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Characterization of Some Genotypic and Phenotypic Traits of Biofilm Producing Clinical Isolates of Methicillin Resistant *Staphylococcus Epidermidis*

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Abstract *Staphylococcus epidermidis* is a major commensal bacterium. Various strains of methicillin resistant *S. epidermidis* are capable of forming biofilms and it is found to be associated with many hospital-acquired infections. Bacterial biofilms, which are micro-colonies encased in extracellular polysaccharide material, mediated by gene products of the *icaADBC* operon, are the sources of many bacterial infections which is so difficult to respond to routine treatments. In this research, we investigated the biofilm forming capacity of a 100 methicillin resistant *staphylococcus epidermidis* isolates, isolated from different clinical specimens delivered to the Diagnostic Microbiology Laboratories and Surveillance Laboratory, Faculty of Medicine, Alexandria University, in relation to the *icaADBC* gene cluster. Also, the minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) assays were used to evaluate the antibiotic sensitivity patterns of these MRSE isolates in their planktonic and biofilm phases to Vancomycin and Linezolid. The results showed that only 27 isolates (27%) produced detectable biofilm, and *icaADBC* gene was detected in only 5 of these isolates. Moreover, there was no statistical association between the presence of the gene and the biofilm status. All 27 biofilm producing isolates were susceptible to both Vancomycin and Linezolid in their planktonic state, but the MBEC values of Vancomycin were higher than those of Linezolid in almost all strains, with an agreement between both MBEC values in 15/27 (55.5%) of isolates and disagreement in 12/27 (44.5%) of isolates, and this was statistically significant ($p<0.05$). In conclusion, this study indicates that the presence of *icaADBC* gene is not always associated with *in-vitro* formation of biofilm. Although Vancomycin and Linezolid continued to be effective for planktonic MRSE infection, their sub-MIC concentrations can induce biofilm formation.

Keywords: *methicillin, S.epidermidis, biofilm, planktonic, vancomycin, Linezolid, icaADBC*

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1. Introduction

Staphylococcus epidermidis (*S. epidermidis*) is now considered one of the most frequent causes of nosocomial infections. In immune competent humans, *S. epidermidis* mainly become pathogenic when associated with indwelling medical devices, such as arteriovenous shunts, contact lenses, urinary and central venous catheters, orthopedic devices, and peritoneal dialysis catheters. [1] *S. epidermidis* infections are seldom lethal, but they significantly contribute to morbidity and health care costs [2].

Hospital-acquired *S. epidermidis* often display resistance against many antimicrobials in use today, such as methicillin and aminoglycosides. [3] The methicillin resistance in staphylococci is mediated by the *mecA*-gene encoding a penicillin-binding protein (PBP2a) with reduced affinity for all beta lactam antibiotics. [4] The *mecA* gene is integrated in the *SCCmec* element. [5] Glycopeptide resistance in *S. epidermidis* is still relatively rare and vancomycin is the drug of choice for methicillin-resistant *S. epidermidis* (MRSE). [6] However, the efficacy of high-dose vancomycin therapy is not confirmed and its toxicity is a major concern. Therefore, combination or alternative therapies may be considered for invasive infections caused by these strains. [7] Some novel drugs with excellent in vitro activity, including linezolid and tigecycline, have been approved for vancomycin resistant strains. [8] However, haematological side effects of long-term linezolid use and low serum level of tigecycline limit their clinical application [9].

It has been widely accepted that the virulence of CoNS infections depends mostly on their ability to form biofilms on polymer surfaces. [2,10] Biofilm is a structured community of bacterial cells adherent to an inert or living surface and/or embedded in a self-produced extracellular polymeric substances (EPS) matrix. [11] The bacteria

within the biofilms are protected from physical, chemical and biological stresses, including antimicrobial agents, antibodies and the antimicrobial products of phagocytic cells [12].

The genes encoding the most important substances and proteins, especially a polysaccharide adhesin, that participate in biofilm formation belong to the intercellular adhesin (*ica*) operon. This operon contains the *icaADBC* genes and the regulatory *icaR* gene. The synthesis of the polysaccharide capsule is mediated by the *ica* operon. When this operon is activated a polysaccharide intercellular adhesin (PIA) and poly-succinyl-glucosamine are synthesized and support cell-to-cell contacts by means of a multilayer biofilm [13].

The mechanism of biofilm resistance is multifactorial. [14] Treatment with antibiotics may kill planktonic bacteria (free living bacteria) shed from the biofilm surface; however, they fail to eradicate those embedded within the biofilm, which can then subsequently act as a nidus for recurrent infection [15].

Minimum inhibitory concentration (MIC) is the standard laboratory method by which planktonic bacterial susceptibility to antibiotics is quantified. However, MIC does not provide a true estimation of the concentration of antibiotics required to treat a bacterial biofilm. The minimum biofilm eradication concentration (MBEC) is a measure that allows that determination to be made for a biofilm [16].

