Egyptian Journal of Medical Microbiology Volume 27 / No.1 / January 2018 65-73 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Phenotypic and Genotypic Characterizations of Staphylococcal Biofilm Formation from Neonatal Infection Isolates

[1]Eman A. Abd Al Rhman, [1]Naglaa F. Abd El Haliem*, [1]Effat M. Hassan, [1]Lamia I. Al Seadawy, [1]Daad F. El Fouhil, [2]Zienab F. Asheiba

Departments [1]Microbiology & Immunology, [2]Pediatrics Faculty of Medicine for Girls, Al Azhar University, Cairo, Egypt

Key words:
Neonatal sepsis, Staphylococci-biofilm formers, icaAD genes and multidrug resistance

Corresponding Author:
Naglaa Farouk Abd El Haliem
Faculty of Medicine for Girls, Al Azhar University, Cairo, Egypt
Tel: 01222279566-01022338787
Naglaafarouk97@gmail.com

ABSTRACT

Background: *Staphylococci are implicated as the main causes of biofilm-associated neonatal infections (NIs).* ***Objectives:*** *This study aimed to investigate biofilm formation by Staphylococci isolated from newborns with neonatal sepsis and comparing and analyzing Staphylococcal biofilm-forming capacity and multidrug resistance.* ***Methodology:*** *One hundred blood cultures and thirty endotracheal tube specimens of neonates with septicemia were comprised in this study. Staphylococcal isolates were detected by conventional methods and investigated for biofilm formation by Microtiter plate (MTP), Tube method (TM) and Congo red Agar (CRA) detection methods followed by detection of icaA & icaD adhesin genes by Multiplex-PCR.* ***Results:*** *Among the isolated strains, fifty Staphylococcal strains were studied for biofilm formation and antibiotic susceptibility. They were 56%, 68%, 88% positive biofilm formers by MTP, TM and CRA detection methods, respectively. While, they were 28 (56%) positive biofilm formers by PCR. Out of the 28 icaAD positive staphylococcal isolates 27 (96.4%) were TM and CRA-positive, but only 15 (53.5%) were MTP-positive. The correlation between the sensitivity and specificity of phenotypic biofilm detection methods with PCR, was 96.4%, 96.4% and 53.5% sensitivity for TM, CRA and MTP methods, while they were 68.1%, 22.7%, 40.9% specificity comparing with PCR, respectively. There was a statistical significant difference between antibiotic resistances among them whereas strong biofilm formers were more resistant to all tested antibiotics compared to moderate and weak biofilm formers.* ***Conclusion:*** *TM is easy, reliable with excellent sensitivity method for detection of biofilm in neonatal nosocomial infections, as compared to CRA and MTP methods. Further investigation of the genetic variations of adhesion genes are needed for better evaluation of staphylococci biofilm forming capacity and multidrug resistance.*

INTRODUCTION

Staphylococci are considered a common causes of neonatal nosocomial infections, particularly in premature newborns or those hospitalized in a neonatal intensive care unit (NICU) for a long time with invasive procedures and antibiotic management [1]. Coagulase-negative staphylococci (CoNS) are main nosocomial pathogens producing late onset sepsis in neonates frequently associated with use of indwelling vascular catheters. Neonatal CoNS infections cause significant morbidity, particularly between infants with very low birth weight [2]. Moreover, CoNS commonly show multi-resistance to antimicrobials. Though, the correlation between clinical effect of CoNS infections and antimicrobial resistance is unknown. *Staphylococci* are also implicated as the most common causes of biofilm-associated infections [3] .

Biofilms are highly organized communities of microbes made up of one or more species attached to biotic or a biotic solid surface and often encased in an extracellular matrix which composed of proteins, polysaccharides and nucleic acids [4]. It has been found to be involved in 80% of all microbial infections in the body. It have been implicated in middle ear infections, urinary tract infections and endocarditis [5]. Biofilms can also be formed on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices[6]. *S. epidermidis* was the first species to be described as a biofilm producer; however, the same ability is encountered in *S. aureus* and other coagulase negative *Staphylococcus* species [7].

Polysaccharide intercellular adhesin (PIA) encoded by the icaADBC operon is the most important characterized constituent of staphylococcal biofilms [3,8]. Just ica operon is activated, icaA, ica D, icaB and icaC proteins are transcribed. The icaA expression produces

low enzymatic activity; but, the expression of icaA and icaD simultaneously promotes a clear increase in the polysaccharide amount [8,9].

The current study aims to investigate the biofilm formation by *Staphylococcal* clinical isolates of newborns suffering from neonatal sepsis by different phenotypic methods and detect genes associated with biofilm formation. Comparing these methods of detection of biofilm formation to reach the most appropriate method. And analyzing the association between biofilm forming capacity and multidrug resistance in staphylococci.

METHODOLOGY

Patients

One hundred and thirty neonates with clinical symptoms and signs of sepsis admitted to Al-Zhraa and Sayed Galal NICUs are enrolled in this study. They were 83 (63.8%) males and 47 (36.2%) females. They were complaining from poor reflexes, lethargy, respiratory distress, bradycardia, apnea, or bleeding, increased C-reactive protein, increased or decreased WBCs. Neonates having major surgically uncorrectable lethal anomalies, preterm < 28 weeks and birth weight < 750 gm were excluded.

