

Detection of biofilm formation among the mastitis isolates of Staphylococci by evaluation of three different screening methods

Hadeel M. Fiadh

College of Veterinary Medicine, Al-Anbar University

Abstract:

Internalization and biofilm formation is an important step in staphylococcal mastitis pathogenesis . Twenty five staphylococcal isolates of coagulase negative and coagulase positive were collected from clinical mastitis in cows, and biofilm production were studied for these isolate by three different methods. Tube method , tissue culture plate and Congo red agar method. The first two methods were used to study effect of glucose addition as source of polysaccharides that important in biofilm formation. The results showed significant increase in biofilm formation by addition of glucose in staphylococci coagulase negative diagnosed by tissue culture plate method. Congo red agar method results showed increase number of positive isolates by increasing incubation time. The results showed that tissue culture plate method (Spectrophotometric method) was the best method for the diagnosis of biofilm formation in comparison with both tube method and Congo red agar method.

الكشف عن إنتاج الغشاء الحيوي في المكورات العنقودية المعزولة من التهاب الضرع باستخدام تقييم ثلاث طرق مسح مختلفة

هديل محمد فياض

كلية الطب البيطري، جامعة الأنبار

الخلاصة:

التداخل مع النسيج تكوين الغشاء الحيوي يعتبر خطوة مهمة في امراضية التهاب الضرع. خمس وعشرون عينة من جراثيم المكورات العنقودية السالبة والموجبة للكواكيليز جمعت من ابقار مصابة بالتهاب الضرع ودرست صفة انتاج الغشاء الحيوي باستعمال ثلاث طرق مختلفة وهي طريقة الانبوب وطريقة طبق الزرع النسيجي وطريقة هلام كونغو الاحمر الطريقتان الاوليتان استخدمتا لدراسة تاثير اضافة سكر الكلوكوز كمصدر لتكوين السكر المتعدد المهم في انتاج الغشاء الحيوي. اظهرت النتائج زيادة معنوية في انتاج الغشاء الحيوي عند اضافة الكلوكوز في جراثيم المكورات العنقودية السالبة للكواكيليز والتي شخّصت بطريقة الطبق الزرع. كما اظهرت نتائج هلام الكونغو الاحمر زيادة العزلات الموجبة لتكوين الغشاء الحيوي بزيادة فترة الحضان. واطهرت النتائج ان طريقة الطبق النسيج الزرع هي الافضل في تشخيص انتاج الغشاء الحيوي عند مقارنتها مع الطريقتان الاخرتان.

Introduction:

Bovine mastitis is a disease characterised by mammary gland inflammation, usually caused by intramammary infections. This condition is difficult to eradicate and responsible for severe economic losses for dairy producers.(1). *Staphylococcus aureus* remains a major pathogen of chronic mastitis, but also *Staphylococcus epidermidis* has emerged as a relevant mastitis pathogen.(2). Internalization is an important step in staphylococcal mastitis pathogenesis (3). In vitro studies have shown that *Staphylococci* are able to adhere to and invade bovine mammary epithelium [4]. Biofilm production by *Staphylococci* an important virulence factor(5),(6) .During intramammary infection bacterial clusters may develop within the udder, and biofilm structures may facilitate *Staphylococci* adherence and colonization of the mammary gland epithelium (3). Biofilms are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, medical implant materials, and tissue, biofilms are characterized by “attached for survival” because once they are formed, they are very difficult to destroy, depending on their locations, biofilms can either be beneficial and detrimental to the environment, for instance, the biofilms found on rocks and pebbles underwater of lakes and ponds are an important food source for many aquatic organisms; on the contrary,

those that developed on the interiors of water pipes might cause clogging and corrosions (7,8). Biofilm formation is considered a selective advantage for *staphylococci* mastitis isolates, facilitating bacterial persistence in the udder, it requires attachment to mammary epithelium, proliferation and accumulation of cells in multilayers and enclosing in a polymeric matrix, being regulated by several loci, as biofilm formation can proceed through different pathways and time ranges, its detection may differ according to the time of observation(9) ,(10).

Bacterial biofilms may impair eradication of chronic mastitis, rendering antibiotherapy less effective. Detection of biofilm-forming ability in mastitis isolates may provide useful information for the establishment of a more adequate therapeutic regimen(9) wherever, biofilm are not susceptible to macrophage phagocytosis and they become resistant to certain antibiotics(11). Our research point toward studying ability of *staphylococcus* spp. to form biofilm and evaluated the reliability of three different method in order to determine most suitable screening method.

