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Detection of Biofilm in Surgical Site Infection by Microtiter Plate Method and Its Correlation with icaD, and icaA genes in *Staphylococcus* Spp

Mostafa I. EL-Amir[1,*], Asmaa Gaber R[2], Mohamed Yousef A[2], Tunis Gamal[1], Mohamed Ali El-Feky[1,3]

[1]Department ofMedicalMicrobiology and Immunology, Faculty ofMedicine, South Valley University, Qena, Egypt
[2]Department of general surgery, Faculty of Medicine, South Valley University, Qena, Egypt
[3]Department of Medical Microbiology and Immunology, Faculty of Medicine, Assiut University, Assiut, Egypt
*Corresponding author: mostafaismail81@yahoo.com

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Abstract Purpose: To determine the bacteriological pattern of bacteria causing surgical site infection (SSI) in Qena University Hospitals and detection of biofilm producing organisms. Methodology: Wound swab from 114 patients with SSI were collected for identification of bacteria and detection of biofilm producing organisms by microtiter plate (MTP) method and tube method (TM). icaA and icaD genes were detected in *Staphylococcus spp.* by polymerase chain reaction (PCR) from extracted bacterial DNA Principal Findings:Patients mean age was 33.6 ± 12.7 years. 56.1% of the patients were enrolled to emergent surgeries. Diabetes mellitus was the most common risk factor detected in 17.5%. The highest infection rates were noticed after appendectomy, then followed by cholecystectomy (45.6% and 36.8% respectively). Most of bacteria isolated were gram-negative bacteria 67.2%. *Escherichia coli* was the most frequent bacteria (32.7%), followed by *Klebsiella pneumonia* (26.2%), *Staphylococcus aureus* (22.9%), *Staphylococcus epidermidis* (9.8%), *Pseudomonas aeruginosa*(5.7%), *then Proteus spp* (2.5%). Significant biofilm production were detected between *Staphylococcus aureus* and *E. coli*spp by MTP method (P value <0.001 and 0.001 respectively). Significant difference between MTP method and TM in detection of biofilm producers (77.8% and 32.7% respectively), (P value <0.001). icaD gene was detected in 62.5% of *Staphylococcus* strains, While all *Staphylococcus strains* were negative for icaA gene. All positive strains for icaD gene were strong biofilm producer by MTP method. Conclusion: MTP was better than TM in detection of biofilm formation. icaD gene were positive in strong biofilm producer *Staphylococcus* by MTP method while icaA not.

Keywords: *surgical site infections, biofilm production, microtiter plate method, icaD gene, icaA gene*

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1. Introduction

Surgical site infection(SSI) is defined as infection which occur within one month after the surgery or within one year if an implant is present . It considered the 3rd commonest cause of hospital acquired infections (HAI). In addition to its association with high morbidity and mortality , it also increase the length of hospital stay [1]. Several patient related risk factors are associated with SSI as obesity, diabetes mellitus, smoking, malnutrition, malignancy, and immunosuppressive medications [2]. Diabetes mellitus and nicotine use increased the risk of SSI for 2-5 folds and the two conditions cannot be controlled during emergency situations [3]. Site of surgery as abdominal operations are associated with high incidence of SSI due to high concentration of bacteria in intestine [4].

Biofilm is a matrix produced by bacteria containing extracellular polymeric substance (EPS) which composed of polysaccharides, glycoproteins, and nucleic acid [5]. It allows bacteria to survive in hostile environment not affected by antibiotics or host defense causing delay in the wound healing rate. Extracellular DNA can be exchanged easily from bacteria to other inside the biofilm allowing resistance characters to spread easily [6].

Several methods are used to detect bacterial biofilm in the wound as qualitative congo red agar, tube test, microtiter plate test, and modified microtiter plate test [7,8]. Modified microtiter plate method considered of high accuracy than other methods as it allows to measure the bacteria attached to the bottom and the walls of the wells.

Different bacterial genes are responsible for regulation of biofilm forming through expression of specific genes. *Staphylococcus aureus* and *Staphylococcus epidermidis* contain intracellular adhesion (icaADBC) operon especially icaA and icaD regulate the production

of biofilm. Formation of N-acetylglucosamine oligomer by N-acetylglucosaminyltransferase encoded by icaA gene. While icaD has an critical role in expression of capsular polysaccharides [9].

In this study we aimed to determine different bacterial species causing SSI in Qena university hospitals. And to compare the accuracy of different techniques of biofilm detection in addition to determine the presence of icaA and icaD genes in *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from our patients.

