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ORIGINAL ARTICLE OPEN ACCESS

Evaluation of Phenotypic Methods in the Clinical Isolates for Biofilm Detection of Staphylococcus aureus and Escherichia coli in Mukalla city, Hadhramout, Yemen

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ABSTRACT

Introduction: Biofilms represent a complex structure comprising prokaryotic cells, proteins, sugars, and DNA as major constituents. Bacteria can grow in biofilms and associated with human infections and considered to be highly resistant to antibiotics. There are various methods to detect biofilm production like tissue culture plate (TCP), tube method (TM), and Congo red agar method (CRA).

Objectives: This study aimed to compare three methods for the detection of biofilm formation in the clinical isolates *Staphylococcus aureus and Escherichia coli.*

Methods: A total of 60 clinical isolates of S. aureus and E. coli were subjected to biofilm detection methods by TM, CRA and TCP.

Results: Out of the total 60 clinical isolates of S. aureus and E. coli, TCP method detected 33 (55%) as strong, 15 (25%) as moderate and 12 (20%) as weak/non-biofilm producers. The sensitivity of TM and CRA was 56.3% and 60.4%, and the specificity was 58.3% and 66.7% respectively. In our validation of the diagnostic biofilm production tests, the TCP method was superior to TM and CRA methods for biofilm detection.

Conclusion: The study concluded that the TCP method is accurate and reliable method for the detection of biofilm formation in the clinical isolates S. aureus and E. coli compared to TM and CRA methods, and can be recommended as a general screening method for the detection of biofilm producing bacteria in clinical laboratories.

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

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Introduction

Biofilms defined as an organized bacterial community that characterized by cells are irreversibly attached to a substratum or to each other [1]. Bacterial biofilm embedded in an extracellular polymeric substance (EPS) attached to biotic or abiotic surfaces, they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription [2]. Infections with biofilms producing by microorganisms are one of the greatest challenges in the modern medical world [3], and they are found to be important in different types of infections and is now a widely accepted bacterial mode of growth [4]. Currently, about 80% of human infections are based on biofilms forming microorganisms [5].

Bacterial biofilm formation is an important virulence factor expressed by various types of bacterial pathogens [6]. Staphylococci species, E. coli and Pseudomonas aeruginosa are considered the most common etiological agents of medical devices infections [7]. These bacteria are responsible for much healthcare-acquired infection and associated with many medical conditions including dental plaque, indwelling medical devices, upper respiratory tract infections and urogenital infections [8, 9, 10].

Biofilm producing bacteria have certain advantages compared to planktonic bacteria that including; detection of quorum-sensing (QS), increased interspecific metabolic cooperation, increased tolerance to immune responses of the host, requiring the high concentrations of antibiotics and increased capability for bacterial conjugation [11]. Biofilm producing bacteria provide a protective degree of homeostasis and constancy in an environment changing [12]. Pathogenic bacteria producing biofilms often results in low sensitive antibiotics and chronic infections development. Therefore, bacterial biofilm formation is considered an important virulence factor [13].

Recently, many phenotypic methods for biofilm detection are used. Tube method (TM) [10], Congo red agar (CRA) method [6], tissue culture plate (TCP) method [14], bioluminescent assay [15], piezoelectric sensors [16], and microscopical examination methods such as light microscope, scanning electron microscope (SEM), transmission electron microscope (TEM), and fluorescent microscopy [17] are the most common, as well as genotypic techniques such as polymerase chain reaction (PCR) [18].

Biofilms formed by pathogenic bacteria are a serious challenge in clinical practice due to several reasons, among which resistance to physical eradications, decreased antibiotics susceptibility, high resistance to microbicidal agents, etc. Due to the high medical importance, analytical methods directed to accurate measurements of biofilm parameters are subjected to rapid development and improvement. In the current research, the clinical isolates of E. coli and S. aureus were screened by TM, CRA and TCP methods to determine their ability to produce biofilm.