Materials and methods:

1. Twenty –five isolate of *staphylococcus* species were taken from mastitis infected cows from the period between January to March of 2011 in Al-Faluja.

All study isolate were well bacteriologically identified and confirmed by biochemical tests. Bacteria were stored in brain heart infusion broth (BHI) medium containing 20% glycerol. Before each experiment one aliquot was thawed at 37°C for 24hr.

2. Qualitative biofilm formation assay: (Adhesion assay or Tube method):

The procedure was made as described by Christensen *et al.* (12)

A. two to three colonies of the isolates were inoculated into 5ml of BHI broth in plastic white tube in the presence of glucose which is the substrate for polysaccharides.

B. Saccharide free basal medium (BHI) broth without glucose that lacks the substrate for polysaccharides was used as control. Both groups of cultures were incubated at 37°C for 18-20hr. The contents were aspirated, one tube was examined unstained and one each stained with crystal violet and safranin. Slim positivity was judged by the presence of the tube

3. Quantitative biofilm formation assay : (Spectrophotometric method):

The procedure was made as described by Christensen *et al.* (13).

working culture were prepared by inoculation on blood agar and incubated aerobically at 37°C for 24hr the culture. The culture used to prepare bacterial suspension in sterile distilled water adjacent to a 0.5 McFarland standard. The suspension obtained were inoculated

to brain heart infusion broth after that poured in to the wells of plastic microplate.

The well of sterile 96-well flat-bottomed plastic microplate (tissue culture plate) were filled with 250µl of BHI broth. Negative control wells contained the broth only. 20µl of bacterial suspension was then added to each well. The plate were incubated at 37°C for 24hr. following incubation the content of each well was aspirated and each well was washed three times with 300µl of sterile distilled water. The remaining attached bacteria were fixed with 200µl of methanol per well, and after 15 min. the plate were emptied and left to air dry. after that the plates were stained for 5min with 160µl per well of crystal violet . Excess stain was rinsed off placing the plate under running tap water. After the plate were air dried, the dye which was bound to the adherent cells was resolubilized with 160µl of 33%(V/V) glacial acetic acid per well. The optical density (OD) of each well was measured at 630 nm By ELISA reader.

4. Congo red agar method:

Phenotypic characterization of biofilm production was performed by culture of the staphylococci isolates on CRA plates as proposed by Freeman *et al.* (14).

According to the authors, biofilm producers form black colonies on CRA, whereas non-producers form red colonies. The Congo red dye directly interacts with certain

polysaccharides, forming colored complexes.(15).

Statistical analysis was conducting according to (16).

Results:

Our results show obvious increase of bifilm formation in same isolates

of staphylococci coagulase negative after addition of glucose , this is was clearly showed in both tube method and Spectrophotometric method(or tissue culture method), as illustrated in table No.1 .

Table No.1 : Biofilm formation in Coagulase negative staphylococci evaluated by two method : tube method and tissue culture method.

Nutritional supplement	No. of isolate	Tube method		Tissue culture	
		No. of +Ve	% of +ve	No. of +ve	% of +ve/
without glucose	17	8	47%	10	59% a*
with glucose	17	11	64%	13	76% b

*Different small letters refer to significant variation between different nutritional supplement.

Coagulase positive staphylococci show no difference of biofilm formation before and after addition of glucose evaluated by tube method while tissue culture method showed

significant increase in biofilm formation of same isolate after glucose addition. Table No.2 . demonstrate our results apparently.

Table No.2 : Biofilm formation by Coagulase positive staphylococci evaluated by two methods : tube method and tissue culture method.

Nutritional supplement	No. of isolate	Tube method		Tissue cultur	
		No. of +Ve	% of +ve	No. of +ve	% of +ve
without glucose	8	5	62%	6	75%
with glucose	8	5	62%	7	87%

Congo red agar method was used to evaluate biofilm formation in staphylococci of mastitis isolates in

different periods of incubation and our results summarized in table No.3

Table No.3: biofilm formation in staphylococci evaluated by Congo red agar method in two different periods of incubation.

	No. of isolate	After 24 hrs		After 48 hrs	
		No. of positive	% of positive	No. of positive	% of positive
Coagulase -ve	17	5	29	7	41
Coagulase +ve	8	2	25	2	25

The results showed that tissue culture plate method (Spectrophotometric method) was the best method for the diagnosis of biofilm formation in comparison with both tube method and Congo red agar method. The results also showed that there are significant increase in diagnostic results of the same isolates of staphylococci

diagnosed by tissue culture plate method in comparison with the results of diagnosis by tube method and Congo red agar method, furthermore, tube method showed significant increase in the diagnostic results in comparison with Congo red agar method, these results well summarized in the table No.4 :

Table No.4 : comparison among three different methods used for evaluation of biofilm formation.