Specimens collection

Blood cultures for 100 neonates suffering from sepsis and end tracheal tube cultures (ETT) for 30 neonates with ventilator associated pneumonia (VAP) were collected. Two mls of blood were taken by vein puncture under complete aseptic conditions and inoculated immediately into blood culture bottles (Hexa-Biotech, Italy). Tips of endotracheal tubes were aseptically cut by using sterile scalpels and were immediately transferred to sterile cups containing trypticase soya broth (TSB) (Difco, Detroit, Michigan) as a transport medium.

Bacterial cultures

Blood culture bottles and cups containing the tips of endotracheal tubes were immediately transferred to microbiological lab for overnight incubation under aerobic condition at 37°C. Subcultures on blood agar, nutrient agar (Lab M, IDG, UK), MacConkey's agar (Difco, Detroit, Michigan) and sabouraud dextrose agar (Biolife, Italy) were done and were incubated at 37°C for 24h under aerobic condition. Subcultures were repeated every other day and they were discarded after two weeks.

Staphylococci were identified by conventional method and the isolates were kept at –20°C in brain heart infusion broth (BHIB) containing 12% glycerol.

Biofilm detection

Biofilm formation by the staphylococcal isolates was detected by three phenotypes and one genotypic method.

Congo red Agar (CRA) method:

Screening of biofilm formation by Staphylococcal isolates were completed as the method described by [10]. Briefly, Congo red Agar medium [BHIB (37 gms/L), agar (10 gms/L), sucrose (50 gms/L) and congo red stain (0.8 gms/L)] were used. Staphylococcal isolates were cultured and incubated aerobically at 37°C for 48 hours. Black colonies appearing indicating a positive result, while non-biofilm producers give pink colonies.

Microtiter plate method (MTP):

MTP method for detection of biofilm formation is a quantitative method. The procedures were done as described by[11]. The Microtiter-plate reader at 630nm was used for reading of optical densities (OD) of stained adherent bacterial films. The mean OD values were exactly calculated for all tested isolates in comparison with negative controls. The cutoff value (ODc) was then established.

Tube method (TM):

Tube method one of the important qualitative methods used for biofilm detection. The complete procedures were completed as [12].

Genotypic method:

icaA and icaD genes are associated with biofilm formation. Coexpression of these genes was done according to [13].

DNA extraction

DNA was extracted by heat shock [14]. Aliquotes (5µl) of the extracted DNA were subjected to the detection of staphylococcal 16S rRNA. The total reaction mixture was 50µl contained 25 µl of master mix (Qiagen- Germany), 5 µl of the extracted DNA, and 2 µl of each primer with 16µl of distilled water. Primers used for icaA detection were, 5'-TCTCTTGCAGGAGCAATCAA as the forward primer, and 5'-TC AGGCACTAA CATCCAGCA as the reverse primer, (Qiagen- Germany). Primers of icaD were, 5'-ATGGTCAAGCCCAGACAGAG as the forward primer and 5'-CG TGTTTTCAAC ATTT A ATGCA A as the reverse primer, (Qiagen- Germany).

Deatection of icaA and icaD

PCR was performed to detect icaA and icaD genes using the methodology previously described by [13]. The amplification was carried out by using a DNA Thermal cycler (Biometra-Germany) with an initial incubation at 94°C for 5 min for one cycle, denaturation at 94°C for 30s, annealing at 54°C for 30 second, extension at 72°C for 30 second repeated for 35 cycles and a final extension step at 72°C for 8 minutes after the last cycle. Full precautions were taken to prevent contamination and internal positive and negative controls were included.

Evaluation of the pattern of DNA amplicons

After amplification, 17 µl of the PCR products were electrophoresed in 1% (W/V) agarose gel (Bioline, London, UK) containing 1µl/mL ethidium bromide and run in a horizontal gel electrophoresis unit (Mini-Sub

DNA cell, BioRad). The bands were visualized by ultraviolet illumination (Foto/PhoresisI) using a transilluminator and gels were recorded as digital TIFF images using a gel documentation system (UVITech). The amplicon was checked for size using 50 bp ladder (Qiagen-Germany), the expected size of the amplicon of icaA was 188bp and the expected size of the amplicon of icaD was 198bp. This was confirmed by the positive control and marker molecular weight.

Antibiotic susceptibility testing for biofilm forming staphylococcal isolates:

This is performed on Mueller-Hinton agar as described by [15] and test samples were incubated for 18h at 35±1°C. Vancomycin (30 µg), meropenem (10 µg), oxacillin (1 µg), gentamycin (10 µg), erythromycin (15 µg), ampicillin (10 µg), ceftriaxone (30 µg), cefazoline (30 µg), and ampicillin/sulbactam (20/10 µg) were used. The diameter of the clear zone of growth inhibition was measured after incubation and the samples showed that halos sensitivity ≥ 13 mm and ≥ 18 mm were classified as sensitive.