In the present study, we investigated the biofilm forming capacity of a group of MRSE isolates, in relation to the *icaADBC* gene cluster. Also, the MIC and MBEC assays were used to evaluate the antibiotic sensitivity patterns of these MRSE isolates in their planktonic and biofilm phases to vancomycin and linezolid.

2. Material and Methods

- *Bacterial strains:* One hundred isolates of (MRSE) isolated from different clinical specimens delivered to the Diagnostic Microbiology Laboratories and Surveillance Laboratory, Faculty of Medicine, Alexandria University, constituted the material of this study. The isolates were mostly from blood (48%), followed by pus (32%), urine (9%), BAL (9%) and (2%) were from nasal swabs. Identification of *S. epidermidis* was carried out using the standard biochemical tests including catalase, DNase and coagulase production, growth and fermentation of mannitol on mannitol salt agar [17] and susceptibility to novobiocin (≥ 16 mm) by the disc diffusion test [18]. Bacteria were maintained in brain heart infusion (BHI) 15% glycerol broth and immediately frozen at -20ºC.
- Methicillin resistance was identified by:
  a. Resistance to cefoxitin (30µg) disc by standard disc diffusion susceptibility test, with zone diameter ≤ 24 mm. [19]
  b. Polymerase chain reaction to detect the 310 bp product of *mecA* gene:

  DNA extraction was performed as follows [20]: few S. *epidermidis* colonies from subculture on blood agar plate were suspended in 50 µl of lysis solution (0.02 M NaOH/SDS and 0.1% SDS (Sodium dodecyl sulphate)), heated at 95^0C for 15 min. and 450 µl sterile distilled water was added to the suspension and centrifugation at 9000 rpm for 5 min was performed. Three µl of the supernatant were used as a template in the PCR. The following primers were used: forward (5'- TGG CTA TCG TGT CAC AAT CG-3') and reverse (5'- CTG GAA CTT GTT GAG CAG AG-3'). *MecA* positive strain (ATCC 33591) was included as positive control. Amplification reaction was carried out in 25µl volume, under the following conditions: Initial denaturation at 92°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 30 seconds, followed by final extension at 72°C for 3min.
- The identified MRSE were subjected to the following:
  a- *Study of biofilm production* by microtitre plate method (MTP), as follows [13]: isolates of staphylococci were cultured overnight in Tryptone-Soya broth (TSB) (Oxoid, UK)) supplemented with 1% glucose (TSB_{glu}). A volume of 200µl was transferred to each of two wells of the sterile 96 wells microtiter polystyrene tissue culture plate. (Becton Dickinson, Franklin Lakes, NJ, USA), so that each isolate was tested twice. After cultivation for 24 hr at 37°C, the contents of the wells were discarded and the wells were gently washed three times with 200 µl sterile phosphate-buffered saline PBS (pH 7.2). Sodium acetate (2%) (200 µl) was added to each well for 5 min. for biofilm fixation then washed using tap water, followed by 200 µl crystal violet (0.1%) for 30 min. at room temperature for biofilm staining, followed by washing 3 times with tap water. The absorbance (optical density OD) of the remaining surface-adsorbed cells of the individual wells was read on a spectrophotometer (EL_X 800 Universal Microplate Reader Bio-TEC Instruments, INC.) at 630 nm. OD < 0.120 was considered absent or weak biofilm, OD 0.120 - 0.240 as moderate biofilm, and OD >0.240 as strong biofilm.

  b- Polymerase chain reaction to detect the 546 bp product of the icaADBC gene cluster

  DNA extraction was performed as previously mentioned [20]. The oligonucleotide primers (Geno-Mechanix) were used: forward (5'-TTATCAATGCCGCAGTTGTC-3'), reverse (5'-AGTTTAACGCGAGTGCGCTAT-3'). Amplification reaction was carried out in 25µl volume, using (Techne Genius, Cambridge, UK) thermal cycler, under the following conditions: An initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1min and a final extension at 72°C for 7 min [21].

  c. Determination of MIC and MBEC of vancomycin and linezolid on MRSE isolates planktonic and biofilm states:

i) MIC determination: [18]

MICs of linezolid (range 0.25-64 mg/ L double fold dilutions) and vancomycin (range 0.25-1024 mg/ L in double fold dilutions) for the MRSE strains selected for biofilm studies were determined using cation adjusted Mueller Hinton broth (CAMHB) according to the CLSI broth microdilution method. Results were interpreted as follows: for vancomycin (Sensitive: ≤ 4µg/ml, Intermediate: 8-16 µg/ml, Resistant: ≥ 32 µg/ml), for linezolid (Sensitive: ≤ 4 µg/mL, Resistant: ≥ 8 µg/mL).

ii) MBEC determination: [22]

