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RESEARCH ARTICLE

Comparison of different methods for detection of biofilm formation in staphylococcus aureus and epidermidis isolates from central venous catheters

Manal M. El-Amin[1]; Hanan Essa Mohamed[1]; Ghada Abd-Elrazek[2] and Marwah G. Amer[1]

1. Clinical Pathology Department, Faculty of Medicine, Zagazig University

2. Anathesia and intensive care Department, Faculty of Medicine, Zagazig University

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Corresponding Author

Marwah Gomah M. Amer

Abstract

Background: The predominant species isolated on indwelling medical devices are Staphylococcus aureus &epidermidis (Staph. aureus & epidermidis). Biofilm formation ability is recognized as an important virulence factor in both of them.

Objective: this study was conducted to compare three different methods for detection of biofilm formation ability (virulence factor) in Staph. aureus & epidermidis isolated from central venous catheter (CVC).

Methods: Staph. aureus and epidermidis strains were isolated from trypticase soya broth (TSB) of CVC from 156 pateints . Two phenotypic methods were used for detection of biofilm production; qualitative Congo red agar (CRA) and quantitative Microtiter plate (MTP). PCR (genotypic method) was used to determine the presence of icaA/D gene.

Results: A total of 50 staphylococcal (32 Staph. aureus and 18 epidermidis) strains isolated and identified from 156 pateints with infected CVC. Biofilm production was detected in 25(50%) isolates by CRA and 26 (52%) by MTP method (36% strong, 16% intermediate) .The icaA/D gene was detected in 28(56%) staphylococcal isolates. the ability of Staph. epidermidis for biofilm formation was(61.1%) which was higher than Staph. aureus strains (53.1%) but with no significant difference($p<0.4$).There was excellent agreement between PCR &CRA methods (Kappa value of 0.72, $P< 0.001$) , also between PCR& MTP (Kappa value of 0.796, $P<0.001$).**Conclusion**: Staphylococci isolated from CVC showed high rate of biofilm production. All slime producing staphylococci were positive for ica A /D genes indicating the important role of ica genes as virulence marker in staphylococci biofilm. CRA is qualitative, Simple, inexpensive and easily reproducible method and convenient as screening method. MTP is semi quantitative method reflects the severity of the condition and so may help in the determination of suitable line of management and remain a precious tool for in vitro screening .

INTRODUCTION

Staphylococci are the most associated organisms with chronic infected medical devices. The predominant species are staphylococcus aureus and Staphylococcal epidermidis ***(Mathour et al., 2006).*** For treatment of critically ill patients in intensive care unit (ICU), the use of central venous catheter (CVC) is very important and biofilm formation on its polymeric surfaces is a considerable problem causing blood stream infection ***(Storti et al.,2005),*** which was difficult and high cost treatment and cause long stay in hospital **(Seif El-Din et al.,2009*).*** Biofilm consist of layers of cell clusters enclosed in a matrix of extra-cellular polysa-ccharide (PIA) which is very important for biofilm formation mediating cell to cell adhesion and gene production ***(Cafiso et al.,2004).*** Biofilm makes the adherence of these microorganisms more easily and protect them from immune system and antibiotic reach ***(Taj et al., 2012).***

The intracellular adhesion (ica) locus consisting of the genes ica ADBC encoding protein which synthesis PIA. Among these genes icaA and icaD played important role for biofilm formation in staphylococcus aureua and epidermidis **(Seif El-Din et al.,2009)**

Detection of biofilm formation tests can be performed including congo red agar (CRA) ,tissue culture plate (TCP),Tube method (TM), bioluminescent assay and fluorescence microscope ***(Hassan et al.,2011).*** The Aim of this study is to compare three different methods for detection of biofilm formation in staphylococcus aureus and epidermidis in order to determine the most reliable method. As the awareness of the presence of this virulence factor (biofilm formation) in a particular strain may help to choose an optimal therapy and to control the infection overcome**.**

Materials and Methods

This study was carried out in clinical pathology department and Intensive care units (ICUs) of Zagazig University Hospitals in the period from February 2012 to october 2014. Approval for the study was obtained from research administration and research ethics committee, faculty of medicine, Zagazig University.