Minimal inhibitory concentration (MIC) and Minimal biofilm inhibitory concentration (MBIC)

MIC and MBIC were used for determination of biofilm forming staphylococcal isolates by broth micro-dilution method using 96 well microtiter plates and the results were interpreted according to [15]. Meropenem and vancomycin were used, as the biofilm forming staphylococcal isolates were commonly sensitive to them by disk diffusion method. MIC is defined as the lowest concentration of an anti-microbial agent that inhibit the visible growth of a microorganism after overnight incubation, while MBIC which corresponds to the lowest concentration of antibiotic which inhibit the growth of biofilm cells as indicated by the absence of observable growth. A positive control and a negative control were included in all experiments.

Statistical Analysis:

Summary of measures was reported as mean ± standard deviation (SD) for quantitative variables, and percentages for categorical variables, and. P value was considered statistically significant at ≤ 0.05. Analyses were performed using the SPSS (Statistical package for social science) computer program version 17.0 software. The sensitivities and specificities of the phenotypic methods for biofilm production were calculated as described by [16].

RESULTS

Current study was conducted to determine the frequency of Staphylococcal biofilm formation associated with sepsis among neonatal infection isolates. One hundred and thirty neonates with neonatal sepsis who were admitted to Al zahraa and Sayed Galal NICUs, 63.8% of them were males and 36.2% were females were conducted in this study. Of the 130 neonates, 50.7% had very low birth weight, and the majority of cases 63% were preterm (Table 1).

Table 1: Patient Demographics:

Data of neonates	(No. =130)	%
Gender:		
Male	83	(63.8%)
Female	47	(36.2%)
Birth weight:		
<1500g	66	50%
1500-2500g	45	36%
>2500g	19	14%
Gestational age:		
<38ws (preterm)	82	63%
≥38ws (full term)	48	37%
Delivery type:		
Caesarian section (CS)	79	60.7%
Normal vaginal delivery	51	39.3%

One hundred and twenty five isolates were isolated from 130 clinical samples (100 blood samples and 30 endotracheal tubes). As regard type of bacterial growth, 74% of blood cultures had monomicrobial growth, while 6% had mixed growth (2 isolates/sample) and no growth was detected in 20% of blood cultures. In endotracheal tube cultures 86.6% of cultures showed monomicrobial growth, while 13.4% were had mixed growth.

Ninety one organisms were isolated from positive blood cultures (BC), *staphylococci* were the most prevalent microorganism, followed by Gram negative bacilli, Gram negative cocco-bacilli, *Candida* species and *Streptococci* isolates were detected. While thirty four organisms were isolated from positive endotracheal tube (ETT) cultures, the *staphylococci* were the most prevalent microorganisms also, followed by Gram negative bacilli and *Candida* isolates were detected (Table 2).

Table 2: Types of organisms isolated from blood and endotracheal tube cultures:

Isolates	Isolates of BC (No.=91)		Isolates of ETT cultures (No.=34)	
	No.	%	No.	%
Staphylococcus aureus	19	20.8%	15	44.1%
Gram negative bacilli	26	28.6%	14	41.1%
Gram negative cocco-bacilli	12	13.1%	-	-
Candida spp.	15	16.5%	2	5.9%
Staph epidermidis	13	14.2%	3	8.8%
Streptococci	6	6.5%	-	-

In the current study, fifty isolates of staphylococci were evaluated for their ability to form biofilms by four screening methods. Three phenotypic methods; Congo red agar method (figure 1), Micro titer plate method (figure 2), Tube method (figure 3) and one genotypic method (PCR) for detection of icaA & icaD genes, (figures 4 & 5).

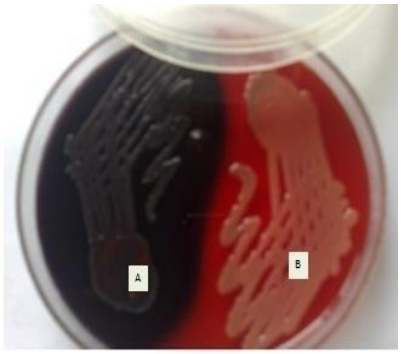

Fig. 1: Staphylococcal biofilm formation screening by Congo red agar method. A: Positive (black colonies), B: negative (pink colonies)

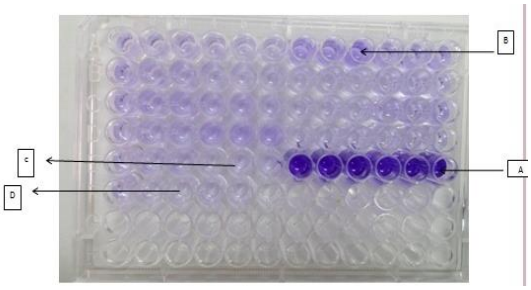

Fig. 2: Biofilm formation detection of by microtiter plate method, A: strong, B: weak, C: non biofilm formers, D: negative control

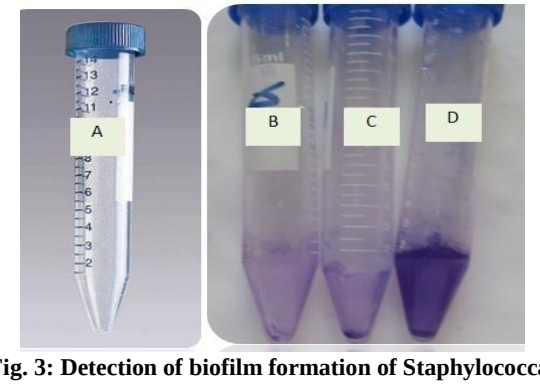

Fig. 3: Detection of biofilm formation of Staphylococcal isolates by tube method. A: Negative control, B: Weak, C: Moderate, D: Strong biofilm former.