Tube method		Tissue culture		Congo red agar method	
without	with	without	with	After24hrs	After48hrs
52% *B	64% AB	64% AB	80% A	28% C	36% C

*Different capital letters refer to significant variation among different diagnostic method.

Considering TCP as standard, data from TM and CRA methods were compared. Parameters of sensitivity and specificity were calculated as explained in the table No.5 below:

Table No.5 : Sensitivity and specificity of Tube method and Congo red agar method in compared with tissue culture plate method.

	T_M	CRA_M
sensitivity	84%	76%
specificity	100%	100%

Discussion:

Staphylococcus spp. utilizes the biofilm mode of growth to initiate and establish recalcitrant infections (17). The prevalence of bovine staphylococcus mastitis range from 7% to 40% of all dairy cattle and this infection is associated with bacterial biofilm (18). In the view of the large number of infections caused by biofilm-producing bacteria, a reliable method for their diagnosis is necessary. The present study examined biofilm formation in three different method. In first two method (tube method and tissue culture method), the study found that addition of glucose increase biofilm formation in staphylococci in both groups coagulase positive & negative, this is because of that the addition of large amounts of sugar to a medium induces a stress condition which, in turn, stimulates fermentation, thus increasing the production of polysaccharide intercellular adhesion and consequent biofilm production (19).

In agreement with that of (20). which was done on *Pseudomonas aeruginosa* and *klebsiella* spp., The results show no significant variation between presence or absence of glucose ,and this is probably due to small size of clinical isolates in both researches, while Tissue culture method succeeded to reach the level of significancy in variation between absence and presence of glucose this may be due to highly sensitivity level of this method in compared with other two methods, this result is

in agreement with that of kim (21) and T. Mathur *et.al.* how found that addition of glucose increase biofilm formation in staphylococcus isolates using relatively large size samples (152 isolates) diagnosed by tissue culture plate method . (22).

Congo red agar method show increasing number of positive cases by increasing time of incubation this result is in agreement with that of (9) and this is due to that not all staphylococcus spp. are heavy biofilm producer , some isolate are moderate and weak (23). , and longest time of incubation give largest chance to these isolate to produce the stain.The results show that TCP method is highly sensitive & specific method and can be recommended for biofilm identification, These observations are entirely in agreement with observations reported by the Adilson Oliveira et al (19) and T Mathur et al (22) and BOSE S. *et al.* (24) and (25)and that probably because this method depend on ELISA reader to analyze the positive & negative results while both other test depend on naked eyes.

References:

1. Bradley, A.J.; Leach K.A.; Breen, J.E.; Green, L .E. & Green, M.J. (2007). Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet Rec.*;160:253–257.

- 2.Oliveira, M. ; Bexiga, R.; Nunes, S.F.; Carneiro, C.; Cavaco, L.M.; Bernardo, F.& Vilela, C.L. (2006). Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Veterinary Microbiology* Vol. 118, Issues 1-2, 26 November, P. 133-140
- 3.Hensen, S.; Pavičić, M.; Lohuis, J.; de Hoog, J.& Poutrel, B.(2000). Location of *Staphylococcus aureus* within the experimentally infected bovine udder and the expression of capsular polysaccharide type 5 in situ. *J Dairy Sci.*;83:1966–1975.
4. Almeida, R.A. & Oliver, S. P. (2001). Interaction of coagulase-negative *Staphylococcus* species with bovine mammary epithelial cells. *J.Microb. Pathog.*;31:205–212.
5. Dhanawade, N. B. ; Kalorey, D. R. ; Srinivasan, R. ; Barbuddhe, S. B. & Kurkure, N. V. (2010). Detection of intercellular adhesion genes and biofilm production in *Staphylococcus aureus* isolated from bovine subclinical mastitis, *Veterinary research communications*; ISSN 1573-7446 , vol. 34, no.1, pp. 81-89
6. Nayak, N.; Satpathy, G.; Nag, H.L.; Venkatesh, P.; Ramakrishnan, S.; Nag, TC. & Prasad S. (2011). Slime production is essential for the adherence of *Staphylococcus epidermidis* in implant-related infections. *J Hosp Infect.* Feb;77(2):153-6.
7. Davies, D.G.; Parsek, M.R.; Pearson, J.P.; Iglewski, B.H.; Costerton, J.W.& Greenberg, E.P. (1998).The involvement of cell to cell signals in the development of bacterial biofilm. *J Science*; 280:295-8
8. Brown, M. and Simith, A. (2003). *Antimicrobial Agents and Biofilms. Medical Implications of Biofilms.* p. 36-38
9. Oliveira, M.; Nunes, S.F.; Carneiro,C.; Bexiga R.; Bernardo, F.; and Vilela, C.L. (2007). Time course of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *J.Veterinary Microbiology*, Volume 124, Issues 1-2, , P187-191.
10. Oliveira, M.; Bexiga, R.; Nunes, S.F.& Vilela, C.L. (2011). Invasive potential of biofilm-forming *Staphylococci* bovine subclinical mastitis isolates. *J Vet Sci.* March; 12(1): 95–97.
11. Cucarella, C.; Tormo, M. A.; Ubeda, C.; Trotonda, M. P.; Monzon, M.; Peris, C.; Amorena, B.; Lasa, I. & Penade's, J. R. (2004). Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus* Infection. *J. Immun.* 72, 2177–2185.

