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In vitro analysis of Biofilm formation by Staphylococcus aureus and Escherichia coli isolated from Clinical samples

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Abstract

Background: Biofilms are group of microorganisms which are embedded within a self-produced matrix of extracellular polymeric substance which adhere to each other. They are found to be involved in a wide range of infections in the human body like urinary tract infections (UTIs) and surgical sites infections (SSIs). Objective: This study was conducted to analysis the biofilm forming *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) isolated from patients with UTIs and SSIs in some hospitals of Mukalla city, Hadhramout, Yemen during the period from December 2018 to May 2019. Materials and Methods: A total of 60 isolates of *S. aureus* and *E. coli* recovered from clinical samples of urine and wound swabs. *In vitro* biofilm formation was detected by tube method (TM), Congo red agar (CRA) method and tissue culture plate (TCP) method. Results: Biofilm formation of *S. aureus* and *E. coli* was observed in 48(80.0%), 32(53.3%) and 33(55.0%) of the isolates by TCP, TM and CRA methods respectively. Conclusion: *In vitro* methods showed that both *S. aureus* and *E. coli* isolated from clinical samples of UTIs and SSIs have high degree of biofilm forming ability, and the TCP method was a quantitative and reliable method for the detection of bacterial biofilm formation.

Key words: Biofilm formation, Tissue culture plate, Tube method, Congo red agar, Escherichia coli, Staphylococcus aureus.

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تحليل تكوين الغشاء الحيوي لجراثيم المكورات العنقودية الذهبية والايشريشية القولونية المعزولة من عينات سريرية في المختبر

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الملخص

الخلفية: الأغشية الحيوية هي مجموعة كائنات دقيقة مضمنة تلقائياً داخل قالب منتج خارج خلوي لمادة من البوليمر ملتصقة ببعضها البعض. يُعتبر وجود الأغشية الحيوية ذو علاقة واسعة بالعدوى في جسم الانسان مثل عدوى التهابات المسالك البولية وعدوى التهابات المواقع الجراحية. الهدف: أجريت هذه الدراسة لتحليل تكوين الغشاء الحيوي لجراثيم المكورات العنقودية الذهبية والايشريشية القولونية المعزولة من مرضى مصابين بالتهابات المسالك والتهابات الجروح في بعض مستشفيات مدينة المكلا، حضرموت، اليمن خلال الفترة من ديسمبر 2018م إلى مايو 2019م. المواد وطرق العمل: تم عزل 60 عزلة من جراثيم المكورات العنقودية الذهبية والايشريشية القولونية من عينات البول ومسحات الجروح السريرية. تم الكشف على تكوين الغشاء الحيوي في المختبر بطريقة الأنبوب، وطريقة أجار صبغة الكونغو الحمراء، وطريقة طبق الزرع النسيجي. النتائج: تم مشاهدة تكوين الغشاء الحيوي لجراثيم المكورات العنقودية الذهبية والايشريشية القولونية معاً في 48(80.0%)، و32(53.3%)، و33(55.0%) من العزلات بطريقة طبق الزرع النسيجي، وطريقة أجار صبغة الكونغو الحمراء، وطريقة الأنبوب على التوالي. الاستنتاج: أظهرت الطرق المختبرية امتلاك عزلات جراثيم المكورات العنقودية الذهبية والايشريشية القولونية قدرة عالية على تكوين الغشاء الحيوي. تعتبر طريقة طبق الزرع النسيجي طريقة كمية وموثوقة للكشف عن إنتاج الغشاء الحيوي الجرثومي.