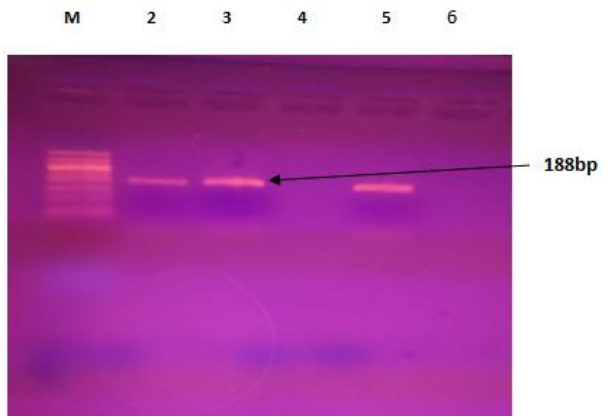

Fig. 4: Ethidium bromide stained 1.5% agarose gel electrophoresis showing: lane 1: MW DNA marker of 50bp ladder, Lane 2&3: positive isolates for icaA gene (188bp), Lane 4: negative isolates for icaA gene, Lane 5: positive control and Lane 6: negative control

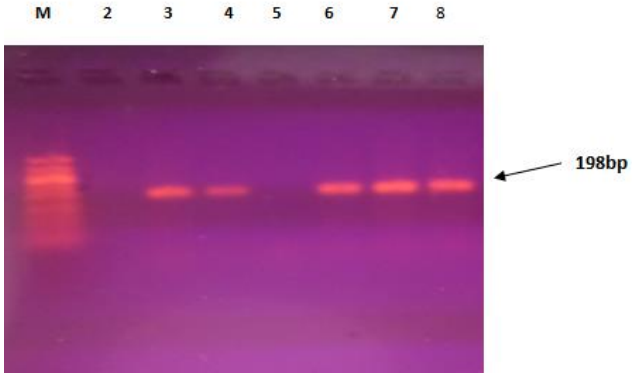

Fig. 5: Ethidium bromide stained 1.5% agarose gel electrophoresis showing: Lane 1: MW DNA marker of 50bp ladder, Lane 2: negative control. Lane3: positive control Lane 4, 6, 7, 8: positive isolates for icaD gene (198bp). Lane 5: negative isolate for icaD gene.

Among fifty isolates of *staphylococci* were evaluated for their ability to form biofilms, 88%, 68%, 56% were positive-biofilm formers by CRA, MTP, and TM detection methods, respectively. While, 56% were PCR-positive (Tables 3 & 4). By comparing the phenotypic biofilm detection methods with icaA & icaD genes detection, 96.4% of staphylococcal isolates were positive by both methods.

Table 3: Detection of staphylococcal biofilm formation by phenotypic methods.

Biofilm formation	Congo red agar (CRA)		Micro titer plate (MTP)		Tube method (TM)	
	No.	%	No.	%	No.	%
Biofilm formers:	44	88%				
Weak	-	-	25	٥٠%	25	50%
Moderate	-	-	-	-	6	12%
Strong	-	-	3	%6	3	%6
Non-biofilm formers:	6	12%	22	44%	16	32%
Total	50	%100	50	100%	50	100%

Table 4: Detection of icaA & icaD genes in Staphylococcal isolates by PCR:

Ica A				Ica D				Total
-ve		+ve		-ve		+ve		
No.	%	No.	%	No.	%	No.	%	
22	44%	28	56%	22	44%	28	56%	50

By comparing the sensitivity of the phenotypic biofilm detection methods with icaA & icaD genes detection method they were 96.4%, 96.4% and 53.5% for TM, CRA and MTP methods, respectively. While the specificity of TM, CRA and MTP methods in biofilm detection when compared to PCR were 68.1%, 22.7%, 40.9%, respectively (Table 5).

Table 5: Statistical evaluation of PCR compared to the phenotypic biofilm detection methods in Staphylococcal isolates:

Method	PCR (Ica+ve) (28)	PCR (Ica-ve) (22)	Sensitivity (%)	Specificity (%)
TM :				
+ve (34)	27	7	96.4%	68.1%
-ve (16)	1	15		
CRA:				
+ve (44)	27	17	96.4%	22.7%
-ve (6)	1	5		
MTP:				
+ve (28)	15	13	53.5%	40.9%
–ve (22)	13	9		

The results of screening methods of biofilm formation were analyzed and antibiogram studies were done by Kirby Bauer disc diffusion method for twenty one staphylococcal isolates (18 *S. aureus* isolates & 3 *S. epidermidis* isolates), which showed biofilm formation by all different methods. Among biofilm formers *S. aureus* isolates the highest resistance was detected against ampicillin and ampicillin-salbactam as all *Staph aureus* isolates 100% were resistant to them, while 94.4% were resistant to ceftrixone. Resistance to cefazoline, gentamycine and erythromycin were detected in 66.6% of isolates, while 55.5% were resistant to oxacillin (MRSA). The least resistance were to meropenem 33.3% and vacomycin as only two isolates 11.1% showed resistance (VRSA). *S. epidermidis* also showed the highest resistance against ampicillin, ampicillin-salbactam and ceftrixone as all of the isolates 100% were resistant to them, while resistance to cefazoline, gentamycin, erythromycin were 66.6%, 33.3% and 33.3% respectively, while all *S. epidermidis* isolates were sensitive to oxacillin, vacomycin and meropenem (Table 6).