MATERIALS AND METHODS

I. Bacterial species

Sixty clinical isolates of E. coli and S. aureus (30 isolates of each) were provided from the culture collection in the Medical Microbiology Department, at the National Center of Public Health Central Laboratories in Mukalla city, Hadhramout, Yemen and subjected to biofilm detection methods by TM, CRA, and TCP.

II. Biofilm detection by tube method (TM)

This qualitative method for biofilms detection was performed as described by Osungunna and Onawunmi [19]. In test tubes, 10 ml of tryptone soya broth with 1% glucose was inoculated with a loopful of the tested bacteria. For 24 hours, the tubes were incubated at 37°C. The tubes were decanted, rinsed with phosphate buffer saline (pH 7.3), and dried after the incubation period. After that, crystal violet (0.1%) was used to stain the tubes, and deionized water was used to remove any surplus stain. The tubes were dried while they were upside down.

When a film could be seen lining the test tube's wall and bottom, biofilm development was thought to be successful. High/strong, moderate, and weak/none biofilm formation were rated according to their strength.

III. Biofilm detection by Congo red agar (CRA) method

The qualitative CRA approach was used in accordance with Triveni et al. [7]. The studied bacteria were put into the CRA media plates, which were subsequently incubated aerobically for 24 hours at 37 °C. On CRA media, black colonies indicate a positive test for a high biofilm production, grayish black to deep red colonies suggest a moderate biofilm development, and red colonies are thought to be weak or non-biofilm producing.

IV. Biofilm detection by tissue culture plate (TCP) method

The quantitative TCP approach was used in accordance with Yadav et al. [14]. In brief, bacterial isolates from freshly prepared nutritional agar were inoculated in 10 mL of trypticase soy broth with 1% glucose, cultured for 24 hours at 37 °C, and then diluted 1:100 with fresh medium. 0.2 ml aliquots of the diluted cultures were placed in each of the 96 sterile polystyrene microtiter plates. In order to keep negative control wells active, broth was added without any culture. Following a 24-hour incubation period at 37°C, the wells were gently removed, tapped three times, and then the free-floating planktonic bacteria were eliminated using 0.2 mL of phosphate buffer saline (pH 7.3).

The wells were allowed to dry for an hour before being stained with crystal violet (0.1% w/v) and having any excess stain is removed with deionized water. The plates were then let to dry. The production of quantitative biofilms was carried out by destaining each well with 150 l of 95% ethanol. Using the ELISA reader of the microtiter plate at wave length 630 nm, the optical density (OD) of the adhering biofilm was determined after 30 min. TCP experiment were performed in triplicate. The optical density cut-off value (ODc) is determined as the average optical density (OD) of the negative control plus three times the negative control's standard deviation (SD). The tested bacterial species were classified into four categories as follows; no biofilm producer, weak biofilm producer, moderate biofilm producer and strong biofilm producer, as presented table (1).

Table 1. Categories of biofilm formation by TCP method

Optical densities values	Adherence	Biofilm formation
OD ≤ ODc	Non	No biofilm producer
ODc < OD ≤ 2 x ODc	Weak	Weak biofilm producer
2 x ODc < OD ≤ 4 x ODc	Moderate	Moderate biofilm producer
4 x ODc < OD	Strong	Strong biofilm producer

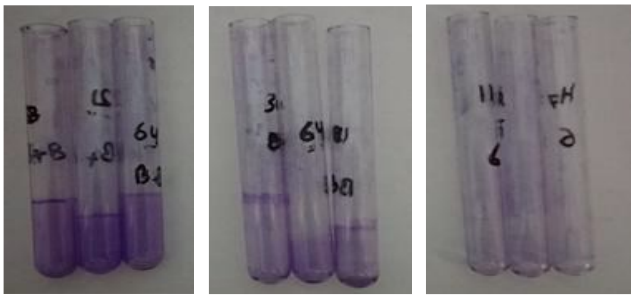
V. Evaluation of diagnostic biofilm production tests

For evaluation of diagnostic biofilm production tests, parameters like sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the TM and CRA methods were calculated and compared with the gold standard TCP method.

