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ORIGINAL ARTICLE

Comparison of two *invitro* phenotypic methods (Tissue Culture plate and Congo Red Agar) for Detection of Biofilm Formation by Enterococci

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ABSTRACT

Background, *Enterococci are Gram-positive bacteria that cause serious nosocomial infections. Bacteria tend to live in a community-like assembly called biofilm. Biofilm production aids bacterial virulence through numerous pathogenic mechanisms.* ***Objective***, *our aim was to Assess the ability of Enterococci to form a biofilm and to compare two in vitro phenotypic methods for detection of Enterococcal biofilm formation.* ***Methodology***, *we used 50 identified enterococcal isolates from clinical specimens to detect biofilm formation by tissue culture plate method (TCP) and Congo red agar method (CRA) then we compared the results of both methods.* ***Results***, *we found that 64% of the isolates were able to form biofilm by TCP method while 38% were positive by CRA method. Congo red agar failed to detect 50% of the positive isolates detected by tissue culture plate method with a sensitivity (40.62%) and a specificity (66.67%).* ***Conclusion***, *we concluded from our study that TCP is the most reliable method to detect biofilm forming bacteria when compared with CRA method.*

INTRODUCTION

Enterococci are Gram-positive bacteria that cause severe nosocomial infections such as blood stream, urinary tract infections and endocarditis[1]. Enterococci are known to form biofilms.

Bacteria tend to live in a community-like assembly called biofilm. Development of bacterial biofilms occurs in a dynamic process that includes attachment of the bacteria to a particular surface, irreversible binding and formation of a hydrated matrix of polysaccharides and protein[2,3]. Surfaces that favor biofilm development include inert surfaces as medical devices and dead tissues as dead bone fragments[2,4]. Antibodies are generated in response to the antigens released by the bacteria located in the biofilm. However, these antibodies are unable to kill the bacteria embedded within the biofilm even among people with excellent immune responses[2,5].

Biofilm production aids bacterial virulence through numerous pathogenic mechanisms as it facilitates attachment to solid surfaces, evasion of phagocytosis and gene exchange between the biofilm´s members generating more virulent strains. Moreover, biofilms can protect bacteria from antimicrobial agents resulting in resistant infections that carry a great clinical significance[2,6]. The mechanisms by which biofilms escape the effects of antimicrobial agents include: inability of the agent to reach the bacteria present at the deep part of the biofilm, the slowly growing bacteria in the biofilm shows decrease susceptibility to the agents, and some bacteria exist in a programmed protected phenotype that is generated after surface attachment[7].

With the appearance of biofilm associated infections, various laboratory methods for detection of biofilms were developed. Phenotypic detection of biofilm formation can be conducted by various techniques as TCP and CRA[8].

The aim of the present study is to evaluate two *in vitro* phenotypic methods (TCPM and CRA) that can be applied for biofilm detection in laboratory settings.

METHODOLOGY

Population of study:

This study involved 50 identified enterococcal isolates obtained from different clinical specimens from the clinical laboratories, Faculty of Medicine, Cairo University.

Isolates identification

- The isolates were cultured on blood agar and bile esculin agar plates and incubated at 37°C for 24‑48h aerobically.
- The isolated organisms were identified by colony characters, Gram staining and the conventional biochemical tests[9].

Phenotypic detection of biofilm-forming isolates by TCP method[10,11].

- Isolates from fresh blood agar plates were inoculated in brain heart infusion broth containing 2% sucrose (BHISuc) and incubated overnight at 37 ºC aerobically.
- On the next day the bacterial suspension were diluted 1:100 with fresh BHI broth.
- Ninety-six wells flat bottom tissue culture single plate ***(Tarson, Kolkata, India)*** was used, fifty wells were filled with 0.2 ml aliquots of the bacterial suspension, two wells containing sterile broth were used as sterility control and one well containing previously known biofilm forming bacteria *Enterococcus fecalis* ATCC 29212 was used as positive control, then the tissue culture plate was incubated for 24 hours at 37°C.
- After incubation the contents of all wells were gently removed through tapping the plates and the wells were washed at least four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) so as to remove free-floating 'planktonic' bacteria.
- Biofilms formed by adherence of organisms in the plate were fixed with sodium acetate (2%) and stained with crystal violet. Excess stain was rinsed off through washing with deionized water then the plate was left to dry.
- Optical density (OD) of stained attached bacteria was determined with a micro ELISA auto reader ***(model 680, Bio rad, California)*** at wavelength of 570 nm (OD570 nm). The OD values were considered as an index of bacterial adherence to the surface and formation of biofilms (table 1).