A statistically significant difference between antibiotic resistance among strong, moderate and weak biofilm formers as strong biofilm formers (2VRSA & 3MRSA) were more resistant to all tested antibiotics compared to moderate and weak biofilm formers.

In the current study, MIC was used for determination of antibiotic susceptibility. Vancomycin and meropenem were used and they were the most sensitive antibiotics. A great difference in antibiotic susceptibility between planktonic populations and biofilm populations, whereas, MBIC>128µg/ml & MIC=2-4µg/ml of 88.8% and 66.6% of *Staph aureus* isolates as regard vancomycin and meropenem susceptibility, respectively.

Table 6: Antibiotic resistance pattern in biofilm forming staphylococci according to the intensity of biofilm formation:

Antibiotics	Weak biofilm forming staph (12)	Moderate biofilm forming staph (6)	Strong biofilm forming staph. (3)	P value
Vancomycin	0	0	2	0.000 (HS)***
Meropenem	0	3	3	0.000 (HS)***
Cefazoline	3	6	3	0.042 (S)*
Gentamycin	3	6	3	0.042 (S)*
Erythromycin	3	6	3	0.042 (S)*
Ceftrixone	9	5	3	0.771 (NS)**
Oxacillin	0	7	3	0.001 (HS)***

P value : Significant ≤ 0.05 ** non significant >0 .05 * Highly significant≤ 0.001*

DISCUSSION

Neonatal sepsis has evolved over the past century to be the commonest cause of neonatal mortality. In developing countries, it is responsible for about 30-50% of the total neonatal deaths [17]. A total of 130 neonates with clinical signs and symptoms of sepsis were enrolled in this study. Among the studied group, 63.8% were males and 36.2% were females. Of the 130 neonates, 50.7% had very low birth weight, and the majority of cases 63% were preterm. Prematurity and low birth weights are considered from the most important risk factors for developing neonatal sepsis. Also, it was observed that the neonatal sepsis was more frequent in neonates delivered by CS as 60.7% were delivered by CS while only 39.3% were delivered by normal vaginal delivery.

A total of 125 isolates were isolated from 130 clinical samples (100 blood samples and 30 endotracheal tubes). As regard type of bacterial growth, 74% of blood cultures were had monomicrobial growth, while 6% were having mixed growth and no growth was detected in 20% of blood cultures. This was in accordance with the result of [18] as they found that 14% of blood culture had polymicrobial growth. And in agreement with that obtained by [19] who detected 25% of clinically diagnosed septic episodes were culture-negative. This may be due to the concurrent use of antibiotics or development of antibodies**.** In contrast, no growth was detected in 59.3% of blood cultures by[20]. In ETT cultures 86.6% of cultures showed monomicrobial growth, while 13.4% were having mixed growth. This result was in agreement with that obtained by [21] as 86.54% of ETT cultures showed monomicrobial growth, while 13.46% showed polymicrobial growth. On the other hand, polymicrobial growth was detected by [22] in 40% of ETT cultures.

As regard types of microorganisms isolated from blood cultures, 91 isolates were isolated from 100 blood culture, *Staphylococci* were the most prevalent microorganism 35.3%, followed by Gram-negative bacilli 28.6%, Gram-negative coccobacilli 13.1%, *Candida* species 16.5% and 6.5% Streptococcal isolates were detected. Similar findings were reported in other studies in Egypt by [20] who found that the *staphylococci* were the most prevalent isolated organism followed by Gram-negative bacilli. In contrast to these results [23] reported that Gram-negative bacilli were the most prevalent followed by Gram-positive cocci. While, 34 organisms out of 30 ETT cultures were isolated, *Staphylococci* were 53%, followed by Gram-negative bacilli 41.1% and 5.9% *Candida* species were detected. However, [24] found that *Klebsiella spp* were the most commonly isolated organisms and represented 32.8% of the isolated organisms.

Biofilm producing bacteria are exhibit resistance to antibiotics by several methods as the biofilms restricted penetration and expression of resistance genes [25] Thus, it was supposed that measuring the biofilm formation can used as a marker for the staphylococcal pathogenicity [26]. In the current study, staphylococcal biofilm formation was detected by three screening methods; MTP, TM and CRA and PCR were used to detect biofilm coding genes. For MTP method, biofilm formation was detected in 56% of the fifty staphylococcal isolates; this result was in agreement with [27] where, 57.8% were biofilm formers by MTP method. However, 83.3% of *S. aureus* and 88.6% of *S. epidermidis* isolated from catheterized patients produced biofilm by the MTP assay [28]. As regard TM method, biofilm formation was detected in 68% of 50 staphylococcal isolates; this result is similar to that found by [29] where 60% of staphylococcal isolates showed biofilm formation by TM. A higher rate of biofilm formation by TM were detected by [5] where, 84% of staphylococcal isolates showed biofilm formation. On the other hand, 88% of 50 staphylococcal isolates were positive by CRA; this result was in accordance with the finding of [5] found that 90% of staphylococcal isolates were positive by CRA.