A single colony from the selected MRSE isolates for biofilm studies was added to a test tube containing 2 ml TSB_{glu}.The tube was incubated overnight at 37°C aerobically. After incubation, 200µl of the bacterial suspension was added to wells of the microtiter plate along with positive control (the biofilm producing strain without adding Vancomycin or Linezolid) and negative control (biofilm negative strain). These were incubated for 24 hours at 37°C aerobically. Planktonic cells were removed by washing with phosphate buffered saline (PBS). The remaining attached bacteria were resuspended in 100 µL of CAMHB (Oxoid, Basingstoke, UK) and challenged with 100µl of Vancomycin at different 2 fold dilutions (2- 1024 µg/ml) or Linezolid at different 2 fold dilutions (0.5- 64µg/ml). Each dilution was tested in triplicate and the plates were incubated for 24 h at 37 ºC. The drug was removed and the wells were rinsed three times with PBS. The subsequent steps (i.e., fixation and staining) were performed as in the biofilm formation assay. The MBEC was defined as the minimum concentration of Vancomycin (or Linezolid) required eradicating the biofilm. Eradication of biofilm gave an OD_{630} reading similar to that of the negative control.

Statistical Analysis: The data was analyzed using computer with statistical Package for Social Sciences (SPSS) version 16.0. The 0.05 level was used as the cut off value for statistical significance. Chi square: (χ^2), Fisher Exact test (FET), McNemar chi square test and Simple correlation (r) were used for statistical analyses.

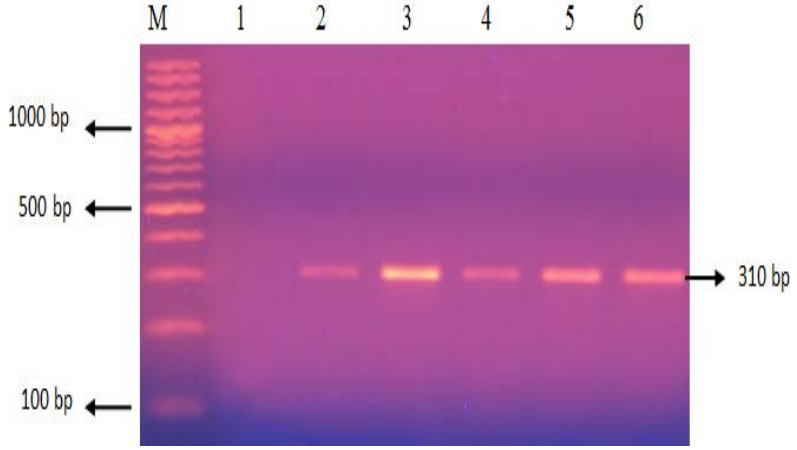

Figure 1. 2% Agarose gel electrophoresis analysis of 310 bp PCR amplification products of *mecA* gene, extracted from *S. epidermidis* isolates. Lane M: DNA molecular size marker (100-bp ladder). Lane 1: *mecA* negative. Lanes 2, 3,4,5,6 were *mecA* positive

3. Results

- A total of 100 MRSE strains were identified as *S. epidermidis* according to the standard microbiological techniques. Methicillin resistance was identified phenotypically by resistance to Cefoxitin and genotypically by the detection of 310 pb PCR product of *mecA* gene (Figure 1).
- **Results of biofilm production by MTP (Figure 2, Table 1)**:

Among the 100 MRSE strains, only 27 (27%) produced detectable biofilm (OD_{630} > 0.120). Regarding biofilm status, 8 isolates (30%) produced strong biofilm (OD > 0.240) and the remaining 19 isolates (70%) produced moderate biofilm (OD 0.120 – 0.240).

Table 1. The biofilm status of the 27 biofilm producing MRSE strains

Biofilm status	Number	OD_{630} value
Strong	8(30%)	> 0.240
Moderate	19(70%)	0.120 – 0.240
Total	27(100%)	