RESULTS

I. Biofilm detection by the tube method (TM)

For the clinical isolates S. aureus and E. coli, TM detected 17 (28%) as strong, 15 (25%) as moderate and 28 (47%) as weak/non-biofilm producers, figure (1). Among S. aureus isolates, 12 (20%) isolates were strong biofilm producers and 9 (15%) isolates were moderate biofilm producers. Weak/non-biofilm producer isolates identified as 9 (15%). Among E. coli isolates, 5 (8.3%) isolates were strong biofilm producer and 6 (10%) of isolates were moderate biofilm producers. Weak/non-biofilm producer bacterial isolates identified as 19 (31.7%), table (2).



Strong producers Moderate producers Weak/non producers

Figure 1: Biofilm production by tube method

II. Biofilm detection by Congo red agar (CRA) method

Among the clinical isolates E. coli and S. aureus, CRA method detected 33 (55%) as biofilm producers and 27 (45%) as non-biofilm producers, figure (2). Among the S. aureus isolates, 10 (16.7%) isolates were biofilm producers and 20 (33.3%) the isolates were non biofilm producers. Among E. coli isolates, 23 (38.3%) bacterial isolates were strong biofilm producers and 7 (11.7%) of isolates were non-biofilm producers, as shown in table (2).

Producer Non-producer

Figure 2. Biofilm production by Congo red agar method

III. Biofilm detection by the tissue culture plate (TCP) method

Of the total clinical isolates of S. aureus and E. coli, TCP method detected 33(55%) as strong, 15(25%) as moderate and 12(20%) as weak/non-biofilm producers, figure (3). Of S. aureus isolates, 18(30%) isolates were strong biofilm producers and 6(10%) of the isolates were moderate biofilm producers. Weak/non biofilm producer isolates identified as 6(10%). Among E. coli isolates, 15(25%) isolates were strong biofilm producers and 9(15%) of isolates were moderate biofilm producers. Weak/non-biofilm producers isolates identified as 6(10%), as shown in table (2).

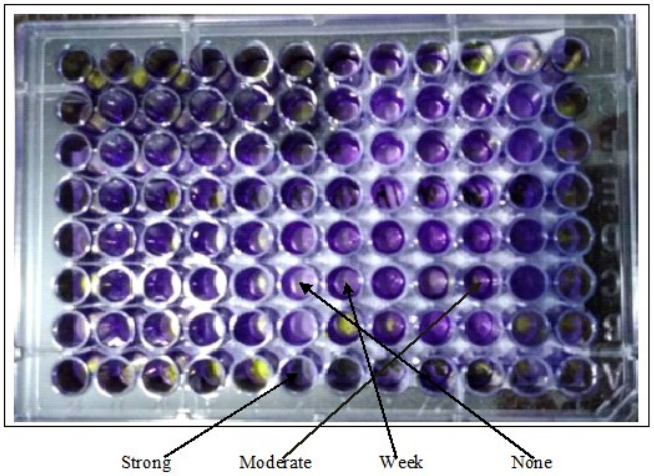


Figure 3. Detection of biofilm formation by tissue culture plate method

Table 2. Biofilm formation of S. aureus and E. coli isolates by TCP, TM, and CRA methods

Bacterial isolates No. (%)			S. aureus	E. coli	Total
TCP	Producer	S	18(30.0)	15(25.0)	33(55.0)
		M	6(10.0)	9(15.0)	33(55.0)
	Non-producer	W/N	6(10.0)	6(10.0)	12(20.0)
TM	Producer	S	12(20.0)	5(8.3)	17(28.3)
		M	9(15.0)	6(10.0)	15(25.0)
	Non-producer	W/N	9(15.0)	19(31.7)	28(46.7)
CRA	Producer		10(16.7)	23(38.3)	33(55.0)
	Non-producer		20(33.3)	7(11.7)	27(45.0)

IV. Evaluation of diagnostic biofilm production tests

In the validation of diagnostic biofilm production tests when compared with the TCP method, out of the 60 tested isolates, the biofilm producers showed in 48 isolates, and 12 isolates were non-biofilm producers according to the results of TCP. Twenty-seven biofilm producers and 7 non-biofilm producers were actually identified by the TM. The

CRA approach also successfully identified 8 non-biofilm producers in addition to 29 biofilm producers, as shown in tables (3) and (4).