Many reports indicated that biofilm formation in staphylococci causing catheter associated and

nosocomial infections is associated with icaA and icaD genes [13]. Expected genes were detected in 56% of staphylococcal isolates. Similary, [27] detected icaA & icaD genes in 54% of staphylococcal isolates. A higher rate of icaA & icaD genes detection was recorded by [9],[13] as 100% of staphylococcal isolates harbored icaA & icaD genes and produced biofilm.

In the cohort study, by comparing the phenotypic biofilm detection techniques with the icaA & icaD genes detection, 96.4% staphylococcal isolates of the icaAD-positive were positive by TM & CRA methods. This finding was in agreement with [13] as all icaA & icaD positive staphylococal isolates 100% were positive by TM. However, in contrast to this finding, [30] found that 69.2% of *S. aureus* icaA-positive were biofilm producers by MTP & CRA methods. Also, [31] stated that only 58% of *S. epidermidis* strains positive for the ica operon were biofilm producers by MTP & CRA methods.

The currant study showed that 3.5%, 3.5%, 39.2% of the icaAD-positive staphylococcal isolates were negative by TM, CRA & MTP, respectively in spite of presence of icaA & icaD genes. However, 12%, 26%, 32% of the staphylococcal isolates were icaA & icaD genes negative but were biofilm formers by TM, MTP & CRA methods, respectively. This finding was in agreement with [7] who reported that the presence of icaADBC gene is not always associated with in vitro formation of biofilm.

The correlation between the sensitivity and specificity of the phenotypic biofilm detection methods with icaA & icaD genes displayed that 96.4%, 96.4% and 53.5% sensitivity for TM, CRA and MTP methods respectively. While they were 68.1%, 22.7%, 40.9% specificity, respectively when compared to PCR. Similar sensitivity but better specificity was 100%, 100% respectively as reported by [13]. In accordance with this finding a similar sensitivity but a better specificity was reported by [31] where the sensitivity and specificity of the CRA when compared to ica genes was 93.3% and 87.5% respectively. While, [3] reported that the CRA method shows 81.8 % sensitivity, 100% specificity. In contrast [27] reported a lesser sensitivity but higher specificity than those detected in the current study, where the sensitivity and specificity of the CRA when compared to ica genes was 31.25% and 47.05%, respectively.

Bacterial identification, screening of biofilm producers followed by susceptibility tests are important for antimicrobial selection for treatment. For this purpose susceptibility pattern of biofilm formers was performed in this study**.** Results revealed that as regard *S. aureus* the highest resistance was detected against ampicillin and ampicillin-salbactam as all *S. aureus* isolates 100% were resistant to them, while 94.4% were resistant to ceftrixone. Resistance to cefazoline, gentamycine and erythromycin were detected in 66.6% of isolates, while 55.5% were oxacillin (MRSA) resistant. The least resistance was to meropenem 33.3% and vacomycin as only 11.1% of isolates showed resistance (VRSA). These were in accordance with [32]. *S. epidermidis* also showed the highest resistance against ampicillin, ampicillin salbactam and ceftrixone as all of the isolates 100% were resistant to them, while resistance to cefazoline, gentamycin, erythromycin were 66.6%, 33.3% and 33.3% respectively, while all *S. epidermidis* isolates were sensitive to oxacillin, vacomycin and meropenem. These were in agreement with results detected by [33] Where resistance to penicillin were 100% while resistance to erythromycin and gentamycin were 75% and 50% respectively were detected in *S. epidermidis* isolates isolated from catheter related blood stream infections. By comparing the pattern of antibiotic resistance of biofilm formers staphylococci according to their intensity in biofilm formation, there was a statistical significant difference between antibiotic resistance among strong, moderate and weak biofilm former as strong biofilm formers (2VRSA & 3MRSA) were more resistant to all tested antibiotics compared to moderate and weak biofilm formers. Similar results were detected by [32] who reported presence of a statistical significant difference between antibiotic resistance patterns among staphylococcal isolates biofilm formers with different intensities. In contrast to this finding, [5] reported presence of non statistical significant difference between antibiotic resistance pattern among biofilm formers and non biofilm formers staphylococcal isolates.

In the present study, antibiotic susceptibility of planktonic cells presented as MIC was compared to MBIC of their counterpart sessile cells. Vancomycin and meropenem were used as they were the most effective antibiotics. A great difference in antibiotic susceptibility between planktonic populations and biofilm populations, whereas, MBIC>128µg/ml & MIC=2-4µg/ml of 88.8% and 66.6% of *Staph aureus* isolates as regard vancomycin and meropenem susceptibility, respectively. Parallel results were described by [34],[7] who compared the vancomycin MIC and MBIC for staphylococcal isolates found that totally isolates existing higher vancomycin MBIC than the MIC. This result may be described by the decreased diffusion of antimicrobial agents through the extensive biofilm matrix, along with the decreased metabolic activity of bacteria within biofilms and the increase in gene transfer. These results confirm that the concentration required to eradicate biofilms is higher than that required to inhibit planktonic cells. The MIC assay is a commonly used method to test antibiotic efficacy because it is quick and reproducible. However, it is not an effective assay for testing antibiotics against adherent biofilm [35].