12. Christensen, G.D.; Simpson, W.A.; Bisno, A.L. & Beachey, E.H.(1982). Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *J. Infect. Immun.*37:318-26.
- 13.Christensen, G.D.; Simpson, W.A.; Younger, J.A.; Baddour, L.M.; Barrett, F.F.& Melton, D.M. (1985). Adherence of coagulase negative *Staphylococci* to plastic tissue cultures:a quantitative model for the adherence of staphylococci to medical devices. *J Clin. Microbiol.*;22:996-1006.
- 14.Freeman, D.J.; Falkiner, F.R. & Keane, C.T.(1989). New method for detecting slime production by coagulase negative staphylococci. *J Clin. Pathol.*42:872-4.
15. Jain, A.& Agarwal, A. (2009). Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci.?? *J Microbiol Methods* 76:88-92.
16. Steel, R. G. D. & Torrie, J. H. (1980). Principle and procedure of statistics. McGraw. Hall Book Companies,2nd ed. 195-233
17. Smith, K.; Gould, K.A.; Ramage, G.; Gemmell,C.G.; Hinds, J. &Lang, S. (2010). Influence of Tigecycline on Expression of Virulence Factors in Biofilm-Associated Cells of Methicillin-Resistant *Staphylococcus aureus*. *J Antimicrobial Agents and Chemotherapy*, January p. 380-387, Vol. 54, No. 1
- 18.Bezek, D.M.(1998). Genus identification and antibiotic susceptibility patterns of bacterial isolate from cow with acute mastitis in a practice population . *J Am Vet Med Assoc*; 212 : 404-406.
19. Oliveira, A.; de Lourdes, M. & Cunha, R.S. (2010) .Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci.
<http://www.biomedcentral.com/1756-0500/3/260>
20. Moteeb, S.H. (2008). Quantitative and qualitative assay of bacterial biofilm produced by *Pseudomonas aeruginosa* and *klebsiella* Spp. *J AlAnbar university for pure science* vol2 No.3
21. Kim, L. (2001). Riddl of biofilm resistance. *Antimicrobial agents and chemotherapy.*; 45(4): 999-1007
22. Mathur,T.; Singhal, S.; Khan,S.; Upadhyay,D.J.; Fatma, T.& Rattan, A . (2006). Detection of biofilm formation among the clinical isolates of staphylococci: An evaluation of three different screening methods. *Indian Journal of Medical Microbiology* vol. 24 Page : 25-29.
23. Eftekhari, F. and Dadaj, T. (2010). Biofilm formation and detection of *IcaAB* genes in clinical isolates of methicillin resistant

Staphylococcus aureus. Iranian Journal of basic medical sciences. Vol. 14, No.2, Mar-Apr, 132-136

24. Bose, S.; Khodke, M.; Basak, S.;& Mallick, S. K.(2009). Detection Of Biofilm Producing Staphylococci: Need Of The Hour. Journal of Clinical and Diagnostic Research, December vol.3Issue: 6 Page:1915 – 1920.

25. Knobloch, J, K.; Horstkotte, M. A. ; Rohde, H. and Mack, D. (2002). Evaluation of different detection methods of biofilm formation in Staphylococcus aureus. Medical Microbiology and Immunology Vol.191, Number 2, 101-106.