The study included 156 critically catheterized ill patients during period of study admitted to ICU. The patients developed localized symptom and sign of infecion around the CVC. Demogra-phic characteristics as age and sex of each patient were collected. Clinical characteristics which included; presence of underlying disease, pervious antibio-tics treatment, hospital and ICU date of admission, CVC duration and also laboratory data are collected.

Sampling:

10 ml Blood samples were withdrawn from peripheral veins under an aseptic condition into bact alert aerobic and anaerobic blood culture bottles using bact alert instrument 3D/60 (Biomerieux France). Subculture was done for positive cases on blood and MacConkey agar under aerobic and anaerobic conditions.

Under aseptic condition, CVCs were collected; a portion of the catheter was cut with sterile forceps into a sterile test tube containing 10 ml trypticase soy broth, sonicated for one minute, and then vortexed for 15 seconds. Sonicated broth was surface plated on blood agar, macConkey, TSB agar, nutrient agar and sabaroud agars.

Methods:

Isolates were initially identified by standard microbiological techniques including gram stain, catalase test and coagulase test .API Staph system (Biomerieux, france) was performed for identification of different staphylococcus strains then biofilm ability formation was detected for each isolate by CRA,MTP and icaA/D gene detection by PCR**.**

- Congo red agar method (Donlan et al., 2002):

Culture of all staphylococci isolates on CRA plates (0.8 g of CRA (Biomark, India) and 36 g of saccharose (Sigma)were added to 1 liter of brain heart infusion agar (Oxoid, Basingstoke, Hampshire, England) then incubated for 24 hours at 37°C. On CRA, slime-producing strains form black colonies, whereas non producing strains develop pink colonies **.**

- Tissue culture plate method (microtiter plate(MTP) (Christensen et al., 1982):

Isolates from fresh agar plates were inoculated in trypticase soy broth with 1% glucose and incubated for 24 hours at 37^{o}C in stationary condition and diluted (1 in 100) with fresh medium (TSB). Individual wells of sterile, polystyrene, flat-bottom tissue culture plates were filled with 200 ul of the diluted cultures, and only broth served as control to check sterility and non-specific binding of media. The tissue culture plates were incubated for 24hours at 37°C. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed well with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free floating planktonic bacteria; then 25 u of 1% crystal violet solution was added to each well (this dye stains the cells but not the polystyrene) plates. The plates were incubated at room temperature for 15 min, rinsed thoroughly and repeatedly with water. Adherent cells, which formed biofilm on all side wells, were uniformly stained with crystal violet. Crystal violet-stained biofilm was solubilized in 200 ul of 95 % ethanol (to extract the violet color), of which 125 ul were transferred to a new polystyrene micro titer dish, which was then read. Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto reader (model 680, Bio rad). Biofilm production According to adherence ability based on ODs, biofilm production is considered; Non/ weak <0.120, Moderately0.120-0.24 and High/strong >0.240.

-PCR for amplification of icaA and ica D (yazadni et al., 2006):

After overnight culture on brain-heart infusion agar plates, one or two colonies were suspended in 20 ml of sterile D.W, and the suspension was then heated at 100ºC for 20 minutes.

***Bacterial DNA extraction:** using Thermo scientific GeneJET Genomic DNA purification kit (Thermo scientific – EU-Lithuania).

***PCR amplification:** 5 µl aliquot was directly used as a template for PCR amplification.

The primers were synthesized by Metabion international AG Martinsried/ Deutchland**.**

***icaA*:**

5′-TCTCTTGCAGGAGCAATCAA (the forward primer).

