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Linezolid Resistance and Biofilm Formation in Invasive and Commensalism *Staphylococcus Epidermidis*

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Abstract: *Staphylococcus epidermidis* is a fundamental reason of catheter-related infections septicemia condition in immunocompromised individuals. To determine if skin-related *S. epidermidis* isolates are distinct from those that cause septicemic illnesses. This study aim to comparative the ability of bacterial to produce biofilm and linezolid resistance genes *S. epidermidis*. Methods 150 specimens were isolated from Urine, wound and blood from different hospitals and 150 specimens from urine and fingerprint of healthy individual in Baghdad city from July 2021 to January 2022. The bacterial isolates were recognized with biochemical test and vitek 2 system. for their potential role in biofilms formed the polystyrene micro-titer plate (MTP) estimate using to measure the capacity to biofilm producer. However, the MTP method capably to categorized the bacterial isolates to strong, moderate, weak, and non-biofilm producers. The biofilm genes (*icaA* and *icaD*) and Linezolid resistance gene *cfr* were detection by PCR method. The results of this study showed 30 (10%) *Staphylococcal epidermidis* strains were isolated and identified from 300 samples. The MTP method was classified the isolates to 11/30(36.6%) strong, 6/30(20%) moderate and13/30 (43.3%) weak biofilm formation. The PCR results revealed that 11/15(73.3%) and 7/15(28.5%) from healthy and clinical *Staphylococcus epidermidis* isolates respectively were complete icaABCD Operon, 4/11(36.3%) and 2/7(14%) were *cfr* gene positive. While 3/15 (20%) and 8/15(53.3%) were defect icaADBC operon, which contain 3/8 (37.5%) *cfr* gene positive and no one from healthy samples. Finally, 1/15 (6.6%) lacking to icaABCD operon and *cfr* positive strains. It was concluded, *icaA*, *icaD* and *cfr* genes have a significant role in staphylococcal infections caused as a virulence marker.

Keywords: ***Staphylococcus epidermidis***, biofilm, linezolid, *icaA*, *icaD*, *cfr*.

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Introduction

Staphylococcus epidermidis (*S. epidermidis)* is a gram-positive, catalase-positive, facultative anaerobe, coagulase-negative bacteria. On human skin or mucosa, it is typically a symbiont and is very prevalent (1). It most frequently affects skin and soft tissue. By producing virulence factors, these bacteria can induce tonsillitis and burn inflammation, among other disorders (2). *S. epidermidis* appears to maintain a little of virulence factors (3), in contrast to *S. aureus*, which typically produces a large number of extracellular enzymes and toxins that allow invasive infections in normal hosts (4, 5). *S. epidermidis*, producing virulence and invading the human body, can quickly transition from a symbiotic to an invasive existence. One of the greatest recurrent causes of nosocomial blood infections is *S. epidermidis*, which has the capacity to colonize medical and

prosthetic equipment. The danger of *S. epidermidis* and other coagulase-negative staphylococci is higher in people who wear contact lenses, have prosthetic joints, cardiac devices, urinary and central venous catheters, endotracheal tubes, orthopedic devices, or who are receiving intravenous medication (6). The important virulence component of *S. epidermidis* is biofilm. Teichoic acids, extracellular DNA, polysaccharide intercellular adhesin (PIA), many of proteinaceous factors (Bhp, Aap, and Embp), in addition other sticky molecules make up the biofilm; nevertheless, it has been demonstrated that not totally *S. epidermidis* isolates encode components critical for biofilm development. For instance, although most clinical isolates include the icaADBC operon, some clinically significant isolates are PIA negative (Antibiotic resistance is provided by biofilm, which holds bacteria to inert surfaces while allowing them to evade the host's immunological response (7). Despite the fact that the minority of *S. epidermidis* isolates are still susceptible to modern antibiotics such as dalbavancin, tigecycline, linezolid, and daptomycin (8).