Table 3. Biofilm formation of S. aureus and E. coli isolates by TCP, TM, and CRA methods.

Method		TCP method		
Tube method		Positive	Negative	Total
	Positive	27	5	32
	Negative	21	7	28
Total		48	12	60

Table 4. Comparison between TCP and CRA method for biofilm detection.

Method		TCP method		
Congo red gar method		Positive	Negative	Total
	Positive	29	4	33
	Negative	19	8	27
Total		48	12	60

The characteristics of TM and CRA when compared with TCP method for biofilm detection were reported as the following: sensitivity, specificity, PPV, NPV and accuracy of TM were 56.3%, 58.3%, 84.4%, 25% and 56.7% respectively, and for CRA method, sensitivity, specificity, PPV, NPV and accuracy were 60.4%, 66.7%, 87.9%, 29.6% and 61.7%, respectively as presented in table (5).

Table 5. Assessment parameters of the tube method and Congo red agar method for biofilm detection using the tissue culture plate method as gold standard.

Screening method	Sensitivity	Specificity	PPV	NPV	Accuracy
TM method	56.3%	58.3%	84.4%	25%	56.7%
CRA method	60.4%	66.7%	87.9%	29.6%	61.7%

DISCUSSION

The current study demonstrated superior detection sensitivity and specificity for biofilm production by S. aureus and E. coli by TCP as compared with TM and CRA method. Sensitivity and specificity of TM were 56.3% and 58.3% respectively in this study, and for CRA method, sensitivity and specificity were 60.4% and 66.7% respectively. Other studies recorded slightly better sensitivity and specificity results of 78.3% and 79.9% respectively [20]. Other studies demonstrated better sensitivity 80.6% and 91.1% and specificity 89.68% and 100% for TM respectively, while CRA specificity was 93.01% and 100% respectively [7, 21]. Similarly, CRA sensitivity reported as 77.7% [21], while lower sensitivity 33.3% and 18% were reported in other studies, respectively [7]. The variations in these results reported sensitivity and specificity of TM and CRA can be explained by subjective errors during interpretation of these qualitative phenotypic tests, and the variations of media used can affect their results [22].

However, we found that PPV, NPV and accuracy were 84.4%, 25.0% and 56.7% respectively for TM and 87.9%, 29.6% and 61.7% respectively for CRA. Other studies reported that the PPV for TM was 79.3% and 64.0% respectively, while for CRA, the PPV was 60.1% [7] and 86% [24]. Another study showed a PPV higher than our study 100% [21]. Also, another study revealed that NPV was 20% and 33.4% for TM and CRA methods [23] similar the present study, while other study showed higher NPV results than our study 90.40 % and 81.59% for TM and CRA methods, respectively [21]. Another study revealed that the precision was 86.7% and 78.82 for TM and CRA respectively [7].

In this study, the TCP method was considered to be the gold standard phenotypic test, and it was the most specific test for biofilm production based on the available literature and the availability of TCP data compared with TM and CRA methods. Additionally, it was a simple test to carry out in the

lab and it provided both qualitative and quantitative evidence of biofilm creation. Additionally, an ELISA reader interprets the TCP data, eliminating the subjectivity associated with other phenotypic assays.

Overall, our study showed that TM and CRA correlated with TCP method in regarding some results biofilm detection. This could be accredited to the subjective evaluate used in TM and CRA methods in comparison to the TCP method. Therefore, if we compared between three methods TCP, TM and CRA for biofilm production, this study revealed that TCP method was the highest biofilm production than other two methods, the CRA found higher in biofilm production than TM. In accordance, some previous studies showed that TCP was most reliable and easy method for biofilm detection as compared to other methods such as TM and CRA, and it can be used as a general screening method for detection of bacterial biofilm producing [20, 24, 25]. Other studies showed that CRA is better for biofilm detection than TM [26, 27], while other studies showed otherwise [14].