5′-TCAGGCACTAACATCCAGCA (the reverse primer),
The two primers include a 188-bp region.
icaD:
5′ATGGTCAAGCCCAGACAGAG (the forward primer) .
5′CGTGTTTTCAACATTTAATGCA (the reverse primer).
PCR was performed in a DNA thermal cycler (Gene Amp, PCR system 9700).
The two primers include a 198-bp region.

The reaction was in a 25-µl volume containing the above-mentioned primers (1 µM each), together with 150 ng of the extracted DNA, 100 µM each of dATP, dCTP, dGTP, and dTTP, 1 U of *Taq* DNA polymerase, and buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, and 2.5 mM $MgCl_2$).One cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec (denaturation), 56-60°C for 30 sec (annealing), 72°C for 30 sec-3 min (extension), and 72°C for 3 min final extension for 1 cycle. After amplification, 10 µl of the PCR mixture was analyzed by agarose gel electrophoresis (2% agarose in Tris-acetate-EDTA). DNA molecular marker100-1000 bp ladder was included.

Statistical analysis:

Data analysis was done using the computer software statistical package for social sciences (SPSS) version 17.0.

Validity of screening test measured by: sensitivity, specificity, positive and negative predictive values. P-value ≤0.05 was considered significant and ≤0.001 was considered highly significant.

Results:

A total of 156 infected CVC &blood culture received from ICUs were collected from 86 male and 70 female in our study. The mean age of the patients was (41.92±15.32) years, the most underlying diseases was polytrauma, cancer &hepatic complication. All Patients were received antibiotic and most of them have a CVC for duration of 1-2 weeks **(table 1).**

the most frequent isolated organism from infected CVC were Staph. aureus (20.5%), Klebseilla pneumoniae (18.6%), Acinetobacter (15.4%) and among CONS staphylococci, Staph. epidermidis was the predominant (11.5%) **(table 2)**.

Among fifty staphylococcal isolated strains; 32 (64%) were Staph. aureus and 18 (36%) Staph. epidermidis strains .

The ability of Staph. epidermidis for biofilm formation(61.1%) was found to be higher than Staph. aureus strains (53.1%) but with no significant difference($p<0.4$) as shown in **(table 3).**

CRA showed 25 positive cases (50%) for slime production detected by black dry crystalline colonies, 26 (52%) were positive by MTP plates, while 28 (56%) cases were positive by PCR method **(table 4).**

Screening of the strength of biofilm formation of the isolated staphylococci by tissue culture plate assay (MTP) revealed that 18(36%) were strong adherent, 8 (16%) were moderate adherent and 24 (48%) were non/weak adherent **(table 5).**

PCR technique was used as standard method for evaluating the other techniques (CRA and MTP).
28 cases were positive by PCR, 23 of them were positive by CRA also, while 5 cases were negative. There was excellent agreement between both techniques, Kappa value was 0.72, P< 0.001**(table 6)**.

Table (7) showed 28 positive cases by PCR, 26 of them were positive by MTP, there was excellent agreement between them as Kappa value was 0.796, P<0.001.

Table (1): Demographic & Clinical characteristic data of patients.

Age		
X±SD	41.92±15.32	
Range	18-75	
Sex	**No**	**%**
Female No (%)	70	44.9
Male No (%)	86	55.1
	No	%
Underlying disease:		
Cancer	23	14.7
Cancer bladder	4	2.6
Cardiac complication	15	9.6
Head trauma	6	3.8
Hepatic complication	23	14.7
Laryngeal oedema	9	5.8
Obst jaundice	11	7.1
Polytrauma	34	21.8
RD	27	17.3
Retroerotoneal cyst	2	1.3
RTA	2	1.3
Antibiotic administration:		
Amikacin		
Ampicillin	9	5.8
Cefataxime	9	5.8
Ceftrixone	42	26.9
Ciprofloxacin	36	23
Colistin	3	1.9
Meropnem	3	1.9
Tetracycline	9	5.8
Tigecyclin	6	3.8
Unasyn	12	7.8
	27	17.3
ICU duration:		
1week	18	11.5
1-2 week	54	34.6
2weak-3week	36	23.1
More than 3 week	48	30.8
CVC duration:		
1week	24	15.4
1-2week	90	57.7
2-3week	33	21.1
More than 3week	9	5.8

Table (2): prevalence of different microorganisms isolated from CVP& blood culture.