The US Food and Drug Administration initially gave the linezolid (LZD) molecule which is belong to the oxazolidinone family the seal of approval for commercial use in 2000. It equates to one of the last-resort medications for methicillin-resistant Staphylococcus infections, Vancomycin-resistant enterococci (VRE), *Staphylococcus aureus* (MRSA), and a number of other resistant gram-positive bacteria. By interacting to the 23S rRNA, oxyazolidinones restrict protein synthesis, which in turn prevents bacterial growth. Linezolid has therefore been shown to have antibacterial action both *in vivo* and *in vitro* (9). However, *S. epidermidis* has been related with plasmid *cfr*-mediated linezolid resistance in Italy (10).

Materials and methods

Specimen collection

A total of 150 specimens were collected from patient referring to Al-Forat and Medical City Hospitals in Baghdad, Iraq and 150 specimens were collected healthy individuals from July 2021 to January 2022. These specimens show in (Table 1).

Table (1): Sample collected in this study

Sample location	Patient	Healthy individual
Urine sample	50	50
Blood sample	50	50
Wound sample	50	0
Finger print	0	50

The specimens were cultured on Mannitol salt agar, staph 110 agars often the growth of bacteria, the isolates were identified by microscopic examination, traditional biochemical tests and confirmed identified using Vitek 2 system (11).

Antibiotic susceptibility test

A disc diffusion routine following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) was used to test the antibiotics' susceptibility (12). Vancomycin (VA- 30µg), cefoxitin (CFX- 30µg), gentamicin (CN-10µg), tetracycline (TE-10 µg), levofloxacin (LEV- 5µg), oxacillin (OX- 5µg), penicillin G (p-10 µg), Rifampin (RA-5µg) and clindamycin (DA-10 µg) were

tested the antibiotic by the disc diffusion methods.

Biofilm formation assay

As previously mentioned, the quantification of *S. epidermidis* biofilm development on abiotic surfaces was evaluated (13). Briefly stated, 200µ l of an overnight TSB with 0.5% glucose as biofilm formation inducer (bacteria concentration was adjusted to in equivalency to McFarland standard no. 0.5) was additional to per well of sterile 96-well U-shaped bottomed polystyrene microplates before the plates were enclosed and incubated aerobically at 37°C for 24 h. Each microbe was examined three times. In order to do control wells, TSB free of bacteria was added. The residual adhering bacteria were fixed with 200 µl of methanol for 15 minutes after the wells had been aspirated and rinsed three times with 200µ l of sterile phosphate-buffered saline (PBS). The wells were marked by 200 µl of 0.1% crystal violet solution after they had dehydrated in the air at room temperature, 15 minutes. The plate was submerged in flowing water tap to remove the extra discoloration. The plates were subsequently dried. The adhering cells were then re-solubilized for 15 minutes with 200µ l of 33% glacial acetic acid. Using a microplate reader (Biotek, UK), then the micro-titer plate measured at 600 nm optical density (OD). The *Staphylococcus epidermidis* isolates were organization according to their ability to generate biofilms. tried to develop standards for classifying *S. epidermidis* isolates according to their ability to produce biofilms. A cut-off OD ($_{ODcut}$) was measured by averaging all of the ODs of the negative control and adding three times the negative control's SD value to it.

OD_{cut} = OD_{avg} of negative control + 3 × standard deviation (SD) of ODs of negative control

The typical OD of the negative control in this study was calculated to be **0.062.** The stander deviation OD value was **0.0026,** therefore the cut off rate was calculated as 0.0698 and the subsequent criteria (Table 2) were involved for biofilm progression in clinical isolates (14).

Table (2): The biofilm produced classification by *Staphylococcus epidermidis* isolates

Optical density values OD	Criteria for classification
OD ≤ 0.0698 *	Non biofilm formation **(NBF)**
0.0698< OD≤ 0.1396	Weak biofilm formation **(WBF)**
0.1396 < OD ≤0.2792	Moderate biofilm formation (**MBF**)
0.2792 <OD	Strong biofilm formation (**SBF)**