Table 1: Interpretation of results of Tissue Culture Plate method

Optical Density Value	Biofilm Formation
<0.120 nm	Non/Weak-biofilm producer
0.120-0.240 nm	Moderate biofilm producer
>0.240 nm	Strong biofilm producer

CRA Method:

- CRA is a specially prepared medium composed of BHI broth (37 g/l) supplemented with sucrose (5 g/l), agar No 1 (10 g/l) and Congo red dye (0.8 g/l).
- Isolates from fresh blood agar plates were inoculated onto CRA plates and incubated at 37˚ C for 24 to 48 hours aerobically. Black colonies that were crystalline dry in consistency denoted biofilm production[12,13].

Statistical analysis

- Using clinical sample size calculator for analytic study; with 0.05 alpha error and power of the study 0.80, CI of 95%, According to literature Exopolysaccharide producers exhibited antibiotic resistance to an average of 7.28 (95% IC 1⁄4 6.50, 8.06) of the 16 antibiotics, while CRA-negative strains were resistant to an average of 5.88 (95% IC 1⁄4 5.32–6.44); sample size calculated to compare between the 2 phenotypic methods was 50 isolates.

RESULTS

Our present study was carried out on 50 previously identified enterococcal isolates retrieved from several clinical specimens at Kasr Al-Ainy school of medicine.

Detection of biofilm production by TCP method:

According to the criteria of Stepanovic et al.[14], it was demonstrated that out of the 50 enterococcal isolates that were screened for biofilm-formation by TCP method. Eighteen strains (36%) were non-biofilm forming, and 32 strains (64%) were biofilm forming.

Detection of biofilm production by CRA:

Cultivation of enterococcal isolates on CRA revealed that 19/50 strains (38%) showed positive growth indicating biofilm production.

Correlation between TCP and CRA results:

The value of Kappa (statistical measurement of agreement) indicates no significant correlation between TCP method and CRA results (p □ 0.001). Interestingly, CRA failed to detect 50% of the positive isolates detected by TCP method with sensitivity 40.62% and specificity 66.67%. the result is shown in table 2.

Table 2: Correlation between tissue culture plate and Congo red agar results

		Congo red agar results				
		Positive		Negative		P value
		Count	percent	Count	percent	
Biofilm by tissue culture plate method	Positive	13	68.4%	19	61.3%	0.610
	Negative	6	31.6%	12	38.7%	
	Total	19	100%	31	100%	

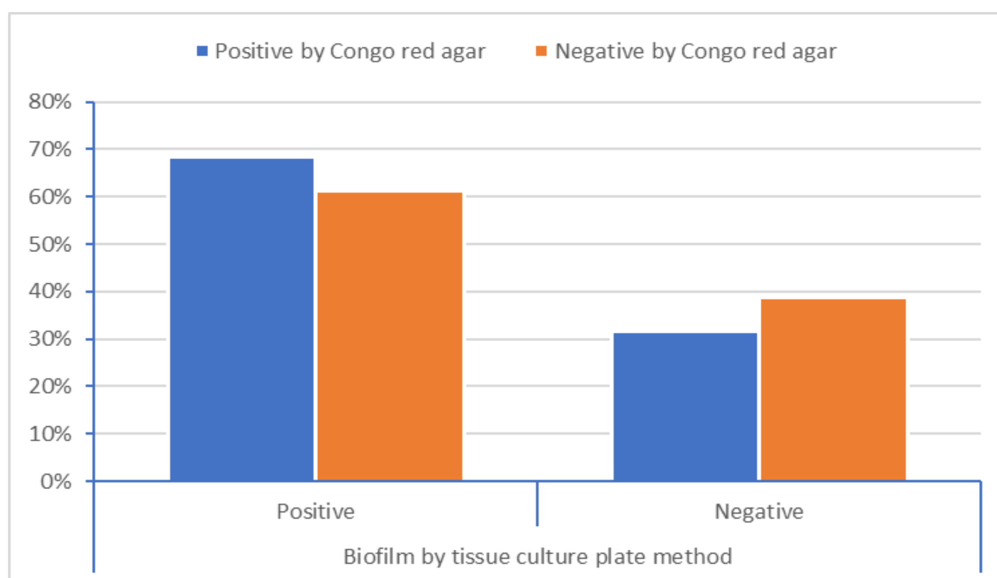

Fig. 1: Correlation between tissue culture plate and Congo red agar results

DISCUSSION

Biofilm producing bacteria are a major cause of many infections and they are known to be difficult to eradicate as the organisms involved in biofilm production are highly resistant to antimicrobial substances. The antibiotic concentration required to kill biofilm forming bacteria is higher than that required to inhibit planktonic cells.[8]