	No	%
CVP culture:		
Gram –ve bacilli:		
Acinetobacter	24	15.4
k.pneumonia	29	18.6
proteus	7	4.5
pseudomonas	9	5.8
Gram +ve cocci:		
staph. aureus	32	20.5
staph. epidermidis	18	11.5
strept.hemolyticus	2	1.3
sterile	35	22.4
Blood culture:		
k.pneumonia	44	28.2
Acinitobacter	14	9
Staph hominis	6	3.8
Candida	1	0.6
E .coli	3	1.9
k.pneumonia+strept hemolyticus	1	0.6
k.pneumonia+strept hominis	1	0.6
pseudomonas	6	3.8
staph.aureus	21	13.5
staph.aureus+ecoli	1	0.6
staph.epidemidis	11	7
strept hemolyticus	2	1.3
sterile	39	25

Table (3): Association between Staphylococci strains and icaA/D gene by PCR.

		PCR		P
		+ve%	-ve%	
CVP culture	**S.aureus**	53.1	46.9	
	S.epidermidis	61.1	38.9	0.403

Table (4): Results of biofilm production in staphylococcal isolates

according to

CRA,MTP methods and PCR.

Biofilm	Positive		Negative		Total
	No	%	No	%	
Congo red agar	25	50	25	50	50
Microtitre plate	26	52	24	48	50
Ica A &D PCR	28	56	22	44	50

N.B : microtiter plate –ve has OD<0.12, +ve has OD >0.12

Table (5): Screening of the strength of biofilm formation of the isolated staphylococci by tissue culture plate assay (MTP)

Micro-organism	Number of isolates	Biofilm formation (OD:680nm)					
		High (strong)		Moderate		Non/weak	
		No	%	No	%	No	%
Stapylococcal biofilm	50	18	36	8	16	24	48

Table (6): Statistical evaluation of the CRA phenotypic method compared to detection of icaA/D genes in staphylococcal isolates.

		PCR		total	Sensitivity	Specificity	+ve predictive value	-ve predictive value
		+ve	-ve					
CRA	+ve	23	2	25	82.1%	90.9%	92%	80%
	-ve	5	20	25				
Total		28	22	50				

Table (7): Statistical evaluation of the MTP method compared to detection of icaA/D genes in staphylococcal isolates.

		PCR		Total	Sensitivity	Specificity	+ve predictive value	-ve predictive value
		+ve	-ve					
MTP	>0.12	26	3	29	92.9%	86.4%	89.7%	90.5%
	<0.12	2	19	21				

Discussion

The infection associated with the use of intravascular devices represents nosocomial infections .It is one of the most frequent causes of morbidity and mortality as it is a source of bacteremia and sepsis in hospitalized patients (**Cunha & Pazzini, 2011).**
The most commonly isolated organisms from central venous catheters are Coagulase negative Staphylococcus, Staph. aureus, Pseudomonas, Klebseilla and Enterococcus species, which are responsible for biofilm production **(Donlan etal.,2001)**
Our study compared the different methods for detection of biofilm formation in Staph. aureus and epidermidis in order to determine the most reliable method that may help to choose an optimal therapy and improve the prognosis of the infection.

