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Basic Science

Detection of Biofilm Producing Staphylococci among Different Clinical Isolates and Relation to Methicillin Susceptibility

Rania M. Abdel Halim*, Nevine N. Kassem, Basma S. Mahmoud

Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

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Keywords: Biofilm; Staphylococci; Methicillin; TCP; TM; CRA

***Correspondence:** Rania M Abdel Halim. Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt. E-mail: rona169@yahoo.com

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Abstract

AIMS: To evaluate three in vitro phenotypic methods; tissue culture plate, tube method, and Congo red agar for detection of biofilm formation in staphylococci and assess the relation of biofilm formation with methicillin resistance and anti-microbial resistance.

METHODS: The study included 150 staphylococcal isolates. Biofilm detection in staphylococci was performed using tissue culture plate, tube method, and Congo red agar.

RESULTS: Tissue culture plate, tube method, and Congo red agar detected 74%, 42.7%, and 1.3% biofilm producing staphylococci respectively. *S. aureus* isolates were more common biofilm producers (53.2%) than CONS (46.8%). Biofilm production in CONS species was highest in *S. hemolyticus* (57.7%). Tube method was 51.4% sensitive, 82.1% specific. As for Congo red agar, sensitivity was very low (0.9%), but specificity was 97.4%. Biofilm producers were mostly; isolated from blood specimens (82.6%) and detected in methicillin-resistant strains 96/111 (86.5%). They were resistant to most antibiotics except vancomycin and linezolid.

CONCLUSIONS: Tissue culture plate is a more quantitative and reliable method for detection of biofilm producing staphylococci compared to tube method and Congo red agar. Hence, it can still be used as a screening method for biofilm detection. Vancomycin and Linezolid are the most sensitive antibiotics among biofilm producing staphylococci.

Introduction

Staphylococcus aureus is a virulent organism that is resistant to most of the conventionally available antibiotics. This is attributed to the fact that they are capable of biofilms formation [1]. Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide, which facilitate the adherence of microorganism [2]. The interior of the bacterial biofilms presents greater resistance to the opsonisation by antibodies and phagocytosis. This explains the chronic character of these infections such as endocarditis, osteomyelitis and especially those infections associated with implanted medical devices that are difficult to be treated [1].

Coagulase-negative staphylococci especially *S. epidermidis* is the most frequent cause of hospital-acquired infections. Most *S. epidermidis* infections are subacute or chronic and occur mainly in immunocompromised individuals or patients with indwelling medical devices. Biofilm formation on the surface of indwelling devices is often involved in the pathogenesis [3]. Biofilms can resist antibiotic concentration 10-10,000 folds higher than those required to inhibit the growth of free-floating Staphylococci. Biofilm producing staphylococci have also been isolated from various clinical samples like blood, urine, pus, skin surface etc. The differentiation of staphylococci concerning its biofilm phenotype might help in their diagnosis and thereby, prevention of infections [4]. Biofilm is an increasing cause of morbidity and mortality associated with chronic and nosocomial

infections, so a greater understanding of the nature of intracellular bacterial communities in infections, their early detection and management will aid in the development of new and more effective treatments [5]. A number of tests are available to detect slime production by staphylococci; which include quantitative methods such as tissue culture plate (TCP), which is considered as the gold-standard method for biofilm detection [6], and qualitative methods such as tube method (TM) [7], and Congo red agar (CRA) [8].

Materials and Methods

This study was conducted on 150 staphylococcal isolates randomly selected from different clinical specimens submitted to the Microbiology Laboratory of Ain Shams University Hospitals. They were isolated from different specimens; 30 pus, 46 blood, nine (9) wound, 15 urine, 22 sputa, 17 central line, five body fluids and six others (two ear swabs, two throat swabs, one bile drain and one radivac). All the isolates were identified morphologically by Gram stain, colonial morphology on culture, catalase test to differentiate it from Streptococcus species and DNase test to differentiate *S. aureus* from coagulase- negative staphylococci (CONS). Identification of CONS species and antibiotic susceptibility testing for all isolates were made using a n automated identification system (Vitek 2, bioMérieux, France) according to CLSI guidelines 2015 [9].