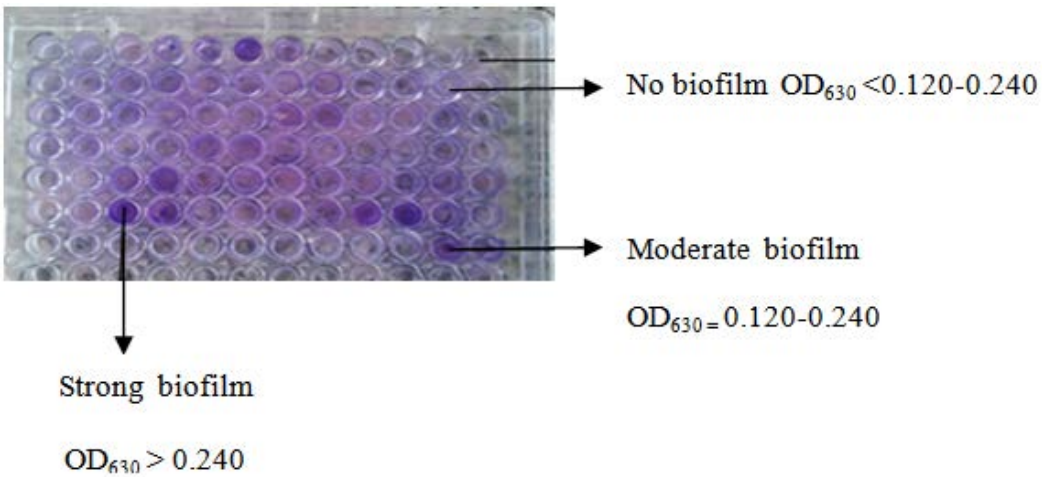

Figure 2. Quantitative detection of biofilm production by MTP. Strong, moderate and non- biofilm producers differentiated by crystal violet staining in 96 well microtiter plates

- **Results of PCR for *icaADBC* gene cluster (Figure 3)**

Among the 100 MRSE strains, 14 (14%) demonstrated the 546 bp PCR product of *icaADBC* gene cluster. Among them, 5/14 (35.7%) isolates were biofilm producers and the remaining 9/14 isolates (64.3%) were non- producers. On the other hand, 22/86 (25.6%) of the 86 *icaADBC* gene negative MRSE isolates produced biofilm.

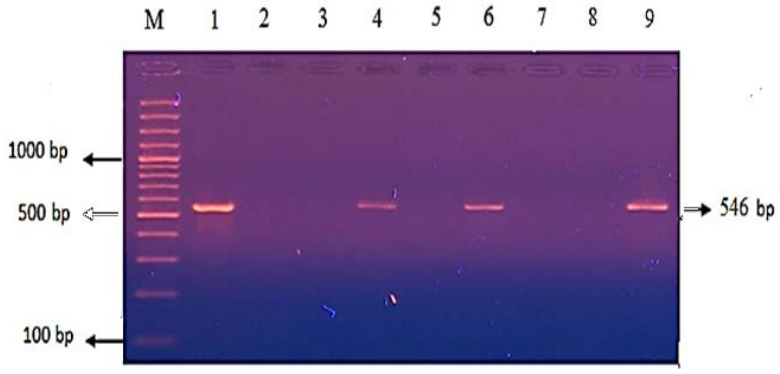

Figure 3. 2% Agarose gel electrophoresis analysis of 546 bp PCR amplification products of the *icaADBC* gene. Lane M: DNA molecular size marker (100-bp ladder). Lanes 2,3,5,7 and 8 are *icaADBC* gene negative. Lanes 1, 4, 6 and 9 are *icaADBC* gene positive

Studying the association between the *icaADBC* gene and the biofilm status among the 27 biofilm producing MRSE strains, there was no statistically significant association between the presence of the gene and the biofilm status (FETp=0.136). Moreover, the 5 *icaADBC* gene positive isolates consisted of 3 strong biofilm producers and 2 moderate biofilm producers (Table 2).

Table 2. Relation between *icaADBC* gene cluster and biofilm status of the 27 biofilm producing MRSE strains

IcaADBC	Biofilm status						Test of significance
	Moderate		Strong		Total		
	No.	%	No.	%	No.	%	
Negative	17	77.3	5	22.7	22	100.0	FETp = 0.136
Positive	2	40.0	3	60.0	5	100.0	
Total	19	70.4	8	29.6	27	100.0	

- **Determination of vancomycin and linezolid MICs:** all 27 biofilm producing isolates were susceptible to both Vancomycin (MIC range 0.25-2 µg/ml) and Linezolid (MIC range 0.25- 4µg/ml).
- **Determination of vancomycin (MBEC):** According to the CLSI guidelines, 16 (59.3%) out of the 27 biofilm producing isolates had MBEC for Vancomycin greater than the defined planktonic MIC breakpoint for resistance ≥ 32 µg/ml, 4 isolates (14.8%) had MBEC values ≤ 4 µg/ml and 7 isolates (25.9%) had MBEC values 8-16 µg/ml.

Table 3. Relation between vancomycin MIC and MBEC values among the 27 biofilm producing MRSE isolates

		Vancomycin MBEC				Total		Test of significance	
		Sensitive		Intermediate / Resistant					
		No.	%	No.	%	No.	%		
Vancomycin MIC	Sensitive	4	14.8	23	85.2	27	100.0	Mc Nemar X^2=	21.04
	Resistant	0	0.0	0	0.0	0	0.0	p=	0.000
Total		4	14.8	23	85.2	27	100.0		

P<0.05 = significant.