TM and CRA methods are qualitative and reliable methods used as a general screening of bacterial biofilm production in the laboratories [6]. In contrast, statistical analysis the biofilm formation indicated that TCP method was the most sensitive and specific method for screening the bacterial biofilm production [7]. The variability in biofilm detection methods has been observed by various authors, which positively reflects the different used protocols in many hospitals and differences in the geographical locations from which the bacterial isolates have been obtained.

This study has several limitations; lacking of reference bacterial strains used as positive and negative controls for biofilm production, as well as lacking confirmation the biofilm production using molecular techniques because high coast in Yemen, and we have not studied the bacterial virulence factors or antibiotic resistance patterns associated with biofilm production.

CONCLUSION

In terms of detecting biofilm, the CRA approach outperformed TM and showed improved results in terms of sensitivity and specificity. The TCP approach was the best quantitative, accurate, and reliable phenotypic biofilm detection method among the three examined methods, according to S. aureus and E. coli. The TCP can be recommended as a general screening method for the detection of biofilm producing bacteria in the clinical laboratories when compared to TM and CRA methods.

CONFLICT OF INTEREST

The authors declare that no conflict of interest associated with this work.

REFERENCES

1. Donlan RM, Costerton W. Biofilms: Survival mechanisms of clinically relevant Microorganisms. Clinical Microbiology Review. 2002; 15(2):167-93. DOI: 10.1128/CMR.15.2.167–193.2002.
2. Macià MD, Rojo-Molinero E, Oliver A. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clinical Microbiology and Infection. 2014; 20(10): 981-990. 10.1111/1469-0691.12651.
3. Moser C, Pedersen HT, Lerche CJ, et al. Biofilms and host response – helpful or harmful. Acta pathologica microbiologica et immunologica scandinavica. 2017; 125(4): 320-338. DOI:10.1111/apm.12674
4. Paharik AE, Horswill AR. The staphylococcal biofilm: adhesins, regulation, and host response. Microbiology Spectrum. 2016; 4(2): 1-27. doi:10.1128/microbiolspec.VMBF-0022-2015.
5. Shrestha B, Shrestha B, Poudel A, et al. In-Vitro Biofilm Detection among Uropathogens and Their Antibiogram Profile. Tribhuvan University Journal of Microbiology. 2018; 5(1): 57-62. DOI: https://doi.org/10.3126/tujm.v5i0.22313.
6. Rewatkar AR, Wadher BJ. Staphylococcus aureus and Pseudomonas aeruginosa- Biofilm formation Methods. Journal of Pharmacy and Biological Sciences. 2013; 8(5): 36-40. www.iosrjournals.org.
7. Triveni AG, Kumar MS, Manjunath C, et al. Biofilm formation by clinically isolated Staphylococcus aureus from India. The Journal of Infection in Developing Countries. 2018; 12(12): 1062-1066. doi:10.3855/jidc.10671.