CONCLUSION

Our findings reinforce the importance of both genotypic and phenotypic methods for optimum evaluation of the biofilm producing ability of *Staphylococci*. Moreover, TM is recommended as a general screening method for detection of biofilm in neonatal nosocomial infections because of its easy, reliable results with excellent sensitivity, as compared to CRA and MTP methods. Large scaled studies are needed for better evaluation of *staphylococci* biofilm forming capacity and multidrug resistance that would surely help the biofilm formation fighting.

Conflicts of interest:

The authors state that there are no conflicts of interest.

REFERENCES

1. Oliveira A, Sanches P, Lyra JC, Bentlin MR, Rugolo LM, and Cunha MLR. Risk Factors for Infection with Coagulase-Negative Staphylococci in Newborns from the Neonatal Unit of a Brazilian University Hospital Clin Med Insights Pediatr. 2012; 6:1–9.
2. Mendoza-Olazaran S, Morfin OR, Vilianeal TL, Rodrigeuez NE, Liaca DJ, Ortiz A, et al. Antibiotic susceptibility of biofilm cells and molecular Characterization of of *Staphylococcus* hominis isolated from blood. PlosOne Dol: 2015; 10.1371/Jornal.Pone.0144684
3. Hashem A. A., Elazab S. Z. , Metwally L. A., Nasr El Islam H. and Mansour M. K. Biofilm Production and Antibiotic Resistance of *Staphylococcus epidermidis* in Catheter Related Bloodstream Infections. Egyptian Journal of Medical Microbiology. 2017; 26(4): 73-80.
4. Zarnowski R, Westler WM, Lacmbouh GA, Marita JM, Bothe JR, Bernhardt J, Sahraoui AL-H, Fontaine J, Sanchez H and Hatfield RD. Novel entries in a fungal biofilm matrix encyclopedia. MBio. 2014; 5(4) e01333-14.
5. Rewatkar AR & Wadher BJ. *Staphylococcus aureus* and *Pseudomonas aeruginosa*- Biofilm formation Methods Journal of Pharmacy and Biological Sciences. 2013; 8(5), P: 36-40
6. Zubair M, Ashraf M, Arshad M, Raza A ,Mustafa B and Ahsan A. Formation and Significance of Bacterial Biofilms. Int. J.Curr.Microbiol.App.Sci. 2014; 3(12): 917 -923
7. El Moufti MF, Baddour M, Harfoush RA, Owais HM. Characterization of Some Genotypic and Phenotypic Traits of Biofilm Producing Clinical Isolates of Methicillin Resistant *Staphylococcus* Epidermidis. American Journal of Infectious Diseases and Microbiology. 2015; 3(3): 95-103
8. Barbieri R, Pesce M, Franchelli S, Baldelli I, De MA and Marchese A. Phenotypic and genotypic characterization of Staphylococci causing breast peri-implant infections in oncologic patients. BMC Microbiology. 2015; 15:26
9. Serray B, Oufrid S, Hannoui I, Bourjilate F, Soraa N, Mliji M, Sobh M, Hamouni A, Timinouni M, El Azhari M. Genes encoding adhesion factors and biofilm formation in Methicillin Resistant *Staphylococcus* in Moracco. J. Infect. Dev. Ctries. 2016; 10(8):863-869
10. Arciola CR, Campoccia D, Baldassarri L, Donati ME, Pirini V, Gamberini S. Detection of biofilm formation in *S.epidermidis* from implant infections. Comparison of a PCR-method that recognizes the presence of ica genes with two classic phenotypic methods. J Biomed Mater Res A. 2006; 76(2): 425–4230.
11. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T& Rattan A. Detection of biofilm formation among the clinical isolates of *staphylococci*: an evaluation of three different screening methods. Indian J Med Microbiol. 2006; 24:25–29.
12. Christensen GD, Simpson WA, Anglen JO, and Gainor BJ. Methods for evaluating attached bacteria and biofilms: an overview, Handbook of bacterial adhesion: principles, methods, and applications, Humana Press, Totowa, N.J. 2000; pp. 213–233.
13. Oliveira A & Lourdes CM RS. Comparison of methods for the detection of biofilm production in coagulase-negative *staphylococci*. BMC Res Notes. 2010; 3:260-2.
14. Roth PE, Martín CF, Villar J and Álvarez MS. Multiplex PCR For simultaneous identification of *staphylococcus aureus* and detection of mithicillin and mupirocin resistence .J.Clin Microbio. 2001; 39(11):4037-4041
15. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. 9 th ed. CLSI Document M7-A7. Wayne, PA. 2012.
16. Ilstrup DM. Statistical methods in microbiology. *Clinical microbiology reviews*. 1990; 3(3):219-226.
17. Chan GJ, Lee ACC, Baqui AH, Tan J and Robert E Black. Prevalence of early-onset neonatal infection among newborns of mothers with bacterial infection or colonization: a systematic review and meta-analysis: BMC Infectious Diseases. 2015; 15:118-202