Biofilm detection was performed using TCP [6], TM [7] and CRA [8]. *S. aureus* (ATCC 25923) was used as negative control.

Tissue culture plate method was performed as described by Christensen et al., 1985 [6] for quantitative measurement of biofilm production in Staphylococcus spp. Using a microtiter assay. A single colony from each subcultured plate on blood agar was inoculated in a glass tube containing two ml TSBglu. The tubes were incubated overnight at 36°C ± 1 under aerobic conditions. Two hundred microlitres from each of the inoculated TSBglu tubes were aseptically transferred in the wells of a flat-bottomed microwell plastic plate. The inoculated microwell plastic plate was incubated overnight at 36°C ± 1 without sealing of the plate for proper oxygenation. Next day, the contents were discarded by inverting the plate and striking it on filter paper. The microwell plastic plate was washed once by adding 200 µl PBS (pH 7.2) into each well and then discarded. Then 200ul of freshly prepared sodium acetate (2%) was added to each well (for biofilm fixation) for 10 minutes and then discarded. This was followed by adding 200 µl crystal violet (0.1%) to each well for biofilm staining. The Plates was kept at room temperature for 30 minutes, and then the stain was discarded. The washing step was repeated once more. Finally, the plate was left to dry at room temperature for one hour, after which, the absorbance was read on a spectrophotometer at 620 nm OD (Figure 1).

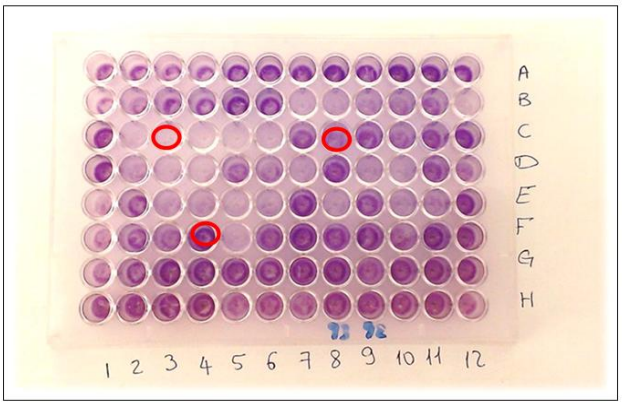

Figure 1: Tissue culture plate showing different biofilm intensities; C3: Non-biofilm producer; C8: moderate biofilm producer; F4: strong biofilm producer

The optical density (OD) value of each isolate was interpreted according to the following table to assess the degree of the biofilm (Table 1).

Table 1: Interpretation of results of Tissue Culture Plate method

OD Value	Biofilm Formation
<0.120	Non-biofilm producer
0.120-0.240	Moderate biofilm producer
>0.240	Strong biofilm producer

Tube method was done as described by Christensen et al., 1982 [7] for qualitative assessment of biofilm production. A loopful inoculum was inoculated on 10 ml TSBglu in plastic tubes. Tubes were incubated aerobically at 36°C ± 1 for 24 hours. Tubes content was discarded, and tubes were washed once with 9 ml phosphate buffer saline pH 7.2 and then discarded. For biofilm fixation, 10 ml of freshly prepared sodium acetate (2%) was added to each tube for 10 minutes and then discarded. For biofilm staining, 10 ml crystal violet (0.1%) was then added to each tube, and tubes were left at room temperature for 30 minutes after which the stain was discarded. The washing step was repeated, and tubes were left to dry in an inverted position at room temperature. Biofilm formation was detected by the presence of visible film on the wall and bottom of the tube. The amount of biofilm formation was interpreted according

to the results of the control strain and graded visually as absent, moderate and strong biofilm formation respectively (Figure 2).