* The cut off value for the current study

Polymerase chain reaction

For total DNA extraction obtain by cultured each isolate overnight at 35 C with shaking in brain heart infusion broth (BD, San Jose, CA, USA). 200 ml of sterile water were added to the sediment then re-suspended with 100 ml of sterile water after that one milliliter of each culture was centrifuged for 12 minutes at 13,000 rpm. The samples were centrifuged at 13,500 rpm for 10 min after being centrifuged at 20 ℃ for 30 min, boiled for 5 min, then frozen once more for min. Following the recovery of the supernatant, 250 ml of cold isopropanol was used to precipitate the DNA. The DNA sediment were dried and re-suspended in of one milliliter water, after that washed with 250 ml of 70% ethanol. PCR master mix component in (Table 3) according to (GoTag Green Master Mix, Nuclease Free Water, TAE 40X, Quantifluor dsDNA System) promaga/USA. The

conditions were explaining (Table 4) Electrophoresis in 2% agarose gel was used to analyze amplicons. The positive and negative controls were *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 29213, respectively. The National Center for Biotechnology Information's GenBank sequence database was used to obtain the *icaA* and *icaD* and *cfr* gene sequences. The primers were created by the Microgene company (15). All *S. epidermidis* isolates were inspect for the existence the biofilm formation *icaA* and *ica D* genes and linezolid resistance gene *cfr* using specific primers, respectively (Table 5).

Table (3): PCR master mix to detect (icaA, icaD and cfr) gene of *S. epedermidis*

Component	Concentration	Amount (µl)
Master mix	1X	12.5
F- primer	0.5µM	1
R- primer	0.5 µM	1
Nuclease free water		8.5
DNA sample		2
Total value		25

Table (4): PCR program to detect icaA, icaD and cfr genes of S. epidermidis isolates.

Step	Time	Temperature	No .of cycle
Initial denaturation	2min	95℃	1
Denaturation	20sec	95	30-40
Annealing	20sec	……	
Extension	1min	72℃	
Final extension	5min	72℃	1

Table (5): Primer sequence of icaA, icaD and cfr genes and their product size.

Name	Abbreviation	Primer Sequence (5 to 3)	AmpliCon Size (bp)	Reference	Annealing temperature
Intercellular adhesin A	*IcaA*:F *IcaA*:R	TCTCTTGCAGGAGCAATCAA TCAGGCACTAACATCCAGCA	188	17	50℃
Intercellular adhesin D	*IcaD*:F *IcaD*:R	ATGGTCAAGCCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	198	17	50.9℃
Linezolid resistance	*Clr*: F *Clr*:R	GAGATAACAGATCAAGTTTTA CGAGTATATTCATTACCTCAT	458	28	46. ℃

Results and dissection

Settings and clinical isolates

The traditional biochemical test and Vitek 2 system was used for identification process. The findings indicated that 30(10%) of isolates were detection as *S. epidermidis* from 300different samples.

Assessment of antibiotic susceptibility

In clinical samples, *S. epidermidis* was the most frequently isolated CONS. Additionally, among the discovered CONS, we noted variations in antibiotic reluctance. *S. epidermidis* had higher rates of resistance to cefoxitin (80%), oxacillin (90%), penicillin (86,6%), Clindamycin (60%), Gentamycin (76.6%) levofloxacin (80%) Tetracycline (26.6%). The most effective antibiotic the vancomycin (90%) and Rifampin (86.6%).

Biofilm formation assay

The most popular test for identifying the emergence of biofilms is the Microtiter plate assay, which was once thought to be the gold standard. This method has the benefit of being a numerical instrument for evaluating the adherence of distinct strains, and has been discovered to be the most accurate, precise, and repeatable screening method for determining the

development of biofilm by clinical isolates consists of *S. epidermidis* (16). reached to just 11/30 (36.6%) strong, 6/30(20%) moderate and 13/30 (43.3%) weak biofilm formation. The result was recorded in the (Table 6) and (Figure 1).

Table (6): Biofilm formation by *S. epidermidis* isolates.