- The paired comparison between vancomycin MIC and MBEC showed that there was agreement between the results of both in only 4/27 (14.8%) isolates (both MIC and MBEC showed susceptible results) and disagreement between the results in the remaining 23/27 (85.2%) isolates (MIC showing a susceptible result and MBEC showing an intermediate/resistant results) and this difference was statistically significant (p<0.05) (Table 3).
- **Determination of linezolid (MBEC) :** According to the CLSI guidelines, 15 (55.6%) out of the 27 biofilm producing isolates had MBEC values for Linezolid greater than the defined planktonic MIC breakpoint for resistance ≥ 8 µg/ml, and 12 (44.4%) had MBEC values ≤ 4 µg/ml.
- Studying the paired comparison between Linezolid MIC and MBEC results showed an agreement in 12/27 (44.4%) isolates (Both MIC and MBEC showed susceptible results) and disagreement in 15/27 (55.6%) isolates (MIC showing a susceptible result and MBEC showing an intermediate/resistant results) and this difference was statistically significant (p<0.05) (Table 4).
- By comparing vancomycin and linezolid MBEC values, it was found that MBEC values of Vancomycin were higher than those of Linezolid in almost all strains (Figure 4). At the same time, there was an agreement

between the results of both MBEC values in 15/27 (55.5%) of isolates (Both values showed susceptible results in 7.4% and both showed intermediate/resistant results in 48.1% of isolates) and disagreement in 12/27 (44.5%) of isolates (Vancomycin MBEC showing susceptible results whereas Linezolid MBEC showed intermediate/resistant results in 7.4%. Vancomycin MBEC showed intermediate/resistant results whereas Linezolid MBEC showed susceptible results in 37.1% of isolates) and this difference was statistically significant (p<0.05) (Table 5).

Table 4. Relation between linezolid MIC and MBEC values among the 27 biofilm producing MRSE isolates

		Linezolid MBEC				Total		Test of significance	
		Sensitive		Resistant					
		No.	%	No.	%	No.	%		
Linezolid MIC	Sensitive	12	44.4	15	55.6	27	100.0	Mc Nemar X^2=	13.07
	Resistant	0	0.0	0	0.0	0	0.0	p=	0.000
Total		12	44.4	15	55.6	27	100.0		

P<0.05 = significant.