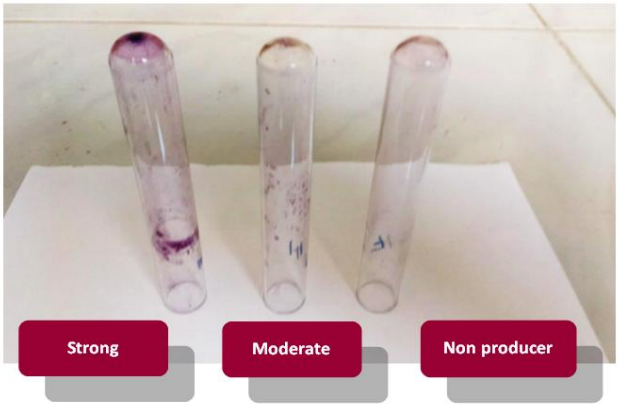

Figure 2: Tube method with different degrees of biofilm formation

The Congo red method was done as described by Freeman et al., 1989 [8] for qualitative assessment of biofilm production. Congo red stain (Research lab fine chem. Industries, India) was prepared as a concentrated aqueous solution of 0.8 g/200 ml distilled water and autoclaved separately from other medium constituents. The dye directly interacts with certain polysaccharides in the biofilm forming coloured complexes. Brain heart infusion agar (37 g) and sucrose (50 g) were dissolved in 800ml distilled water and autoclaved. Congo red stain (200ml) was then added when the agar cooled to 55˚C. Staphylococcal strains were inoculated on the prepared media and incubated aerobically at 37°C for 24 hours. Black colonies with dry crystalline consistency indicate strong biofilm formation. Red colonies with occasional darkening at the centre of the colonies were considered non-biofilm producers (Figure 3).

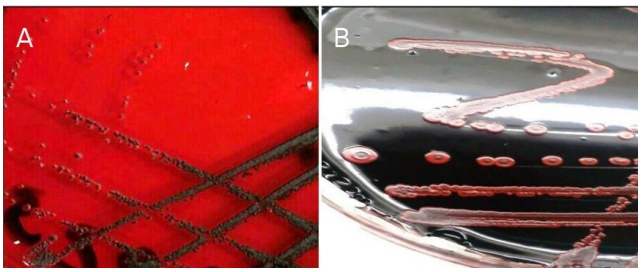

Figure 3: A) CRA showing black colonies with dry crystalline consistency (positive for biofilm formation); B) CRA showing red colonies with darkening at the centre (negative for biofilm formation)

Results

In this study, 150 clinical isolates of staphylococci were isolated; 78 (52%) were *S. aureus,* and 72 (48%) were CONS. Identification of CONS by Vitek 2 system revealed 41 *S. hemolyticus,* 18 *S. epidermidis,* 11 *S. hominis,* one *S. simulans* and one *S. warneri.* Biofilm detection in staphylococci was performed using TCP method, TM and CRA method.

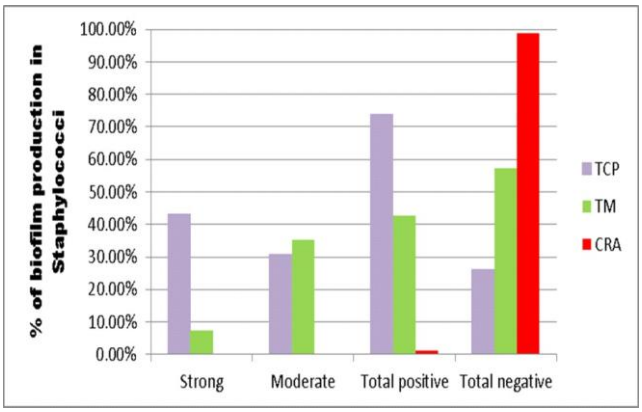

Figure 4: Comparison between TCP, TM and CRA as regards detection of biofilm formation in Staphylococci

The TCP method detected total positive biofilm production in 111 (74%) staphylococcal isolates, t h e strong positive was 65 (43.3%), and moderate positive were 46 (30.7%). As for the TM method, total positive biofilm production was 64 (42.7%), the strong positive was 11 (7.3%), and 53 (35.3%) were moderately positive. CRA method detected biofilm production only in 2 isolates (1.3%) (Figure 4).

