen the *Staphylococcus aureus* isolates for *in vitro* biofilm forming abilities from cases of acute and chronic mastitis.

II. MATERIAL AND METHODS

2.1 Bacterial isolates

A total of 150 mastitis milk samples were collected from Veterinary Clinics of FVSc & A.H and local villages of Shuhama in sterile 10ml polypropylene tubes and transported to the laboratory on ice. The samples were subjected to Bromothymol Blue (BTB) test as per protocol of (7) to confirm the clinical status of collected milk samples. The *Staphylococcus aureus* isolates were identified by Gram staining and standard biochemical tests. *S. aureus* isolates were confirmed by *nuc* gene specific PCR as per the protocol of (8).

2.2 Evaluation of Biofilm formation

Staphylococcus aureus isolates were evaluated for their *in vitro* biofilm forming abilities using Tube assay, Liquid interface coverslip assay and Congo red assay.

2.2.1 Tube assay

Staphylococcus aureus isolates were screened for their biofilm forming ability using tube assay of (9). Briefly, 2ml of TSB was inoculated with loop full of *S.aureus* colonies and incubated for 48 hours at 37°C. The contents were decanted and tubes were washed with PBS and left to dry at room temperature. The tubes were stained with 4% crystal violet solution and rotated gently to ensure uniform staining. Stain was decanted and tubes were observed for biofilm formation. Positive result indicated presence of visible film lining the wall and bottom of the tube. Results were interpreted on base of a score card (0 - Absent, 1- Weak, 2 – Moderate, 3- Strong biofilm former).

2.2.2 Liquid-interface coverslip assay

Staphylococcus aureus isolates were screened for biofilm formation using air-liquid interface coverslip assay as described by (9). Overnight cultures of *Staphylococcus aureus* were inoculated in tubes containing 3-5ml TSB and allowed to grow to stationary phase. The stationary phase cultures were diluted in ratio 1:100 in TSB. 100 µl of diluted culture were filled in each well of a flat-bottom 6-well tissue culture plate (Costar, USA). Sterile

coverslips were inserted into each well to achieve a 90° angle relative to the bottom of the well so that the meniscus of the medium was at the centre of the coverslip. Plates were covered and kept in incubator at 37°C for 18 hours. Bacteria were stained by submerging coverslips in 0.1% crystal violet for 10 min. Coverslips were dipped in two successive water baths to rinse off excess dye and allowed to air dry. Bacteria at air-liquid interface were visualized under a microscope. Results were interpreted as: No adherence(-), Low level of adherence and no biofilm line on air-liquid interface (+), Intermediate level of adherence with staining below air-liquid interface(++), High level of adherence with clearly defined line at air-liquid interface (+++).

2.2.3 Congo red assay

Staphylococcus aureus isolates were screened for biofilm forming ability using Congo red assay as per protocol of (9). Congo red agar plates (Hi Media) were inoculated with loop full of *S.aureus* colony followed by incubation at 37°C for 24 hours. Formation of black colonies with a dry crystalline consistency indicated a strong biofilm formation while as formation of red /orange red color indicated no biofilm formation.

III. RESULTS

Bromothymol Blue (BTB) test revealed that 83 out of 150 (55.33%) samples were of clinical mastitis and 67 out of 150 (44.66%) were subclinical mastitis samples. A total of 80 (53.33%) *S. aureus* isolates were recovered from 150 samples of mastitic milk on the basis of their characterization on selective/differential media and biochemical tests. All the 80 *S. aureus* isolates were positive for tube coagulase test, morphological and biochemical characteristics as well as presence of *nuc* gene corresponding to presence of an amplicon size 270bp.

In-vitro biofilm forming abilities of *Staphylococcus aureus* isolates was evaluated by Tube assay, Liquid Interface coverslip assay and Congo red assay. It was observed that of the 80 *Staphylococcus aureus* isolates 29(36.25 %) were strong biofilm formers, 23(28.75%) were moderate biofilm formers and 28(35%) were weak / non biofilm formers by tube assay (Fig 1). Liquid interface coverslip assay revealed that 19(23.75%) isolates were strong biofilm formers, 17(21.25%) were moderate biofilm formers and 44(55%) were weak/ non biofilm formers (Fig 2). Congo red assay for biofilm formation revealed 20 (25%) isolates were moderate biofilm formers and 60(75%) were weak / non biofilm formers (Fig 3). A comparative evaluation of the three *in-vitro* biofilm formation techniques for *Staphylococcus aureus* isolates is summarized in Table 1.