Clinical isolates						Healthy isolates					
Isolates code	**Mean OD_{600}**	**SD**	**Isolates code**	**Mean OD_{600}**	**SD**	**Isolates code**	**MEAN OD_{600}**	**SD**	**Isolates code**	**MEAN OD_{600}**	**SD**
315	0.089	0.000	123	0.1165	0.001	20DFP	0.088	0.001	5DFP	0.102	0.001
21	0.6105	0.268	200	0.099	0.006	15	0.0935	0.008	8DFP	0.1	0.004
107	0.2395	0.072	213	0.215	0.002	14	0.0955	0.008	40	0.6415	0.031
70	0.307	0.439	43	2.4115	0.006	13	0.5435	0.065	12	0.8025	0.051
73	0.0825	0.000	154	0.0895	0.006	32	0.169	0.004	50	0.5435	0.046
92	0.095	0.001	1DFP	2.2835	0.176	58D	0.0925	0.005	30	0.1135	0.011
47	0.3645	0.359	199	0.205	0.023	6	0.2355	0.000	10	0.142	0.000
137	0.3835	0.039	110	0.527	0.045	NC	0.0615	0.002	NC	0.0625	0.002

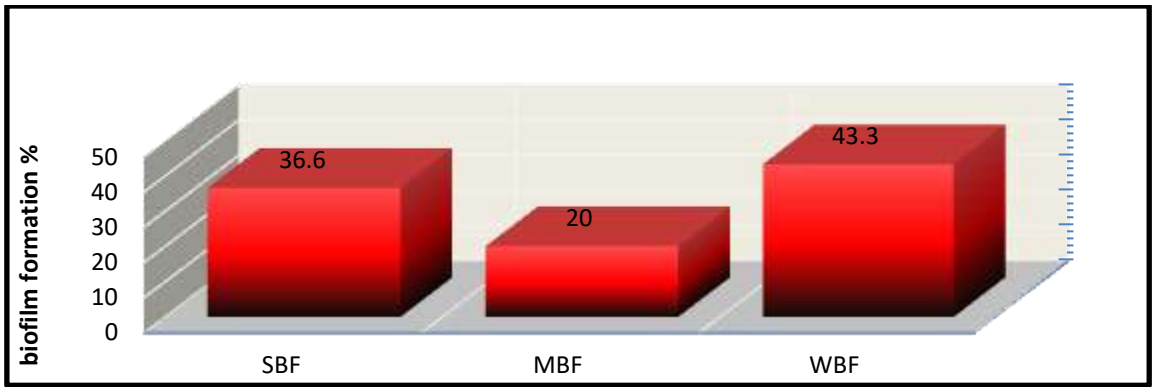

Figure (1): Biofilm formation of *S. epidermidis*.

All *S. epidermidis* isolates which screened for biofilm formation were tested by PCR for biofilm formation genes *icaA* and *icaD* and linezolid resistance *cfr* gene. Using reference primers, these genes were amplified using PCR to identify *icaA* and *icaD* amplicons of 188 bp and 198 bp respectively. In this study 18 (60%) present complete operon, 7/15(46.6%) and 11/15(73.3%) in clinical and healthy isolates respectively, while 11/30(36.6%), defected 8/15(53.3%) in clinical isolates and 3/15(20%) in healthy isolates, whereas 1/15(6.6%) lacking in healthy isolates (Figure 2, 3), through the detection of *icaA* and *icaD*, the icaADBC operon was screened for in all S. *epidermidis* isolates (Table 7). However, the *cfr* gene amplicon 458bp was detected in 10/30(33.3%) of *S. epidermidis*, 5/15(50%) in clinical and 5/15(50%) in healthy isolates (Figure 4) as explain in (Table 8).

Table (7): The icaADBC operon was classified according to present *icaA* and *icaD* genes.