Table 2: Comparison between TM and TCP as regards biofilm production

		TCP					
		Non-producers	Moderate producers	Strong producers	Total Staph. isolates	P value	Chi-square
TM	Non-producers	32	29	25	86	<0.001	20.445
		82.1%	63.0%	38.5%	57.3%		
	Moderate producers	6	13	34	53		
		15.4%	28.3%	52.3%	35.3%		
	Strong producers	1	4	6	11		
		2.6%	8.7%	9.2%	7.3%		
Total Staph. isolates		39	46	65	150		

Table 2 shows a comparison between the results of biofilm production by TM and TCP, using the Chi- square test. TM is considered a highly significant test, P value (< 0.001). As for CRA, it detected a very low number of biofilm producers (two) compared to total positive biofilm producers by TCP (111), according to the P value (0.579) CRA method is considered the non-significant test.

In our study, Sixty- eight out of 78 (87.1%) of *S. aureus* were MRSA, and 63 out of 72 (87.5%) of CONS were methicillin resistant. (Table 3) Shows detection of biofilm formation in staphylococci in relation to methicillin susceptibility. By TCP method, biofilm

production was detected in 73.3% of MRS (75% of MRSA, 71.4% of MRCONS) and 78.9% of MSS were biofilm producers. The 65 strong biofilm producers were; 30 (46.1%) MRSA, 6 (9.2%) MSSA, 24 (36.9%) MRCONS and 5 (7.7%) MSCONS. The 46 moderate biofilm producers were; 21 MRSA, 2 MSSA, 21 MRCONS and 2 MSCONS). As for TM, the 11 strong biofilm producers were 6(54.5%) MRSA and 5 (45.5%) MRCONS. By TM, The 53 moderate biofilm producers were 28 S. aureus [21 (39.6%) MRSA, 7(13.2%) MSSA], 25 CONS [22] (41.5%) MRCONS, 3 (5.7%) MSCONS]. Congo red agar method detected only two MRCONS biofilm producers. Biofilm producers were mostly detected in methicillin resistant strains [96/111(86.5%)]

Table 3: Detection of biofilm formation in staphylococci in relation to methicillin susceptibility

	S. aureus (no 78)		CoNS (no 72)				
	MRSA 68	MSSA 10	MRCoNS 63	MSCoNS 9	MRS 131	MSS 19	Total staphylococci 150
Tissue culture plate method (strong)	30	6	24	5	54	11	65
%	46.2%	9.2%	36.9%	7.7%	36%	7.3%	43.3%
Tissue culture plate method (moderate)	21	2	21	2	42	4	46
%	45.7%	4.3%	45.7%	4.3%	28%	2.6%	30.7%
Tissue culture plate method (negative)	17	2	18	2	35	4	39
%	43.6%	5.1%	46.2%	5.1%	23.3 %	2.6%	26%
Total positive	51	8	45	7	96	15	111
%	45.9%	7.2%	40.5%	6.3%	64%	10%	74%
Tube method (strong)	6	0	5	0	11	0	11
%	54.5%	0%	45.5%	0%	7.3%	0%	7.3%
Tube method (moderate)	21	7	22	3	43	10	53
%	39.6%	13.2%	41.5%	5.7%	28.6 %	6.7%	35.3%
Tube method (negative)	41	3	36	6	77	9	86
Total positive	27	7	27	3	54	10	64
%	42.2%	10.9%	42.2%	4.7%	36.6 %	6.7%	42.7%

MRSA: Methicillin-Resistant Staphylococcus aureus; MSSA: Methicillin-Sensitive Staphylococcus aureus; MRCONS: Methicillin-Resistant Coagulase negative Staphylococci; MSCONS: Methicillin Sensitive Coagulase negative Staphylococci; MRS: Methicillin-Resistant Staphylococci; MSS: Methicillin Sensitive Staphylococci.

Table 4 shows biofilm production in different CONS species. By TCP, biofilm production in CONS species was highest in *S. hemolyticus* (57.7%), followed by *S. epidermidis* (21.2%) and then *S. hominis* (19.2%). By TM, biofilm production in CONS species was highest in *S. hemolyticus* (46.7%), followed by *S. epidermidis* (33.3%) and S. hominis (20%). Whereas, Only two *S. epidermidis* isolates were biofilm producers by CRA method.