2. Patients and Methods

The study was conducted from July 2017 to January 2018 at general surgery department in Qena University Hospitals. One hundred and fourteen patients were included in our prospective case control study. The study included any surgery complicated with SSI within one month after operative procedure or one year if implant was present.

Full history was taken including age, sex, smoking, diabetes mellitus, any chronic debliting disease, malignancy, admission due to elective surgery or emergency, method of hair removal (shaving or clipping of hair in surgical site), and prophylactic administration of antibiotics. The study was approved by the Ethics Committee, Qena Faculty of Medicine, South Valley University. The study was conducted in agreement with the principles of the Declaration of Helsinki.

A total of 114 wound swabs were collected from SSI after wound cleaning with physiological saline. Samples were immersed in nutrient broth then directly sent to our laboratory. All swabs were sub cultured on blood agar and incubated at 37oC for 24hours. Colonies were identified by colony morphology, gram staining, different biochemical tests as triple sugar iron, Simmon's citrate agar ,catalase, and oxidase test [10].

Biofilm production was detected by two different methods. The first one by modified micro titer plate method (MTP) by using 96 wells flat bottom microtiterplate. Biofilm production by bacteria coating the well was detected by ELISA reader at wave length 570 nm. Biofilm production is considered high (OD more than 0.24), moderate (OD equal 0.12-0.24), or weak (OD less than 0.12), as described by Stepanovic*et al. (*2000) [8]. Also biofilm production was detected by tube method as described previously [7,11].

For molecular analysis to detect icaA and icaD genes in *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates, DNA extraction was done by boiling method as described by Gad *et al.(*2009) [12]. The sequence of forward and reverse primers to detect icaA gene were 5'-TCTCTTGCAGGAGCAATCAA-3', and 5'-TCAGGCACTAACATCCAGCA-3' respectively. While the primers used to detect icaD gene were: forward primer 5'- ATGGTCAAGCCCAGACAGAG 3', and reverse primer 5'CGTGTTTTCAACATTTAATGCAA 3'. Reaction volume was 25µl containing;1 µl of each the forward and reverse primer, 12.5 µl of GeneTaq Green PCR Master Mix (GENETIX BRAND, Germany) , 2µl of the extracted bacterial DNA, and 8.5 µl of distilled water. The thermal cycling conditions consist of an initial denaturation step at 94℃ for 5 min, and followed by 50 cycles of 30 seconds at 94℃, 30 seconds at 55.5℃, and 30 seconds at 72℃, lastly final extension step at 72℃ for 1 min.

PCR product was 188 base pairs(bp) for icaA gene and 198bp for icaD gene as shown in (Figure 1). PCR product were separated by agrose gel electrophoresis (1.5%) containing ethidium bromide(5 µl/100ml) then visualized by UV transillumination.

Statistical analysis was done using SPSS software version 22. Categorical variables were presented as proportions and percentages. Numerical variables were presented as mean and standard deviation when normally distributed, and median and interquartile ranges when not normal distribution. Chi-square test or Fisher exact test were performed when appropriate. P value of 0.05 or less was considered significant.

3. Results

From 114 patients, 41(35.9%) of the cases were males and 73(64.1%) were females. The age of the patients varied between 15- 75 years old with mean 33.6 ± 12.7 years. About one third of the patients(36.8%), their age ranged from 25-34 years. 64 patients (56.1%) were enrolled to surgery due to emergency situations, while 50 patients (43.9%) were considered as elective cases and admitted in the hospital one day before the surgery. Diabetes mellitus was detected in 17.5% (20/114) of the patients. Higher infection rates were noted after appendectomy (45.6%), then followed by cholecystectomy (36.8%), hernia surgeries (6.14%), common bile duct exploration (3.5%), perforated peptic ulcer (3.5%), colon cancer surgery (2.6%), and lastly mastectomy (1.75%), (Table 1).

Table 1. Clinical characters of patients with wound infection

	Number of cases = 114 (%)
Age	33.6 ±12.7
Sex Male Female	 41(35.9%) 73(64.1%)
Surgery situation emergency Elective surgery	 64 (56.1%) 50 (43.9%)
Risk factors Diabetes mellitus Smoking obesity Anemia Malignancy	 20 (17.5) 16 (14) 11 (9.6) 10 (8.7) 5 (4.3)
Site of surgery Appendicectomy Cholecystectomy Hernia surgeries Common bile duct exploration perforated peptic ulcer Colon Cancer Surgery Mastectomy	 52 (45.6) 42 (36.8) 7 (6.14) 4 (3.5) 4 (3.5) 3 (2.6) 2 (1.75)