الكلمات المفتاحية: تكوين الغشاء الحيوي، طبق الزرع النسيجي، طريقة الأنبوب، أجار صبغة الكونغو الحمراء، المكورات العنقودية الذهبية، الايشريشية القولونية

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Introduction

Staphylococcus aureus is an opportunistic pathogen implicated as the most comon agent of skin and soft tissue infections. It exists in the nasopharynx, skin, eye, intestine and urogenital tract as normal flora**[12]** *Escherichia coli* (abbreviated as *E. coli*) are bacteria found in the environment, foods, and intestines of people and animals. *E. coli* are a large and diverse group of bacteria. Although most strains of *E. coli* are harmless, others can make you sick. Some kinds of *E. coli* can cause cause urinary tract infections. *E. coli* strain, and spreads via the perineal, vaginal, and periurethral areas to the lower urinary tract (i.e., urethra and bladder) where they may establish colonization **[11].**

Bacterial biofilm defined as an organized bacterial community embedded in an extracellular polymeric matrix attached to biotic or abiotic surfaces **[10].** Biofilms exhibit an altered phenotype with respect to growth rate and gene transcription **[5].** Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing **[19].** Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are some factors which influence biofilm formation **[19].** The higher incidence of biofilm-associated infections is contributed the frequent use of artificial implants and medical devices nowadays **[8].** Both Gram-positive and Gram-negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Pseudomonas aeruginosa **[6].** Several phenotypic methods for biofilm detection are used, the most common are tissue culture plate (TCP) method **[23],** tube method (TM) **[2],** Congo red agar (CRA) method **[15],** bioluminescent assay **[4],** piezoelectric sensors **[1],** and microscopical examination methods such as light microscopy, scanning electron microscope (SEM), transmission electron microscope (TEM), and fluorescent microscopy **[16],** as well as genotypic techniques for biofilm detection such as polymerase chain reaction (PCR) **[9]**. Therefore, our study was aimed to analysis the *in vitro* biofilm forming ability by TM, CRA and TCP methods among *S. aureus* and *E. coli* isolated from patients with UTIs and SSIs in some hospitals of Mukalla city, Hadhramout, Yemen.

Materials and methods

Study design

A cross-sectional study was carried out in Mukalla city/Hadramout, Yemen from December 2018 to May 2019.

Collection of clinical samples

A total of 309 clinical samples were collected. A 200 wound swabs samples were collected from surgical site infections (SSIs) and 109 mid-stream urine samples were collected from urinary tract infections (UTIs).

Microbiological analysis of clinical samples

All samples were analyzed at the medical microbiology department at the national center for public health laboratories, Mukalla/Hadhramout by the standard methods for bacterial culture growth, then the isolates were identified by the observed for the colony appearance, Gram staining and subjected to further biochemical tests **[20].**

Biofilm detection methods

Qualitative assay for biofilm formation

Tube method (TM)

As described by Rewatkar and Wadher **[15]** and Osungunna and Onawunmi **[13],** this qualitative method for biofilm detection was performed as the following: a loopful of tested bacteria was inoculated in 10 ml of tryptone soya broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hours. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. The tubes were stained with crystal violet (0.1%), an excess stain washed with deionized water. Tubes were dried in inverted position. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as weak/none, moderate and high/strong.

Congo red agar (CRA) method

Triveni *et al.* **[22]** have described a simple qualitative method to detect biofilm production using CRA medium as the following: the CRA medium plates were inoculated with tested bacteria and incubated at 37oC for 24 hours aerobically. Black colonies on medium indicates positive test for strong biofilm production, grayish black to deep red indicates moderate biofilm producers and red colonies are considered as weak/non biofilm producers.

Quantitative tissue culture plate (TCP) assay for biofilm formation

Quantitative TCP method was performed as described by Hassan *et al.* **[7],** Ramachandran and Sangeetha **[14]** and Yadav et al. **[23].** Briefly, cultures of the isolates from fresh nutrient agar were inoculated in 10mL of trypticase soy broth with 1% glucose. After incubated for 24 hours at 37ºC, the cultures were diluted 1:100 with fresh medium. Individual wells of sterile 96 polystyrene microtiter plates were filled with 0.2ml aliquots of the diluted cultures. Negative control wells were maintained by adding broth without culture. After incubation for 24 hours at 37ºC, the wells were removed by gentle tapping and washed with 0.2mL phosphate buffer saline (pH 7.3) three times to remove free floating planktonic bacteria. Then the wells were dried for 1 hour and stained with crystal violet (0.1% w/v) and the excess stains were removed using deionized water and the plates were kept for drying. Quantitative analysis of biofilm production was performed by adding 150µl of 95% ethanol to destain each wells. After 30 min, optical density (OD) of stained adherent biofilm was obtained by using microtiter plate ELISA reader at wave length 630 nm. The experiment was performed in triplicate and repeated

three times. Optical density cut-off value (ODc) = average OD of negative control + 3x standard deviation (SD) of negative control. The bacterial species tested were classified into four categories as follows: OD ≤ ODc no biofilm producer; ODc < OD ≤ 2 x ODc weak biofilm producer; 2 x ODc < OD ≤ 4 x ODc moderate biofilm producer; 4 x ODc < OD strong biofilm producer.