18. Zhong MPD, Johnson Y, Revell P and Versalovic J. Polymicrobial blood stream infections in the neonatal intensive care unit are associated with increased mortality: a case-control study BMC Infectious Diseases. 2014; 14:390.
19. Kishk RM, Mandour MF, Farghaly RM, Ibrahim A, and Nemr NA. Pattern of Blood Stream Infections within Neonatal Intensive Care Unit, Suez Canal University Hospital, Ismailia, Egypt. International Journal of Microbiology. 2014; Article ID 276873, 6 pages.
20. Shehab El-Din EM, MohamedM. El-Sokkary A, Bassiouny MR, and Hassan R. Epidemiology of Neonatal Sepsis and Implicated Pathogens: A Study from Egypt: BioMed Research International. 2015; Article ID 509484, 11 pages
21. Golia S, Sangeetha KT., and Vasudha CL. Microbial Profile of Early and Late Onset Ventilator Associated Pneumonia in The Intensive Care Unit of A Tertiary Care Hospital in Bangalore, India. J. Clin Diagn Res. 2013; 7(11): 2462–2466.
22. Petdachai W. Ventilator-associated pneumonia in a newborn intensive care unit. Southeast Asian J Trop Med Public Health. 2014; 35(3):724-729.
23. Labib AZ, Ahmed B ,Mahmoud AB, Eissa NAA, El Gendy FM, Soliman MA and Aly AA. Early Diagnosis of Neonatal Sepsis: A Molecular Approach and Detection of Diagnostic Markers versus Conventional Blood Culture International Journal of Microbiological Research. 2013; 4 (1): ISSN 2079-2093, pp77-85,
24. Tripathi S, D.G.K.M, Amita J and Kohli N. Study Of Ventilator Associated Pneumonia In Neonatal Intensive Care Unit:Charecterisitcs, Risk Factors And Outcome. Internel Journal of Medical Update, 2010. 5(1): p. 12-19
25. Klevens RM, Morrison MA & Nadle J. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States, Journal of the American Medical Association, American Medical Association, Chicago, IL. (2007); 289: pp. 1763-1771,
26. Campoccia D, Montanaro L, Renata Arciolaa C. The significance of infection related to orthopedic devices and issues of antibiotic resistance. Biomaterials. 2006; 27 :2331– 2339
27. Naser RA, AbuShady HA, Hussein HS. Biofilm formation and presence of icaAD gene in clinical isolates of *staphylococci*. The Egyptian Journal of Medical Human Genetics. 2012; 13: 269–274
28. Gad GFM, El-Feky MA, El-Rehewy MS, Hassan MA, Abolella H& El-Baky RMA. Detection of icaA, icaD genes and biofilm production by *S.aureus* and *S.epidermidis* isolated from urinary tract catheterized patients. J Infect Dev Ctries. 2009; 3:342–51.
29. Gunti R,Usha UR and Arava DR. Detection of biofilm production in Staphylococcus aureus and coagulase negative Staphylococci using three different methods. J Pharm Biomed Sci; 2013; 37: 1952-1956.
30. Yousefi M., Pourmand M. R. , Fallah F., Hashemi A., Mashhadi R. and Nazari-Alam A. Characterization of *Staphylococcus aureus* biofilm formation in urinary tract infection. Iran J. Public Health. 2016; 45(4): pp. 485-493.
31. Abou El-Khier NT, El-Kazzaz SS and Elganainy A. Phenotypic and Genotypic Detection of Biofilm Formation in *Staphylococcus epidermidis* Isolates from Retrieved Orthopaedic Implants and Prostheses. British Microbiology Research Journal.. 2015; *9*(4): 1-10.
32. Samie A & Shivambu N. Biofilm production and antibiotic susceptibility profiles of *Staphylococcus aureus* isolated from HIV and AIDS patients in the Limpopo Province, South Africa African Journal of Biotechnology. 2011; 10(65), pp. 14625-14636.
33. Cherifi S, Byl B, Nagant A D C, Nonhoff C, Denis O and Hallin M. Genetic characteristics and antimicrobial resistance of *S.epidermidis* isolates from patients with catheter-related bloodstream infections and from colonized health care workers in a Belgian hospital. Annals of Clinical Microbiology and Antimicrobials. 2014; 4, pp. 13:20
34. Antunes AL, Trentin DS, Bonfanti JW, Pinto CC, Perez LR, Macedo AJ, Barth AL. Application of a feasible method for determination of biofilm antimicrobial susceptibility in staphylococci. APMIS. 2010; 118(11): 873-877.
35. Mostafa MAM, Aly AA, Abdel-Latif W, Alaam RAM. Biofilm production by *Pseudomonas aeruginosa* Clinical Isolates and its Relationship with Pseudomonas Quinolone Signal *(pqsA*) Gene and Antibiotic Resistance. Egyptian Journal of Medical Microbiology. 2017; 26 (4): 19-27.