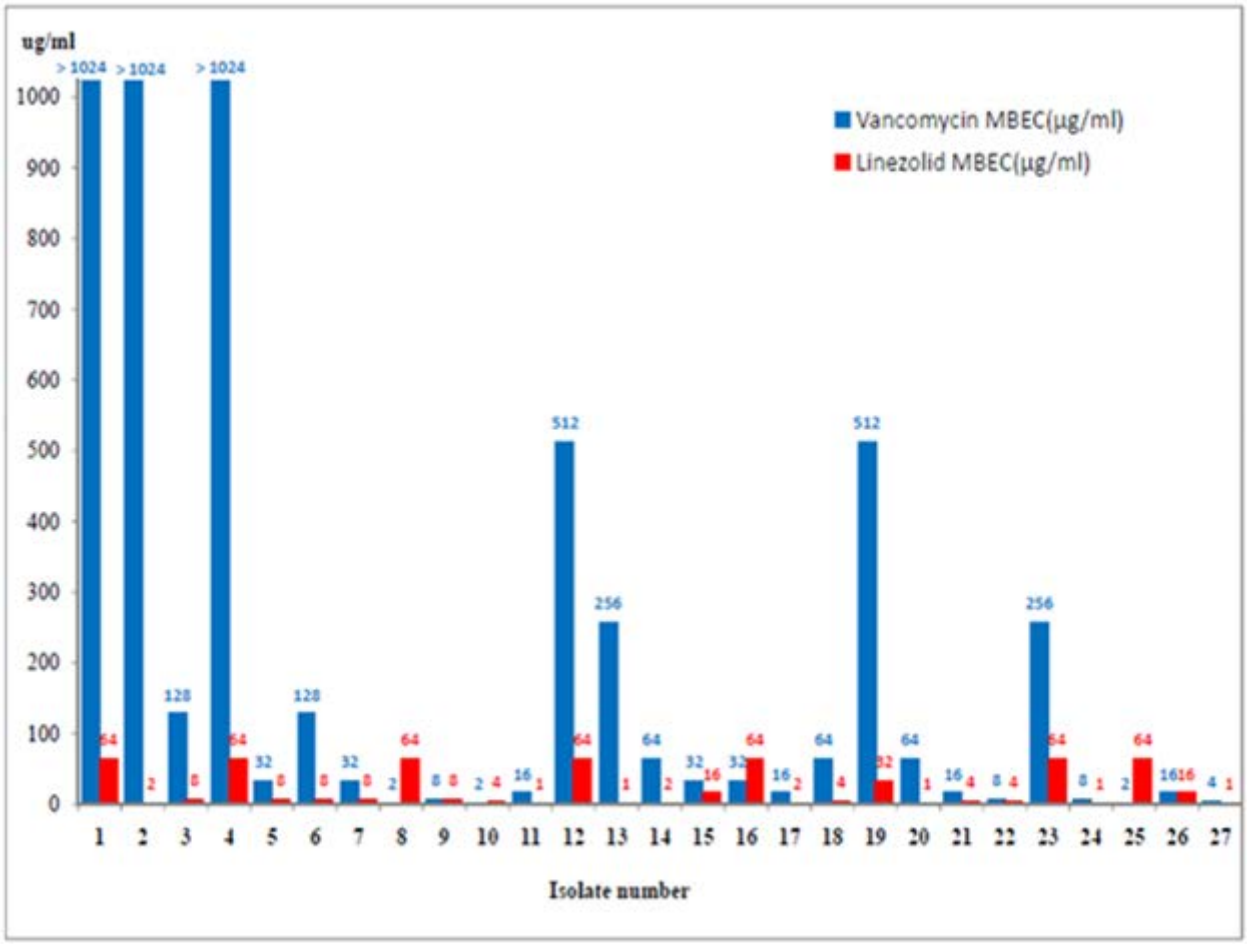

Figure 4. MBEC of Vancomycin and Linezolid on the 27 biofilm producing MRSE strain

Table 5. Relation between Vancomycin MBEC and Linezolid MBEC

Vancomycin MBEC	Linezolid MBEC				Total		Test of significance	
	Sensitive		Intermediate / Resistant					
	No.	%	No.	%	No.	%		
Sensitive	2	7.4	2	7.4	4	14.8	Mc Nemar X^2=	4.08
Intermediate / Resistant	10	37.1	13	48.1	23	85.2	p=	0.043
Total	12	44.5	15	55.5	27	100.0		

P < 0.05 significant.

- By studying the correlation between biofilm OD and MBEC of vancomycin and linezolid, it was noted that vancomycin MBEC showed a strong positive significant correlation with biofilm OD (p<0.05), however the same positive correlation was noted between biofilm OD and linezolid MBEC, yet it was weak and non- significant (p>0.05) (Table 6).

Table 6. Correlation coefficient between biofilm OD_{630} and Vancomycin, Linezolid MBEC

	Biofilm OD	
	r	p
Vancomycin MBEC	0.75	0.000
Linezolid MBEC	0.18	0.378

P < 0.05 significant.

4. Discussion

Staphylococcus epidermidis is a common cause of biofilm-mediated life threatening infections associated with intravenous catheters, artificial heart valves, prosthetic joints and other medical devices. [23] Methicillin resistant *S. epidermidis* (MRSE) isolates that circulate within hospital settings are mostly resistant to other classes of antibiotics and can act as a reservoir of mobile genetic elements transferred to other *S. epidermidis* isolates as well as to other *Staphylococcus* species [24].

In the present study the 48% of MRSE isolates were isolated from blood, followed by pus (32%), then urine and BAL (9%) and only (2%) from nares. This was in agreement with the sudy of Iorio *et al* (2012) [25] in Brazil, who studied MRSE isolates from Rio de Janeiro hospitals and found that 40% of their isolates originated from blood, 20% from nares, 11.4% from surgical sites, 5.7% from catheter tips, 5.7% from throat and 2.8% from other sources.

Biofilm formation, mediated by PIA production, is considered to be the main virulence factor of CoNS. The microtitre plate (MTP) assay is considered an accurate and quantitative tool for comparing the biofilm formation of different strains [13,26].

In the current study, among 100 MRSE isolates, biofilm production by MTP was found in 27% with different intensities, 8 isolates (30%) were strong producers and 19 (70%) were moderate, while in the remaining 73% no biofilm was formed.

These results are close to those obtained by Terki *et al* (2013) [27], in Algeria, who found that 6/21 (28.5%) of the studied *S.epidermidis* isolates from urinary catheters were biofilm producers. Other studies showed discrepant results, such as that of Seif el Din et al (2011) [28] in Egypt and Mathur T et al. (2006) [13] in India, where CoNS biofilm producers constituted 41% and 53.9% respectively. These higher results might reflect the different sources from which the strains were isolated.

The *icaADBC* gene cluster was detected by PCR in this study in 14 (14%) of MRSE.

The presence of this gene was also investigated in 2 Egyptian studies, that of Nasr *et al* (2012) [23] who found the gene in 16 out of the 50 (32%) investigated Staphylococcus isolates. Furthermore Seif El-Din *et al* (2011) [28] studied the presence of *icaA* and *icaD* genes in Staphylococcus isolates among pediatric cancer patients and healthy volunteers. The genes were present in 12/22 (53%) of CoNS strains among cancer patients, but not in the healthy volunteers. According to Eftekhar and Mirmohamadi (2009) [17] study, the *icaADBC* gene carriage was observed in 30% of *S. epidermidis* isolates from symptomatic patients and in 8% among skin isolates from healthy individuals.