In the present study, the most frequent isolated organisms from CVC were Staph. aureus (20.5%), Klebseilla pneumoniae (18.6%), Acinetobacter (15.4%) and among coagulase negative staphylococci(CoNS), Staph. epidermidis was the predominant (11.5%). This was agree with **Donlan etal (2002)** and **Anisio etal (2005)** who found that CoNS and gram negative bacilli were the predominant organisms but **Savas etal (2006) and Gad etal (2009)** reported that Klebseilla pneumoniae was the most relevant organism in catheterized patients while **Arciola etal (2001)** reported that both Staph. aureus and epidermidis are important causes of infections associated with catheters and other medical devices.
In the present study, the ability of Staph. epidermidis for biofilm formation(61.1%) was found to be higher than Staph. aureus strains (53.1%) but with no significant difference($p<0.4$). Our results were consistent with study of **Aricola et al (2002) and Dika (2014)** who reported that the Staph . epidermidis is the major biofilm producer **.** Also our results agree with **Oliveira and Cunha (2010)** who reported that the ability to produce biofilm is observed in coagulase negative staphylococci and Staph. epidermidis especially .The high prevalence may be due to the fact that this microorganism is a skin colonizer that becomes opportunistic in immunocompromised people **(Richard et al.,2008)** . Although **Seif El-Din and his colleagues (2009)** reported higher percentage(83.4% and 88%of staphylococcus aureus and staphylococcus epidermidis respectively) could produce biofilm, but still Staph. epidermidis has the upper hand.

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices **(Aricola et al., 2001)**. Suggested mechanisms by which biofilm producing bacteria cause disease are detachment of cells from medical device biofilm, endotoxin production, resistance to immune system and generation of resistance through plasmid exchange **(Donlan and Costerton, 2002).** Staphylococci are recognized as the most frequent causes of biofilm-associated infections **(Vuong and Otto 2002).**

In the present study we have assayed isolated staphylococcal strains from CVC for qualitative biofilm forming ability by congo red agar test (CRA).
There were 50% positive staphylococci isolates while 50% were negative for CRA test .Similar results were reported by **Namvar (2013)** who reported 65% positive results with congo red agar and **Khan et al. (2011)** reported 47.79% positive results **.** However **Taj etal.(2012)** reported that only four isolates (3.4%) were positive by CRA test .As researchers have only recently found that PIA/PNAG (polysaccharide intracellular adhesins/poly N-actyl glucosamine) have little input in the biofilm matrix of Staphylococcus aureus so cannot be detected by the CRA method **(Knoblochetal.,2002 and Mathur etal.,2006)**.

In the current study we have performed quantitative detection of biofilm using microtiter plate method (MTP) showed that 52% of Staphylococcus strains were biofilm producers while 48 % strains non producers. Biofilm producers can be further classified as 36% strong producers and 16% moderate producers. these were agree with **Mathur et al (2006) and Khan et al (2011)** who found that 57.8% and 58% respectively were positive slime producing staphylococci by microtiter plate but was less than found by **Deka (2014)** who reported 83% positive results. He suggested a strong dependence and variation according to the growth conditions and the use of various sugar supplementations for biofilm formation in staphylococci.

The most important advantage of the microtiter plate assay in addition to the phenotypic biofilm production information presented by CRA is the ability of this method to differentiate between weak and strong biofilm producers. This reflects the severity of the condition and so may help in the determination of suitable line of management **(Handke *et al.*, 2004).**

The presence of the icaADBC operon is essential for PIA production **(Gerke etal.,1998)**: the co- expression of icaA and icaD increases N-acetylglu- cosaminyl transferase activity and slime production **(Arciola etal.,2006)**.

The icaAD gene was detected in 28 (56%) staphylococcal isolates in the present study. **Satorres and Alcaráz (2007)** also reported that of all staphylococci isolated from blood and intravascular catheters, 42.2% were positive for icaA and icaD genes while **Rasha etal.(2012)** detected (32%) positive icaA/D positive staphylococcal isolates. **Arciola et al. (2005) and Mertens & Ghebremedhin (2013)** found 57% and 61% respectively of the staphylococcus epidermidis infected cases were positive for icaA/D genes.