Statistical analysis

Data statistical analysis were conducted using the software of Statistical Package for Social Sciences (SPSS) version 25. The association between different categories was measured and compared using Pearson Chi-square ($\chi 2$) test. The level of significance was set at *P-value* less than 0.05.

Ethical approval

Research ethical approval of this study was obtained from Hadhramout University, Faculty of Science. Written consent was obtained before commencing the study. Permission letter was obtained from the hospital's administrations.

Conflicts of interest

The authors declare that they have no conflict of interest.

Results and discussion

In the present study, we processed all isolates of *S. aureus* and *E. coli* recovered from wound swabs and midstream urine samples and screened the ability to form biofilm *in vitro* by three phenotypic methods TCP, TM and CRA because they can be performed in most laboratories' settings.

Positive results of biofilm produced by the qualitative TM was confirmed by visible thick film obtained inside the wall and the bottom of the tube indicating strong and intermediate biofilm production, other than indicating that not biofilm formed with color, figure (1).

Strong producers Moderate producers Weak/non producers

Figure (1). Biofilm production by tube method

TM detected biofilm formation in 32(53.3%) out of 60 isolates of *S. aureus* and *E. coli*. The highest biofilm production was found among *S. aureus* isolates 21(70%), followed by *E. coli* isolates 11(36.7%). Among *S. aureus* isolates, 12(40%) isolates were strong biofilm producers and 9(30%) of isolates were moderate biofilm producers. Non-biofilm producers isolates identified as 7(23.3%) of isolates were weak biofilm producers and others 2(6.7%) isolates were negative. Of *E. coli* isolates, 5(16.7%) isolates were strong biofilm producers and 6(20%) of isolates were moderate biofilm producers. Non-biofilm producers isolates identified as 15(50%) isolates were weak biofilm producers and others 4(13.3%) isolates were negative. There was significant statistical analysis of TM method for screening biofilm production (P=0.010) as given in table (1).

CRA method detected biofilm formation in 33(55%) out of 60 *S. aureus* and *E. coli* isolates. The highest biofilm production was found among *E. coli* isolates 23(76.7%), followed by *S. aureus* isolates 10(33.3%) with significant statistical analysis (P=0.001) as given in table (1). Of *E. coli*, 23(76.7%) of isolates gave black colour indicating the biofilm production, while 7(23.3%) isolates gave red colour colonies indicating non-biofilm production. Also, 10(33.3%) black colour colonies of *S. aureus* isolates were observed for the biofilm production, while 20(66.6%) isolates gave red colour colonies indicating non-biofilm production by CRA method, as shown in figure (2).

(A) Producer (B) Non-producer

Figure (2). Biofilm production by Congo red agar method

Quantitative TCP results of biofilm production are shown in figure (3). TCP method detected biofilm formation in 48(80%) out of 60 *S. aureus* and *E. coli* isolates. A 24(80%) of isolates were biofilm producers for each *S. aureus* and *E. coli*. Among *S. aureus* isolates, 18(60%) were strong biofilm producers, 6(20%) isolates were moderate biofilm

producers and 6(20%) isolates were non-biofilm producers. While *E. coli* isolates showed 15(50%) were strong biofilm producers, 9(30%) isolates were moderate biofilm producers. Non-biofilm producers isolates identified 1(3.3%) isolate was weak biofilm producer and other isolates 5(16.7%) were negative. There was insignificant statistical analysis of TCP method for screening biofilm production (*P*=1.000) as given in table (1).