Numerous studies have reported that biofilm-positive strains or strains carrying intercellular adhesin genes (*ica*), which encode polysaccharide intercellular adhesin (PIA), are more pathogenic.[29,30] Synthesis of PIA, the main EPS component of biofilms, was found to play an important role in establishing *S. epidermidis* infections in animal models [31] though its essential role is still debated [21,30,32].

In 2009 a study has shown that more invasive and less commensal staphylococcal strains carry *icaADBC* gene. [33] Another study found no specific association between the presence of biofilm genes and pathogenicity. [34] Furthermore, some studies suggested that there was no difference in *icaADBC* carriage between the invasive and contaminating blood culture isolates. [35,36] Moreover, studies using an animal model showed no difference in virulence between wild *S. epidermidis* strains (biofilm-positive) and their biofilm-negative mutants. [37,38] Finally it has been reported that even *icaADBC*-negative CoNS could cause biofilm-related persistent infections depending on other genes and regulatory mechanisms [39].

In the current study by correlating the phenotypic biofilm production methods with presence of *icaADBC* gene cluster, 35.7% (5/14) of the *icaADBC* gene positive staphylococcal isolates were biofilm producers by microtitre plate (MTP).

In accordance with our findings, other studies (Yazdani *et al* 2006 [40] and De Silva *et al*. (2002) [41] demonstrated that the presence of the *ica* genes did not correlate with biofilm production.

However, in contrast to our finding, Gad *et al* (2009), in Egypt [42] studied the biofilm production and the presence of *icaA* and *icaD* genes in staphylococci isolated from urinary catheter segments. It was found that all biofilm producing strains were positive for both genes, which indicates the important role of ica genes as virulence markers in staphylococcal infections associated with urinary catheterization. [17,29] This might explain the lower rates reported in the present study (14%), as the studied MRSE isolates were not obtained from devices or catheters.

Unexpectedly, in the current study, 25.5% (22/86) of the *icaADBC* gene negative isolates produced biofilm by MTP, among which biofilm was strongly adherent in 5 isolates.

Some studies suggested that PIA and its encoding *ica* genes might not be of universal importance in CoNS biofilm formation (Arciola *et al.* (2006) [32] and Rohde *et al*.(2007) [43]). One explanation for these findings might be that PIA production is not critical for biofilm formation but contributes to the distinct biofilm architecture. [44] However its exact role in biofilm formation is not clear. In the PIA/*ica*-negative biofilms, matrices are mainly composed of proteins, teichoic acids and extracellular DNA. Some specific proteins, such as Bap (Biofilm-associated protein), Aae (Autolysin/adhesin protein), and Aap (Accumulation-associated protein), were found to be responsible for the vital step of cell-cell accumulation and biofilm formation in the absence of PIA [45].

On the other hand 64.3% (9/14) of *icaADBC* gene positive isolates did not produce biofilm by MTP.

In Ninin *et al*., 2006 study [46] it was found that 32 of 91(35%) isolates carrying the ica operon did not produce biofilm. Similar to our findings, some investigators have

found no association between the presence of the *ica* operon and biofilm formation by clinical isolates of *S. epidermidis*. [35,38] Regulation of the ica operon appears to be very complex and the production of PIA is subjected to on-off switching [47].

In addition, Dobinsky *et al*., 2003 found that expression of the *ica* m-RNA has been shown to occur in biofilm negative *S. epidermidis* suggesting that biofilm accumulation is controlled by regulatory mechanisms other than the *ica* operon [48].

Despite various efforts, treatment of an infection after biofilm establishment is frequently useless, because of the reduced susceptibility of biofilm to antibiotics.

In the present study, the effects of vancomycin and linezolid on MRSE biofilm and planktonic cells were studied by testing the minimal inhibitory concentration (MIC) and minimal biofilm eradication concentration values (MBEC).

The results of the present study demonstrated that the Vancomycin resistance was higher in the biofilm mode of growth than in the planktonic mode of growth. Among the biofilm-forming isolates, 85.2% showed high Vancomycin MBEC (≥ 8 µg/mL) and cannot be considered susceptible to Vancomycin according to the CLSI breakpoints. [19] Resistance rate for 8 strong biofilm producers was 87.5% (7/8) and that for 19 moderate biofilm producers was 84.2% (16/19). All these isolates, presented Vancomycin MICs of ≤ 4 µg/mL, which is within the "susceptible" category. However, in the cases of biomaterial associated infections, in which biofilm formation is the main characteristic, conventional MIC only predicts the ability of an antimicrobial agent to inhibit the growth of the bacteria released from the biofilm, not the bacterial growth within biofilm matrix [49].

Antunes *et al* 2011 found that the vast majority 89% (58/65) of biofilm-producing Staphylococcus species isolated from central venous catheters could be considered non-susceptible to vancomycin (MBEC values ≥ 8 µg/mL) and the resistance rate for strong biofilm producers was 45% (77/170), that for moderate biofilm producers was 45% (107/240). [50] These MBECs were higher than MICs for vancomycin. A higher MBEC/MIC ratio of ~64 was found in six strong biofilm-producing isolates and two moderate biofilm-producing isolates.