Table 1: Comparative evaluation of three *in vitro* biofilm formation techniques

S.No	Technique	Strong biofilm former	Moderate biofilm former	Weak biofilm former
1.	Tube assay	29(36.25 %)	23(28.75%)	28(35%)
2.	Liquid interface coverslip assay	19(23.75%)	17(21.25%)	44(55%)
3.	Congo red assay	-	20(25%)	60(75%)

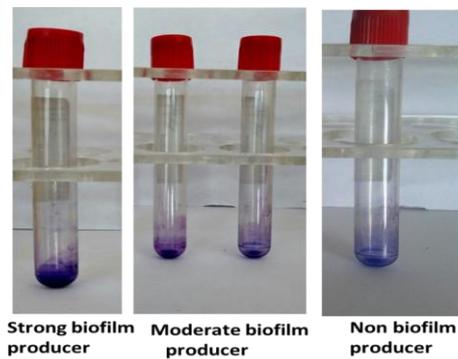


Fig 1. *In vitro* biofilm formation using Tube assay

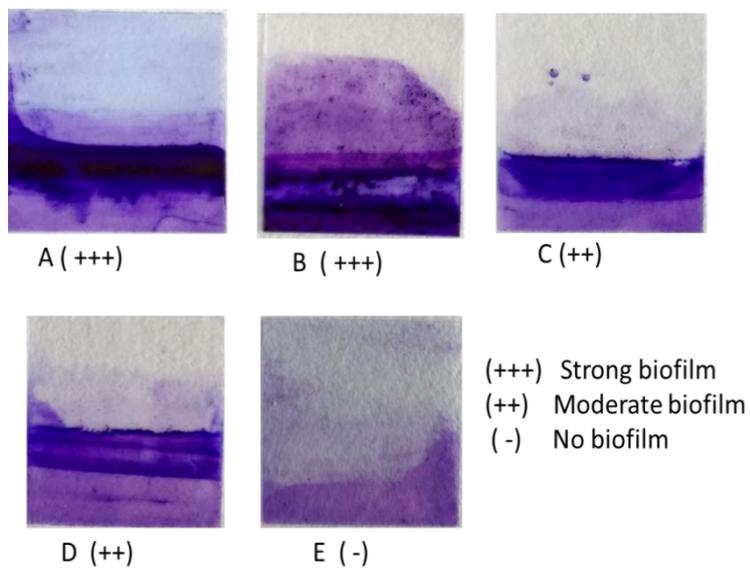
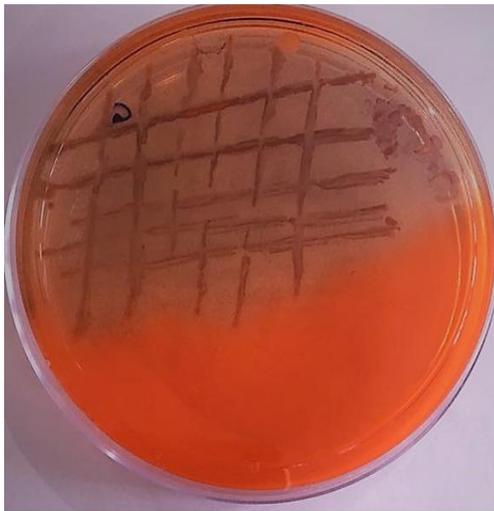


Fig 2. *In-vitro* biofilm formation using Liquid interface coverslip assay



**A. Moderate biofilm
(Black colonies)**



**B. No biofilm
(Orange colonies)**

Fig 3. Congo red assay for *Staphylococcus aureus*

IV. DISCUSSION

Staphylococcus aureus is one of the significant causes of udder infection in dairy animals (3). Intramammary infections (IMI) with this pathogen may lead to clinical and sub-clinical mastitis associated with increase in somatic cell number (SCC). The prolonged infections are related to microbial growth as adhesive colonies are enclosed by a large exopolysaccharide matrix, thus establishing a biofilm (10). Biofilm formation have been identified as virulence factor in *Staphylococcus aureus* infections in clinical settings and has been attributed to severity of urinary tract infections, catheter infections, middle ear infections, dental plaques, gingivitis, endocarditis, cystic fibrosis and infections of joint prosthesis and heart valves (11). Investigations by researchers related to strategies employed by bacteria related to the mechanism and pathogenesis of biofilm formation reveal that certain chemicals produced by biofilm forming bacteria protect them from disinfectants, antimicrobials and phagocytic host immune systems (12). Various conventional methods for biofilm formation have been established, to evaluate the potential of biofilm forming bacteria.