Biofilm production in staphylococci among various clinical specimens as detected by TCP method showed that the highest percentage were isolated from Blood cultures (82.6%) followed by urine (80%) and body fluids (80%) (Figure 5).

Comparative analytical study of TM and CRA methods about TCP method which is considered as the standard gold test showed that tube method was 51.4% sensitive, 82.1% specific for biofilm detection, PPV and NPV were 89% and 37.2% respectively.

Table 4: Biofilm production in CONS species

	CONS species				
	S. hemolyticus	*S. epidermidis*	*S. hominis*	*S. simulans*	*S. warneri*
Biofilm producers by TCP (111)	30	11	10	0	1
% from total biofilm producers	57.7%	21.2%	19.2%	0%	1.9%
Biofilm producers by TM (64)	14	10	6	0	0
% from total biofilm producers	46.7%	33.3%	20%	0%	0%

As for congo red agar method, sensitivity was very low (0.9%), but specificity was 97.4% for biofilm detection, PPV and NPV were 50% and 25.7% respectively.

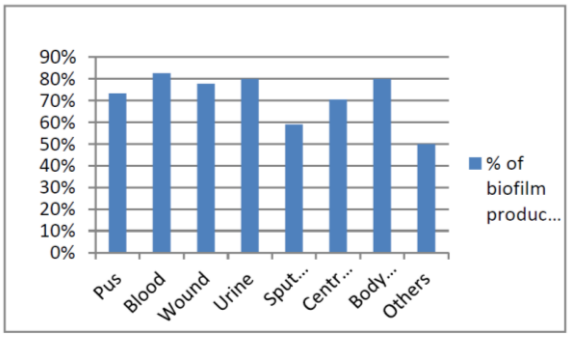

Figure 5: Biofilm production in staphylococci isolated from various clinical specimens (TCP method)

Biofilm producing strains were more resistant to almost all the classes of antibiotics showing resistance to Cefoxitin in 87.4% of staphylococci, Levofloxacin 57.7%, Gentamycin 53.2%, Clindamycin 60.4%, Erythromycin 69.5%, Doxycycline 40.5%, and Linezolid 3.6%. Biofilm non-producers were comparatively less resistant; Cefoxitin resistance was detected in 87.2% of staphylococci, Levofloxacin 43.6%, Gentamycin 23.6%, Clindamycin 53.8%, Erythromycin 61.5%, Doxycycline 18% and Linezolid 1.3%. All isolates were sensitive to vancomycin.

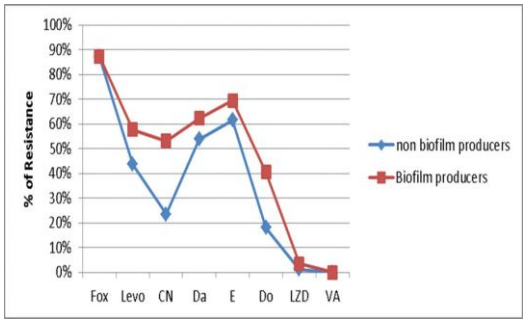

Figure 6: Antibiotic resistance pattern of Biofilm producers and non-producers staphylococci; Fox: Cefoxitin; LZD: Linezolid; levo: Levofloxacin; CN: Gentamycin; Da: Clindamycin; E: Erythromycin; Do: Doxycycline; VA: Vancomycin

Figure 6 shows the antibiotic resistance pattern of Biofilm producers and non- producers as detected by the TCP method.

Discussion

Biofilm formation is considered an important cause of all staphylococcal species associated with the infection of biomedical devices. Biofilm producing staphylococci isolated from other clinical samples are also of clinical significance as biofilm constitutes a reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections. So, a reliable and easy method for their diagnosis is necessary [10].