There are different methods that can be used for screening the biofilm formation. In our study, 50 previously identified enterococcal isolates retrieved from many clinical samples were tested for biofilm production using TCP and CRA methods. TCP method considered the gold standard in our study and was compared with the CRA method.

In the TCP method, we found that 32 isolates (64%) were biofilm producers, while 18 isolates (36%) were non-producers. Approximately similar rates of biofilm production were reported by Hassan et al.,[8] who found biofilm producing organisms were 70 isolates (64.7%), and non or weak biofilm producers were 40 (36.3%). Another data from India also showed that out of 152 isolates tested, the number of biofilm producers identified by TCP method were 53.9 %, and non-biofilm producers were 46%.[12] On the other hand, Sultan and Nabiel[10] tested total number of 145 isolates for biofilm production, the TCP method detected biofilm production in 43 isolates (29.7%). Similarly, Ruchi et al.,[6] detected biofilm production in 27% of the isolates by TCP method.

In the CRA method, we reported that isolates showing biofilm formation were 19 strains (38%). Sultan and Nabiel[10] tested biofilm formation in 63 isolates and perceived biofilm production in (43.4%) of them by CRA method. Similar pattern was also reported by Ruzicka et al.[15] who noted that out of 147 isolates of *S. epidermidis* CRA detected biofilm formation in 64 (43.5%) isolates and by Ruchi et al.,[6] who detected biofilm production in 40.8% by CRA method. in contrast, Hassan et al.[8] found that biofilm producing bacteria were 11(10%) and 99 (90%) could not produce biofilms by CRA method.

Another different studies by Turkyilmaz and his colleagues[16] who studied biofilm production in Staphylococcal species and detected biofilm in 61.1% by CRA method. El. Naghy et al.,[17] reported that total biofilm production was (73.7 %) by CRA method which is higher compared to our study. This difference may be due to that they worked on different number & different types of strains.

In the present work we reported no significant correlation between tissue culture plate method and Congo red agar results (p ⍰ 0.001). Congo red agar failed to detect 50% of the positive isolates detected by tissue culture plate method with a sensitivity 40.62% and specificity 66.67%.

Sultan and Nabiel[10] demonstrated that CRA has a sensitivity of 88.4% and specificity of 75.5%. Ira et al.,[18] reported a CRA specificity of 77%. Studies conducted by Ruchi et al.,[6] Hassan et al.,[8] and Panda et al.,[3] demonstrated higher specificity results for the CRA (81%, 92% and 93.9% respectively). Similarly, the CRA sensitivity reported by Ruchi and his colleagues [6] was 94.5% that was better than our results.[6] These variations in the reported sensitivity and specificity of CRA can be explained by the subjective errors during interpretation of these phenotypic qualitative tests.

Moreover, inter-batch variation of the used media can affect their results.

Knobloch et al.[19] did not recommend the CRA method for detection of biofilm in their study because out of 128 isolates of *S. aureus*, CRA detected only 3.8% of biofilm producing isolates in comparison with TCP which detected 57.1% of them.

On the other hand, El. Naghy et al.[17] found a significant correlation between TCP and CRA method (P value = 0.006). Khalil et al[20] found the two techniques are closely similar in detection of biofilm production by *Enterococcus* species, although TCP method seems to be more precise and dependable.

CONCLUSION

We can conclude from our present study that TCP seems to be the most reliable method to detect biofilm formation by microorganisms when compared to CRA methods.

Ethical committee approval
Faculty of medicine, Cairo university ethical committee approval was obtained at 11th November 2023

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Author contribution:
Mera Mohamed Galal Anan: corresponding author, writing the manuscript, participated in writing the methodology and results parts, working on the practical part.
Abeer Khaled Abu-El-Azayem: participating in the practical part.
Sabrin Mohamed Mohamed Abdelhamed Elkashef: writing discussion part, participate in writing the methodology, working on the practical part.

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