In present study *in vitro* biofilm formation revealed that 36.25% isolates and 23.75% isolates were strong biofilm formers, 28.75% and 21.25% were moderate biofilm former, 35% and 55% were weak / non biofilm formers by Tube and Liquid interface coverslip assay, respectively. In a study by Taj and Essa, (2011) out of 115 isolates of *S.aureus* isolated from samples of urine and pus, 23 (20%) were strong biofilm formers, 40 (34.78%) were moderate biofilm formers and 52 (45.2%) were weak / non biofilm formers. Congo red assay revealed that 25% isolates were moderate biofilm formers producing black colonies with no dry crystalline

morphology and 75% were weak / non biofilm formers producing orange red colonies which correlates well with the findings of (13).

The results of tube assay correlate well with the coverslip assay for evaluating the biofilm producing potential of pathogenic organism. However, Congo red assay is less sensitive method for evaluating the biofilm production of *S. aureus* species in comparison to the other two methods.

V. CONCLUSION

Biofilms are of significant concern due to their involvement in many animal diseases, bovine mastitis being important owing to its great impact on livestock economy. Biofilm formation can also cause many problems in the medical field, particularly with prosthetic devices such as indwelling catheters and endo-tracheal tubes. Obtaining clinical samples from such cases for laboratory examination to identify biofilm formation can help in prevention of fatal and persistent infections. A close association is found between biofilm production with persistent infection and antibiotic therapy failure, thus identification of infections caused by biofilm producing *staphylococci* may be helpful in modifying the antibiotic therapy and prevent infection. In most of the cases, tube assay can be adopted as most suitable and reliable method for detection of pathogenic potential of bacterial strains.

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REFERENCES

- [1] K. Oleveira, General aspects of biofilms and its implication in ruminants, *Star Vac Library*, 2007.
- [2] F. Ross, R.P. Flynn, J. Meaney, W.J. Fitzgerald, and G.F. Coffey, Isolation and characterization of two anti- *staphylococcal* bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections. *Letters in Applied Microbiology*, 41, 2005, 482-486.
- [3] J.W. Costerton, P.S. Stewart, and E.P. Greenberg, Bacterial biofilms: a common cause of persistent infections. *Science*, 284, 1999, 1318-1322.
- [4] C. Cucarella, C. Solano, J.Valle, B. Amorena, I. Lasa, and J.R. Penades, Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol*, 183, 2001, 2888–2896.
- [5] M.B. Melchoir, F.J. Gruenel, and W. Gastra, Extended antimicrobial susceptibility assay for *Staphylococcus aureus* isolates from bovine mastitis growing in biofilms. *Veterinary Microbiology*, 125(1-2), 2007, 141-149.
- [6] A. Abdulmir, and S. Jasim, Potential of bacteriophage cocktail in eliminating methicillin resistant *Staphylococcus aureus* biofilm in terms of different extracellular matrices. *Ann clinical Microbial Antimicrob* 14, 2015, 1-10.

- [7] G. Markey, *Staphylococcus aureus. Book of Veterinary Clinical Microbiology*, 2013.
- [8] O.G. Brakstad, K. Aasbakk, and J.A. Maeland, Detection of *Staphylococcus aureus* by Polymerase Chain Reaction Amplification of the *nuc* Gene. *Journal of Clinical Microbiology*, 30(7), 1992, 1654-1660.
- [9] Y. Taj, and F. Essa, Study on biofilm forming properties of *Staphylococcus aureus*. *Journal Infect Dev Countries*, 5(6), 2011, 403-409.
- [10] W. Costerton, R. Veeh, M. Shirtliff, M. Pasmore, C. Post, and G. Ehrlich, The application of biofilm science to the study and control of chronic bacterial infections. *Journal of Clinical Investigation*, 112, 2003, 1466-1477.
- [11] H.W. Boucher, and G.R. Corey, Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Disease*, 46 (5), 2008, 344-9.
- [12] K. Saitou, K. Furuhta, and M. Fukuyama, Biofilm formation abilities and disinfectant resistance of *Pseudomonas aeruginosa* isolated from cockroaches captured in hospitals. *Biocontrol Sciences*, 14, 2009, 65-68.
- [13] M.D. Mathur, S. Vidhani, and P.L. Mehndiratta, Bacteriophage therapy: An alternative to conventional antibiotics. *Journal of Association of Physicians of India*, 5, 2006, 593-96.