8. Donlan RM. Biofilms and device-associated infections. Emerging Infectious Diseases. 2001; 7(2): 277-81. doi: 10.3201/eid0702.010226
9. Jamal M, Ahmad W, Andleeb S, et al. Bacterial biofilm and associated infections. Journal of the Chinese Medical Association. 2018; 81: 7-11. https://doi.org/10.1016/j.jcma.2017.07.012.
10. Bhardwaj A, Kharkwal AC, Singh VA. A Comparative Appraisal of Detection of Biofilm Production Caused by Uropathogenic Escherichia coli in Tropical Catheterized Patients by Three Different Methods. Asian Journal of Pharmaceutics. 2018; 12(4): 1445-1450.
11. Surgersa L, Boydc A, Girarda P, et al. Biofilm formation by ESBL-producing strains of Escherichia coli and Klebsiella pneumonia. International Journal of Medical Microbiology. 2019; 309: 13-18. DOI:10.1016/j.ijmm.2018.10.008.
12. Torlaka E, Korkutb ET, Uncua A, Sener Y. Biofilm formation by Staphylococcus aureus isolates from a dental clinic in Konya, Turkey. Journal of Infection and Public Health. 2017; 10: 809-813. https://doi.org/10.1016/j.jiph.2017.01.004
13. Landini P, Antoniani D, Burgess JG, Nijland R. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. Applied Microbiology and Biotechnology. 2010; 86(3): 813–823. DOI:10.1007/s00253-010-2468-8.
14. Yadav M, Khumanthem SD, Kshetrimayum MD, Damrolien S. Biofilm production and its correlation with antibiogram among clinical isolates of uropathogenic Escherichia coli. International Journal of Advances in Medicine. 2018; 5(3): 638-643. DOI: http://dx.doi.org/10.18203/2349-3933.ijam20182116.
15. Donlan RM, Murga R, Bell M, et al. Protocol for detection of biofilms on needleless connectors attached to central venous catheters. Journal of Clinical Microbiology. 2001; 39: 750-3. DOI:10.1128/JCM.39.2.750-753.2001
16. Aparna MS, Yadav S. Biofilms: microbes and disease. The Brazilian Journal of Infectious Diseases. 2008; 12(6): 526-30. DOI:10.1590/S1413-86702008000600016.
17. Roy R, Tiwari M, Donelli G, Donelli V. Strategies for combating bacterial biofilms: A focus on anti-biofilm agents and their mechanisms of action. Virulence. 2018; 9(1): 522-554. doi: 10.1080/21505594.2017.1313372.
18. Kırmusaoğlu S. The Methods for Detection of Biofilm and Screening Antibiofilm Activity of Agents. In; Antimicrobials, Antibiotic Resistance, Antibiofilm Strategies and Activity Methods. 2019. DOI: http://dx.doi.org/10.5772/intechopen.84411.
19. Osungunna MO, Onawunmi GO. Antibiotic resistance profiles of biofilm-forming bacteria associated with urine and urinary catheters in a tertiary hospital in Ile-Ife, Nigeria. Southern African Journal of Infectious Diseases. 2018; 33(3): 80–85. https://doi.org/10.1080/23120053.2018.1442192.
20. Nosrati N, Jahromy SH, Karizi SZ. Comparison of Tissue Culture Plate, Congo red Agar and Tube Methods for Evaluation of Biofilm Formation among Uropathogenic E. coli Isolates. Iranian Journal of Medical Microbiology. 2017; 11(3): 49-58. Corpus ID: 55811462.
21. Ramachandran R, Sangeetha D. Phenotypic evaluation of biofilm formation in human pathogenic bacterial isolates from different clinical specimens. European Journal of Biomedical and Pharmaceutical Sciences. 2017; 4(10): 625-632.
22. Sultan AM, Nabiel Y. Tube method and Congo red agar versus Tissue culture plate method for detection of biofilm production by uropathogens isolated from midstream urine: which one could be better? African journal of clinical and experimental microbiology. 2019; 20(1): 60-66. https://dx.doi.org/10.4314/ajcem.v20i1.9.
23. Puja G, Pratima G, Garima M, et al. Detection of biofilm production in blood culture isolates of staphylococci. International Journal of Medical Research & Health Sciences. 2015; 4(1): 22-28. DOI:10.5958/2319-5886.2015.00004.1.
24. Hassan A, Usman J, Kaleem F, et al. Evaluation of different detection methods of biofilm formation in the clinical isolates. Brazilian Journal of Infectious Diseases. 2011; 15(4): 305-311. DOI:10.1016/S1413-8670(11)70197-0.
25. Deka N. Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the detection of biofilm formation by Coagulase Negative Staphylococcus isolated from Non-clinical Isolates. International journal of Current Microbiology and Applied Sciences. 2014; 3(10): 810-815.
26. Deotale VS, Attal R, Joshi S, Bankar N. Correlation between biofilm formation and highly drug resistant uropathogens (HDRU). International Journal of Current Research and Review. 2015; 7(2): 61-65.
27. Ruchi T, Sujata B, Anuradha D. Comparison of Phenotypic Methods for the Detection of Biofilm Production in Uro-Pathogens in a Tertiary Care Hospital in India. International Journal of Current Microbiology and Applied Sciences. 2015; 4(9): 840-849.