The total number of bacteria isolated from our patients were122.Mixed infections were recognized in seven patients (6.14%). Two different bacterial species were isolated from six patients while three different bacterial species were isolated from one patient. Most of bacteria isolated were gram-negative bacteria 82/122 (67.2%) while the remaining were gram-positive

bacteria 40/122 (32.7%). *Escherichia coli* was the most frequent bacteria isolated 40/122 (32.7%), followed by *Klebsiella pneumonia* 32/122 (26.2%), *Staphylococcus aureus*28/122(22.9%), *Staphylococcus epidermidis* 12/122 (9.8%), *Pseudomonas aeruginosa*7/122(5.7%), *then Proteus spp*3/122(2.5%).

As shown in (Table 2) biofilm production by MTP method; all species of *Staphylococcus aureus* and *Proteus* were biofilm producers, 26/28(92.9%) of *Staphylococcus aureus*produced biofilm strongly, while 2/28 (7.1%) formed biofilm moderately. All *Proteus* species were moderately biofilm producers. In 83.3% (10/12) of *Staphylococcus epidermidis*biofilm was detected ,4/12 (33.3%) with moderate biofilm formation, and 6/12(50%) showed strong biofilm formation. According to*Klebseillaspp*, biofilm was detected in 26/32 (81.25%). In 17/32(53.13%) biofilm detected strongly while in 9/32 (28.13%) biofilm detected moderately .In 60% of *E. coli*spp, (24/40) biofilm was detected; 15 species (37.5%) formed biofilm moderately; while 9 species (22.5%) formed it strongly. In *Pseudomonas spp*,4/7 (57.1%) were moderate biofilm producers, while in 3/7 (42.8%) no biofilm was detected and none of species showed strong biofilm formation. Significant biofilm production were detected between *Staphylococcus aureus* and *E. coli*spp (P value <0.001 and 0.001 respectively).

The biofilm formation by different microorganisms was detected also by tube method assay (TM) . *According to E. coli*spp, 15/40 (37.5%) were biofilm producing, while 25/40 (62.5%) were non-biofilm producing. *Also Klebseilla*spp, 12/32 (37.5%) were biofilm producing while 20/32 (62.5%) were non-biofilm producing. In *staphylococcus aureus* 10/28 (35.7%)were biofilm producing while 18/28 (64.2%) were non-biofilm producing. *Proteus*spp, 1/3 (33.3%) were biofilm producing organisms while 2/3 (66.6%) were non-biofilm producing organisms. *Pseudomonas* spp, 2/7 (28.5%) were biofilm producing organisms, while 5/7 (71.4%) were non-biofilm producing. All *Staphylococcus epidermidis* were non-biofilm producing.

As shown in Table 3, Biofilm production detected in 95/122 (77.8%) of the isolates with MTP method while tube method detected biofilm formation in 32.7% (40/122) of the isolates. Significant difference between the two techniques in detection of biofilm production (P value <0.001).

28 strains of *Staphylococcus aureus* and *12 strains of Staphylococcus epidermidis*strains were subjected to molecular study to detect icaA and icaD genes. 23 strains of *Staphylococcus aureus*and two strains of *Staphylococcus epidermidis* were found to be positive for icaDgene Figure 1. All positive strains for icaD gene were strong biofilm producer by MTP method.icaD gene was detected in 62.5% of *Staphylococci* strains, While all *Staphylococci* were negative for icaA gene.

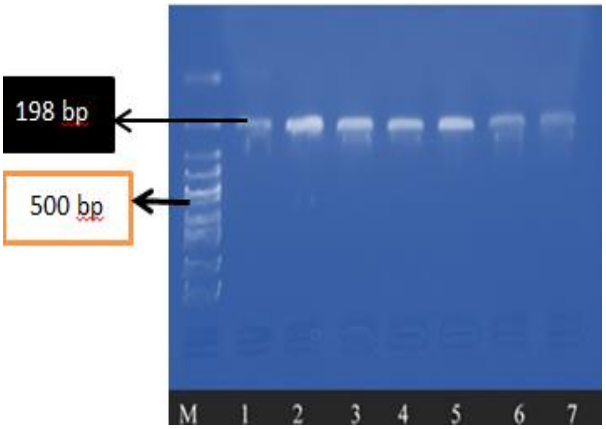

Figure 1. Gel electrophoresis showing bands of PCR product of icaD gene. Lane M showing 100bp DNA ladder from 100-1000 bp. lane 1-7 showing amplified PCR product at 198 b