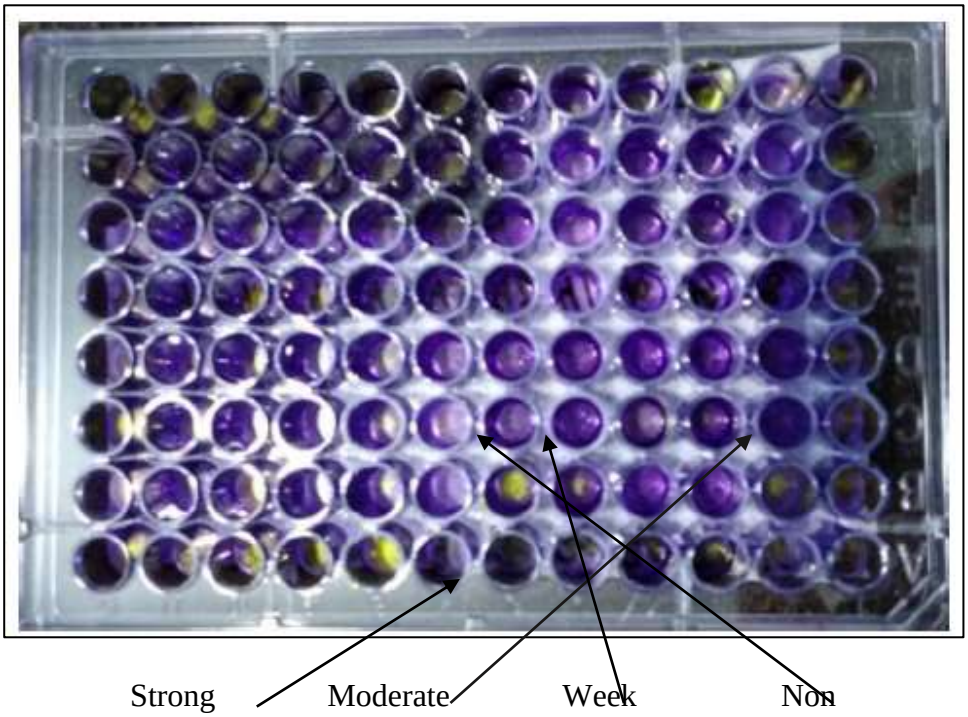

Figure (3). Detection of biofilm formation by tissue culture plate method

Table (1). Screening the biofilm formation of *S. aureus* and *E. coli* isolates by TCP, TM and CRA methods

Bacterial isolates	No. of isolates	TCP				TM				CRA	
		Producer		Non-producer		Producer		Non-producer		Producer	Non-producer
		S	M	W	N	S	M	W	N		
S. aureus	30	18	6	0	6	12	9	7	2	10	20
E. coli	30	15	9	1	5	5	6	15	4	23	7
Total(%)	60	48(80)		12(20)		32(53.3)		28(46.7)		33(55)	27(45)
χ^2 test value		0.00				6.696				11.380	
P-value		1.000				0.010*				0.001*	

**P-value* < 0.05 is considered statistically significant

Key: (TCP) Tissue culture plate, (TM) Tube method, (CRA) Congo red agar, (S) Strong, (M) Moderate, (W) Weak, (N) Negative

According to these results, similar researches revealed TCP method detected 81%, TM detected 59.5% and CRA detected 70.3% bacterial isolates biofilm producer **[3].** Other study found that TCP detected 64%, TM detected 44% and CRA detected 12% as bacterial biofilm producer **[18],** whereas another study showed that TCP detected 27%, TM detected 37.96% and CRA 40.88% as bacterial biofilm producers **[17].** A study revealed that 76% were bacterial biofilm producers detected by TCP method, 83% by TM and 63% by CRA method **[21**]. Other study reported biofilm producer identified by TCP method 22% and CRA method 78% [14]. Also, several studies showed similar results for the detection of biofilm production [6,7,8].

Conclusions

The study concluded that *in vitro* methods showed *S. aureus* and *E. coli* isolated from clinical samples of UTIs and SSIs have high degree of biofilm forming ability. The TCP method was a quantitative and reliable method for the detection of bacterial biofilm formation. TCP method can be recommended as a general screening method for detection of biofilm producing bacteria in the clinical laboratories.

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