In this study, both the icaA and icaD genes were present in all biofilm producing strains. **Arciola *et al*. (2001) and El-Mahallawy *et al.* (2009)** revealed that all strains bearing the icaA gene, a component of the ica locus, also bear icaD. This indicates that the presence of both genes is essential for biofilm production and confirms that both genes are part of one operon, so either the entire operon is present or abscent **Seif El-Din et al (2011)**. This is supported by the results of a study done by **Fluckiger et al. (2005)** who stated that the ica locus and biofilm formation are crucial parameters for staphylococcal colonization and survival on implants.

PCR was used in this study as a reference for the phenotypic method based on several studies **(Cafisoetal.,2004&Seif El-Din etal.,2009)**, With respect to the phenotypic methods, CRA test showed 82.1% sensitivity and 90.9% specificity. While the microtiter plate method (MTP) presented 92.9% sensitivity and 86.4% specificity when compared to PCR recognizing the concomitant presence of the icaA and icaD. These results go hand in hand with results of **Oliveira & Cunha (2010).**

There was an excellent agreement between the results of PCR detection of ica A/ D genes and congo red agar test (Kappa value was 0.72, P< 0.001) which was in line with the results of **Aricola et al (2005), EL-Mahallawy et al,(2009) and Terki et al,(2013)**,who found that CRA method is convenient as it enable visualization of strain phenotype and this method with PCR are two convenient methods for biofilm detection. In our results there was also an excellent agreement between the PCR method and microtiter plate method (Kappa value was 0.796, P<0.001) and this agree with **Aricola et al (2005)** while **Adilson & Maria(2010)** found moderate agreement between them.
In this study, two cases found to be negative by MTP plate method and positive with PCR this could depend on the culture condition in MTP causing variability depending on the type of incubation medium and its production lot .so some strain appear negative because their phenotype is not completely expressed in TSB broth**.**
In our study, positivity at the CRA plate test(used as a phenotypical reference test for slime production) did not always correlate with the presence of icaA and icaD genes, in accordance with **Ruzicka et al.(2004)**, who demonstrated that 20% of strains with ica genes did not express phenotype. **Liberto et al.(2007)** hypothesize a translational or post-translational regulation with production of proteins with low or absent activity, associated with an absent phenotype. As Slime production and association in biofilm are two parameters of great complexity :they are highly correlated with the environment. Indeed, anaerobiosis and low concentrations of iron strongly increase biofilm expression **(Baldassarri et al.,2001; Cramton etal.,2001).** On the other hand ,recent studies highlighted the role of phenol- soluble modulines that can control the passage from biofilm phase to non-biofilm phase, with subsequent dissemination **(Yao etal.,2005)**. More- over, glucose concentration and, even more, glucose uptake of a particular strain ,and/or a peculiar phase of the growth curve ,can influence ica operon transcription and biofilm expression **(Dobinsky etal.,2003).**
In conclusion, both genotypic and phenotypic methods improve identification biofilm ability production by staphylococcus spp . each method has its advantages and drawbacks, as well as their specific indication. CRA is qualitative, Simple, inexpensive and easily reproducible method and convenient as screening method. MTP is semi quantitative method and remain a precious tool for in vitro screening of different biomaterial for the adhesive properties and finally PCR method is simple, rapid , reliable and only requires minimal amounts of DNA so, it can be adopted as most suitable an reproducible method for detection of biofilm**.**

Conclusion

Our results have confirmed data presented by other authors in that the presence of icaADBC operon genes is associated with biofilm formation .Therefore, both genotypic and phenotypic methods improve identification biofilm ability by staphylococcus spp .PCR method can be adopted as most suitable an reproducible method for detection of biofilm.CRA is qualitative, Simple, inexpensive and easily reproducible method and convenient as screening method. TCP is semi

quantitative method and remain a precious tool for in vitro screening of different biomaterial for the adhesive properties .each method has its advantages and drawbacks, as well as their specific indication.

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