Table 2. Detection of biofilm producers organisms by microtiter plate (MTP) method

Micro-organisms	Non-Biofilm producing organisms N=27	Biofilm producing organisms N=95		Chi-square	P value
		Moderate M (%)	Strong S (%)		
E. coli N=40	16 (40%)	15 (37.5%)	9 (22.5%)	(0.095-0.570)	0.001*
		24(60%)			
Klebseilla N=32	6 (18.7%)	9 (28.13%)	17(53.13%)	(0.40-3.16)	0.5
		26(81.25%)			
Staphylococcus aureus N=28	0 (0%)	2 (7.1%)	26 (92.8%)	--------	< 0.001*
		28(100%)			
Staphylococcus epidermidis N=12	2 (16.6%)	4 (33.3%)	6 (50%)	(0.30-7.15)	0.4
		10(83.3%)			
Pseudomonas spp. N=7	3(42.8%)	4 (57.1%)	0 (0%)	(0.07-1.67)	0.18
		4 (57.1%)			
Proteus spp N=3	0 (0%)	3 (100%)	0 (0%)	------	0.35
		3 (100%)			

Table 3. Difference between microtiter plate method (MTP) and tube method (TM) in detection of biofilm producer organisms

		Microtiter plate test		Total N(%)	Chi-square	P Value
		Negative N(%)	Positive N(%)			
Tube method	Negative	26(21.3)	56(45.9)	82(67.3)	0.007-0.42	<0.001*
	Positive	1(0.8)	39(31.9)	40(32.7)		
Total		27(22.1)	95(77.8)	122		

4. Discussion

In our study, diabetes mellitus was the commonest risk factor between our patients as 17.5% were diabetic. This finding was similar to Cheng *et al.*(2015) [13]. Mukagendaneza*et al.(*,2019)found that the commonest age group of SSI was ranged from 26-45 years and less incidence of SSI between elective surgeries [14], which was similar to our results as most of our patients were ranged from 25-35 years and (56.1%) of our patient were in emergency situations for surgery at the time of admission.

In our study, gram-negative bacteria was the commonest cause of SSI (67.2%) and the most frequent microorganism between gram negative bacteria was *Escherichia coli* (32.7%), while *Staphylococcus aureus* was the causative organism in (22.9%) of wound infection. Most of surgeries in our study were abdominal surgeries which contain high concentrations of bacteria in intestine and associated with wound contamination [15]. Alkaaki*et al.,(*2019) found that*Escherichia coli* was the commonest organism causing SSI after abdominal surgery while gram positive bacteria causing 37% of SSI which nearly similar to our result [16].

In this study we examined 122 isolates by two screening methods for their ability to form biofilms. The MTP method and compared with data from the tube method.By MTP method, biofilm production was detected in different bacteria as *Staphylococcus aureus* , *Proteus, Staphylococcus epidermidis, Klebseillaspp, E. coli*spp, *and Pseudomonas spp as described previously* [17,18]. Significant association with biofilm production was associated with *Staphylococcus aureus* and *E. coli*spp aspreviouslydescribed [19,20].

In our study, the MTP method detected biofilm production in (77.8%), while TM detected biofilm production in (32.7%). This data was similar to which was found by previously that MTP method and its modification were more accurate than TM as the later depend on observer interpretation [8]. In our study the sensitivity and specificity of TM were 41% and 96.3% respectively.

In our study, icaD gene was detected in 62.5% of *Staphylococci* strains, While all *Staphylococci* were negative for icaA gene. This result disagreed with Mirzaee*et al.,* (2014) who found that *icaD*gene detected in 80.6% of the isolates carried, whereas *icaA, icaB*and *icaC* were detected in 51.6%, 45.1% and 77.4% respectively [21].

Gad *et al.,* (2009)found that all biofilm producing staphylococci strains were positive for icaA and icaD genes [12]. While Aboelnour*et al.*, (2018) found that the most frequently detected genes among S. aureus were icaR (68.2%), icaC (63.6%), ica D (60.6%), fibA (56.1%), fib (53.0%), icaB (51.5%) and icaA (30.3%). 12% of the isolates with biofilm formation capacity had no any genes of the seven genes studied. other genes or other mechanisms were implicated in formation of biofilm. icaR was detected by higher frequency than other genes [22]. This may be explained by the role played by icaR genes as a regulatory gene for biofilm production.

5. Conclusions

Escherichia coli was the commonest organism causing SSI after abdominal surgery. MTP was better than TM in detection of biofilm formation.icaD gene were positive in strong biofilm producer *Staphylococcus* by MTP method while icaA not.

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