Our study tested 150 clinical isolates of staphylococci by three in-vitro screening procedures for their ability to form a biofilm. TCP method is the standard gold method as reported by Mathur et al., 2006 [11], hence it was considered a standard method for interpretation of our results. Biofilm producing staphylococcal isolates were 74% when TCP was performed (43.3% strong producers and 30.7% moderate producers). This method gave the best discrimination between strong, moderate and non-production of biofilm as it used cut off values. Results of our study were higher than other studies [11] [12] [13] [14] [15] [16] who reported 53.9%, 54.2%, 21.8%, 46%, 43.3%, and 39.7% of staphylococci as biofilm producers respectively.

We detected biofilm production in 53.2% of *S. aureus* and 46.8% of CONS by TCP method. Fatima et al., 2011 [17] also reported a high percentage of *S. aureus* as biofilm producers (64.89%). However, Akinkunmi and Lamikanra 2012 [13] reported 36% of *S. aureus* and 32.9% of CONS as biofilm producers and Ramakrishna et al., 2014 [10] reported 38% of *S. aureus* while 84% of CONS as biofilm producers. This might be attributed to the difference in the sources from which their strains were isolated.

Our results revealed that *S. hemolyticus* was the most frequently isolated species among CONS. However, Oliveira and Cunha, 2010 [18] reported *S. epidermidis* as the most frequently isolated species among CONS. This could be explained by the difference in the type of specimens selected; where half of the specimens collected in their study were catheter tips from which *S. epidermidis* is usually isolated (their study included 100 specimens, 50 catheter tips, 30 blood cultures and 20 nasal swabs).

We detected a high percentage of biofilm production in staphylococci isolated from blood culture specimens (82.6%). On the other hand, Sharvari and Chitra, 2012 [15] found a very high incidence of biofilm production in staphylococcal isolates from patients with artificial devices (89.5%), whereas, biofilm production in staphylococcal isolates from blood culture specimens were 45.9%. Also, Oliveira and Cunha, 2010 [18] detected 54.3% and 28.4% biofilm producing staphylococcal from catheter tips whereas and blood culture specimens respectively.

However, our sputum samples gave the least percentage of biofilm production (59.1%). Sharvari and Chitra, 2012 [15] also detected the least biofilm producing specimens among their sputum samples (26.3%).

Biofilm producers were mostly detected in methicillin resistant strains 96/111 (86.5%). This is discordant with O'neill et al. 2007 [19] who reported biofilm production in 74% among MRSA and 84% among MSSA isolate.

They stated that the significant association between methicillin susceptibility in *S. aureus* and ica-dependent biofilm formation was first reported when PIA production was found to be essential for biofilm formation by MSSA but not MRSA. Furthermore, MSSA biofilms are significantly induced in growth media supplemented with NaCl, which is known to activate ica operon expression.

However, this was not the case in Sharvari and Chitra, 2012 [15] who reported biofilm production in 72.3% of methicillin- resistant and 30.3% methicillin sensitive staphylococci (80.8% of MRSA, 31.6 of MSSA, 60% of MRCONS and 28% of MSCONS). Also, Rewatkar and Wadher 2013 [20] reported biofilm production in 85% among MRSA and 15% among MSSA isolates. Eiichi et al., 2004 [21] found a very high percentage (95.4%) of biofilm production in MRSA and Fatima et al., 2011 [17] reported 87.6% of MRSA as biofilm producers.

In our work, Tube method detected less number of biofilm producers, 42.7% which was lower compared to the TCP method. This difference may be due to the inter-observer variability in the reading of results, also may be due to performing the test using plastic tubes instead of glass, hindering visual interpretation. This was concordant with Saha et al., 2014 [22] where the TCP method detected 69% of biofilm producers, whereas, TM detected only 36%. They further stated that this method could discriminate between strong and moderate biofilm producers. However, the interpretation is observer dependent and there are chances of subjective errors. Our results were nearly similar to Mathur et al., 2006 [11] and Umadevi and Sailaja 2014 [23] who reported 41.4% and 42.5% as biofilm producers by TM respectively. On the other

hand, Oliveira and Cunha, 2010 [18] and Reddy 2017 [24] reported a higher percentage; 82% and 63% biofilm producers by TM respectively.