Class of icaADBC operon	Meaning (34, 35)	Clinical n/15(%)	Healthy n/15(%)
Complete	both icaA and icaD identified	7 (46.6%)	11(73.3%)
Defective	only icaA or icaD detected	8(53.3%)	3(20%)
Missing	both icaA and icaD lacking	0	1(6.6%)

Table (8): Comparatives between biofilm production of the icaADBC operon and LZR *cfr* gene in *S. epidermidis* isolates

	Clinical isolates				Healthy isolates			
MTP classes	**Complete no(%)**	**Defect no(%)**	**Lacking no(%)**	***Cfr* positive no(%)**	**Complete no(%)**	**Defect no(%)**	**Lacking no(%)**	***Cfr* positive no(%)**
Strong	2/6 (33.3%)	4/6 (66.6%)	0	1/6 (16.6%)	5/5 (100%)	0	0	1/5 (20%)
Moderate	2/3 (66.6%)	1/3 (33.3%)	0	2/3 (66.6%)	1/3 (33.3%)	1/3 (33.3%)	1/3 (33.3%)	2/3 (66.6%)
Weak	3/6 (50%)	3/6 (50%)	0	2/6 (33.3%)	5/7 (71.4%)	2/7 (28.5%)	0	2/7 (28.5%)

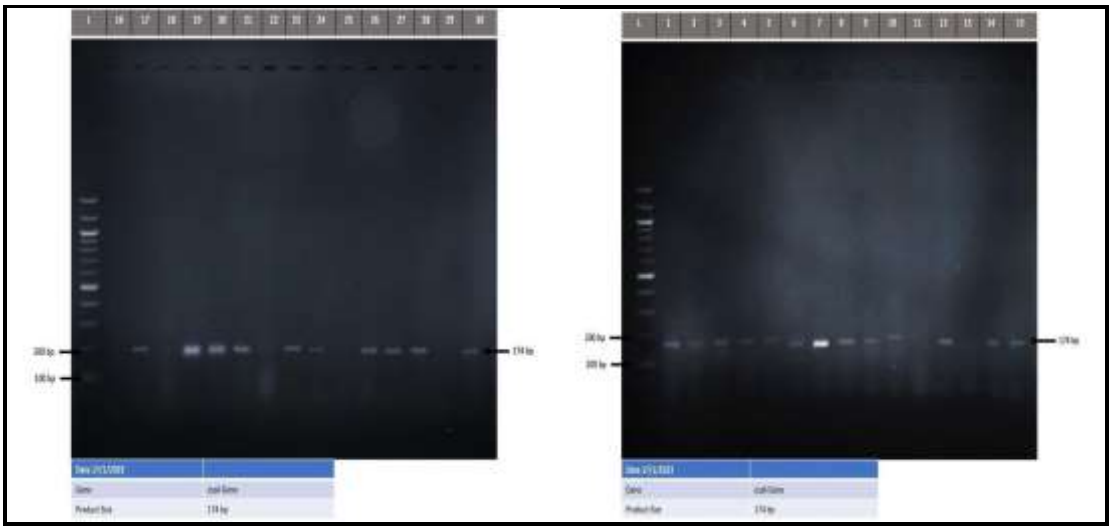

Figure (2): Agarose gel electrophoreses of PCR amplified products for *icaA* gene using 1.5% of agarose at 70 v/cm for 1.5 hour Lan (L) positive result with positive band of *ica* gene ≈ 174pb.

Figure (3): Agarose gel electrophoreses of PCR amplified product for *icaD* gene using 1.5% of agarose at 70 v/cm for 1.5 hour Lan (L) positive result with positive band of *icaD* gene ≈ 215 bp