In Egypt, El-Sheikh *et al* (2010) found that all studied staphylococcal strains in the indwelling vascular catheter patients were susceptible to vancomycin in their planktonic form, and that the MIC was 8-16 times lower than MBEC for vancomycin [51].

Interestingly, Lee *et al*. (2006),(52) found that ciprofloxacin and rifampicin were found to achieve a complete eradication of staphylococcal biofilms after a short-term treatment, and that vancomycin was of much lower effectiveness, even at very high concentrations [51].

Statistical analysis of the current results demonstrated that vancomycin MBEC had shown a strong positive significant correlation with the biofilm status ($p<0.05$). The MBEC was significantly higher in strong biofilm-producing isolates than in moderate biofilm-producing isolates.

This finding can be explained by the decreased diffusion of antimicrobial agents through the extensive biofilm matrix, as well as the decreased metabolic activity of bacteria within biofilms and the increase in gene transfer. [53] It has been reported that vancomycin accumulates at high concentrations in the biofilms of Gram positive bacteria, especially *S. epidermidis*. [54] This may be attributed to the ability of glycopeptides to bind to exopolysaccharides produced by the bacteria. However, such high concentrations of vancomycin are not achievable in clinical practice. [51]

During the present study we observed that sub-minimal inhibitory concentrations (sub-MIC) of antibiotics induced *S.epidermidis* biofilm formation *in vitro*. This was evidenced by the readings of OD of sub-MIC concentrations (0.25, 0.5, 1, 2 mg/L) which were higher than the OD of the positive control (the biofilm producing strain without adding antibiotics) The term "sub-MIC" will be used to refer to concentration of antibiotics below the MIC (<1 MIC).

It has been shown that the sub-MIC concentrations of the cell-wall-active antibiotics oxacillin and vancomycin, and of the translation inhibitor linezolid, have been shown to induce *S. aureus* biofilm formation [55].

Among our biofilm-forming isolates, 55.6% showed high linezolid MBEC (≥ 8 µg/mL) and were considered resistant to linezolid according to the CLSI breakpoints [18]. Resistance rate for the 8 strong biofilm producers was 87.5% (7/8) and that for the 19 moderate biofilm producers was 42.1% (8/19).

All these isolates, however, presented linezolid MICs of ≤ 4 µg/mL, which is within the "susceptible" category. This is similar to Uckay *et al.*, 2009 surveillance which was carried out at the University of Geneva Hospitals and found that 100% of *S.epidermidis* isolates were sensitive to Linezolid [56].

Statistically the positive correlation can be shown between biofilm OD_{630} and linezolid MBEC, yet it was shown to be weak and non- significant. We observed a significant increase in the MBEC/MIC ratio in our biofilm-producing isolates. The high MBEC /MIC ratio of 64 was found in two strong biofilm producing isolates and one moderate biofilm producing isolates.

Linezolid demonstrated better antimicrobial activity in *in-vitro* biofilm formation than vancomycin as 44.4% (12/27) of biofilm producing isolates were sensitive to linezolid whereas only 14.8% (4/27) were sensitive to vancomycin.

This is in agreement with Curtin *et al* (2003) who studied the biofilm eradication by *in-vitro* model for formation of biofilm by *S. epidermidis* and found that linezolid achieve eradication of *S. epidermidis* biofilms more rapidly than vancomycin and gentamicin [55].

We observed a significant increase in the linezolid MBEC/MIC ratio in our biofilm-producing isolates. Although both linezolid and vancomycin showed higher MBEC than MIC in *in-vitro* biofilms, 85% of isolates were resistant to vancomycin and their MBEC were 8 up to 1024 times greater than the planktonic susceptibility breakpoints, while, 55% of isolates were resistant to linezolid with a MBEC up to 64 times greater than the planktonic susceptibility breakpoints.

Although these antibiotics at the highest achievable serum concentrations were effective against bacteria grown planktonically, they were inadequate to eradicate bacterial biofilms. Administration of a single antibiotic to patient with catheter-associated infections, based on the results of *in-vitro* susceptibility tests designed for

planktonic bacteria, is unlikely to reach an effective concentration to eradicate the bacteria adherent to the catheters. This may be one of the reasons that explain the frequent failure of treating CoNS infections with conventional antibiotics in patients with foreign body infections when the devices are not removed [22].

5. Conclusion

In the light of the present study, we can conclude that the presence of *icaADBC* gene is not always associated with *in-vitro* formation of biofilm, vancomycin and linezolid continued to be effective for planktonic MRSE infection, but MIC values alone cannot accurately determine the exact susceptibility of biofilms, and finally, the sub-MIC concentrations of antibiotics can induce bacterial biofilm formation.

Conflict of Interests

The authors have no competing interests.

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