Congo red agar method was found to be easier and faster to perform than other phenotypic methods, but it only detected 1.3% of biofilm producing staphylococci in our study. Knobloch et al., 2002 [25], Mathur et al. 2006 [11], and Taj et al., 2012 [26] also reported very low percentage of positive biofilm producers by CRA method, 3.8%, 5.3%, and 3.4% respectively. The low percentage of positive results by CRA in our work might be attributed to the technique of preparation of the CRA, where congo red stain was autoclaved before being added to the agar. On the contrary, the study performed by Sharvari and Chitra, 2012 [15] gave higher results (25.3%). This could be attributed to congo red stain being prepared separately without autoclaving in sterile distilled water and then added to sterile molten autoclaved agar. Also, modified CRA method as described by Kaiser et al., 2013 [27] can be used instead to increase capacity of biofilm detection (the formula included BHIA with sucrose (5%), Congo red (0.08%), NaCl (1.5%), glucose (2%), and vancomycin (0.5 mg/mL). According to their study, this formula showed a high percentage of correlation among biofilm production in *S. epidermidis* and the presence of the *icaAB* gene (82.9%). The addition of vancomycin at a sub-MIC concentration (0.5 µg/mL) to modified CRA led to phenotype change in 64.8% of their strains, all of which were classified as a non-biofilm producer by the original CRA method and presenting the *icaAB* genes. The presence of a minimum concentration of vancomycin probably acts as a stress factor against the bacterial cells, which may lead to some alterations such as cell wall thickening [28] and may induce an increased expression of genes related to biofilm formation [29] [30].

Mathur et al., 2006 [11] recommended performing CRA method from *S. epidermidis* strains freshly isolated from clinical specimens of patients when the strains still retain their virulence characteristics expressed in “in vivo” conditions.

Also, some studies revealed higher results than ours as [31] [18] [20] who reported 83%, 73% and 90% of positive biofilm producers respectively. Cafiso et al., 2004 [31] explained the detection of a high percentage of biofilm producers (83%) by CRA by the addition of glucose 1%w/v in the congo red medium enhancing the production of biofilm in almost all isolates, where two is-positive non-biofilm producers by TCP became producers in CRA.

In the present study, the sensitivity of tube method was 51.4%, and specificity was 82.1% for biofilm detection which was higher than Saha et al., 2014 [22] who reported 34.21% sensitivity and 58.82% specificity for biofilm detection. However, Mathur et al., 2006 [11] and Bose et al., 2009 [12] reported higher sensitivity and specificity 73.6%, 76.3%, 92.6% and 97.6% respectively.

On the other hand, we report very low sensitivity for congo red agar method (0.9%) but higher specificity (97.4%) for biofilm detection. Also, (Mathur et al., 2006 [11], Bose et al., 2009 [12], and Saha et al., 2014 [22] reported 6.8%, 8.3% and and 21.1% sensitivity, 90.2%, 96.3% and 58.8% specificity respectively. Our results were discordant with the findings of Oliveira and Cunha, 2010 [18] who reported higher sensitivity 89% and specificity 100% for biofilm detection.

Biofilm producing strains in our work were resistant to almost all groups of antibiotics. Among our isolates, 87.4% were resistant to Cefoxitin, Levofloxacin (57.7%), Gentamicin (53.2%), Clindamycin (60.4%), Erythromycin (69.5%) and Doxycycline (40.5%) which was lower compared to biofilm non-producing strains where resistance to Cefoxitin was 87.2%, Levofloxacin 53.6%, Gentamycin 23.6%, Clindamycin %, Erythromycin 61.5%, and Doxycycline 18%. This is concordant with Sharvari and Chitra, 2012 [15], Ramakrishna et al., 2014 [10] and Singh et al., 2017 [32] who found that staphylococci biofilm producers were more resistant to commonly used antibiotics.

In our study, all the strains were sensitive to vancomycin (100%) while only (3.6%) were resistant to linezolid. This is concordant with Sharvari and Chitra, 2012 [15] who reported 100% of their isolates sensitive to vancomycin and (4.1%) resistant to linezolid. However, in Ramakrishna et al., 2014 [10] and Hashem et al., 2017 [33] studies all the strains were sensitive to both linezolid and vancomycin.

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