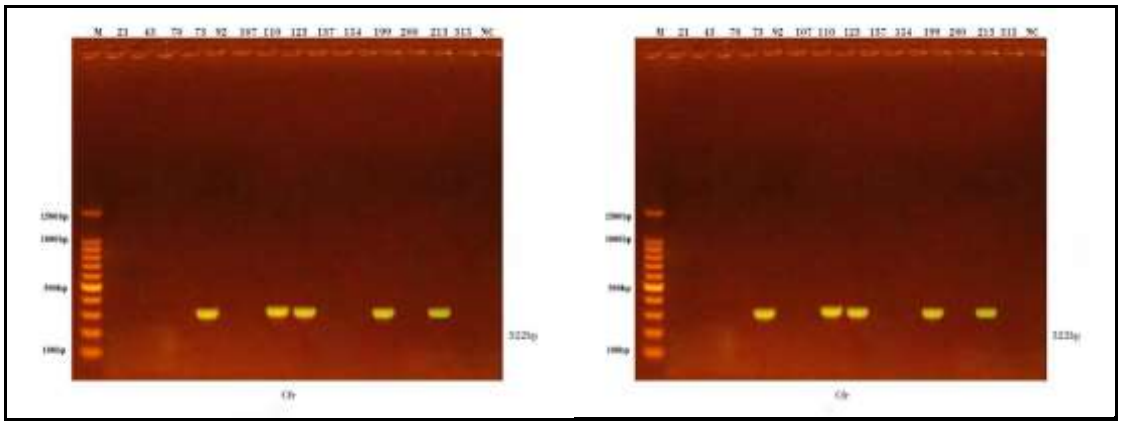

Figure (4): Agarose gel electrophoreses of PCR amplified products for *cfr* gene using 1.5% of agarose at 70 v/cm for 1.5 hour Lan (L) positive result with positive band of *cfr* gene ≈ 322bp.

As biomaterials are used more often in medical practice, *S. epidermidis* infections linked to biomedical implants have significantly grown in frequency. Recent research has revealed that disease-causing strains of *S. epidermidis* are differed from commensal populations with regard to how frequently they carry virulence elements such as those responsible for adhesion and biofilm development (17). The complete genome sequencing and MLST identification for the clade and clonal complex were supported by *S. epidermidis* genotypes' genealogical inference among commensal and pathogenic sources (18). Our results disagree with Martínez *et al*. (19) that only 8 isolates were weak biofilm produce, while eleven isolates non biofilm formation. This served as the foundation for investigating whether variations in adhesion and biofilm development could reproduce genetic variation and identify different lineages of virulent bacterial strains. Harris *et al.* (20). demonstrated that all of the isolates adhered and produce biofilms in vitro, exhibiting microscale variation in their biofilm architectural patterns. Isolates developed patchy, multi-layered biofilms that attached to one another. The loci icaADBC have been linked to the development of biofilms (21). Our research found out that only in its entire form was the icaADBC operon discovered in 7 (23.3%) of the 30 biofilm-positive isolates, meaning that none of clinically important pathogens lacked this operon. It's interesting only *icaA* gene was found in the defective operon while missing *icaD* except two isolate contain *icaD* and lacking to *icaA* gene. Others researches (22) have shown that there are differences in the biofilm/PIA levels across clinical isolates of *S. epidermidis* and that higher biofilm formation is correlated with elevated *ica*A transcript and elevated PIA synthesis. Antimicrobial resistance is frequently linked to *S. epidermidis* infections. Layer *et al.* (23) were showed in two different German hospitals, there were LRSE outbreaks that resulted in the recovery of four and six LRSE isolates. A significant rise in the use of linezolid for the treatment of MRSA infections preceded the larger outbreak. These findings demonstrate the significant level of antibiotic resistance present in clinical MRSE strains, which may make therapy more challenging. The good news is that 5(16.6%)and 5(16.6%) of our clinical and commensal isolates respectively have the *cfr* gene, a synthetic oxazolidinone that is advised for use in the treatment of gram-positive

infections that are resistant to multiple drugs (24). Our study found 10/30(33.3%) of *cfr* gene (5/15%) in clinical and healthy isolates respectively, these results are in line with those of a systematic review and meta-analysis, which discovered a very low frequency of linezolid resistance (0.3%). However, our findings differ from those of a study conducted in Mexico, where the authors reported that clinical isolates of *S. epidermidis* from patients hospitalized in Guadalajara and Monterrey had a frequency of linezolid resistance of 3.6% (3/83) and that the three isolates belonged to ST23 (25). This is supported by the finding that ST23 is better able to take the *cfr* gene, which are encoding linezolid resistance gene and was initially discovered in plasmid pSCFS1 (26). However, complete-genome sequencing (WGS) of 176 *S. epidermidis* clinical isolates from American patients revealed that ST5 and ST22 are linked to linezolid resistance, with a frequency of 22% reported by the authors. All *S. epidermidis* ST5 linezolid-resistant clinical isolates include the plasmid pMB151a, which contains the *cfr* gene in these isolates (27).

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