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Research Article

Expression of Biofilm and Virulence Factors Genes by Commensal and Virulent *Staphylococcus epidermidis* Isolates

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I N F O

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A B S T R A C T

Background: The pathogenicity of *Staphylococcus epidermidis* in community-associated bacterial infections has incredible significance due to its capacity for biofilm production. The goal of this investigation was to compare the expression of different virulence factors among clinical and healthy isolates.

Methods: Six *S. epidermidis* isolates were confirmed by *16S rRNA*, and then phylogenetic tree analysis was used to detect the relationship between clinical and healthy isolates. Quantitative PCR (qPCR) was used to compare the *S. epidermidis* gene expression level between clinical and healthy isolates. The expression levels of the *icaA, clf*, and *IgG* genes were compared in planktonic cells and biofilm form for the same *S. epidermidis* isolates.

Results: All clinical isolates appeared highly capable of expressing all three genes compared to healthy isolates. The isolate SP137 with robust biofilm formation exhibited significantly higher expression of *icaA, clf,* and *IgG* genes (27, 3783.3, and 247.6, respectively) compared to the other isolates ($p < 0.01$).

Conclusion: Clinical isolates harbor more genes related to virulence factors than healthy individual isolates.It was discovered that all genes (*icaA, clf, and IgG)* are expressed more strongly in clinical isolates than in healthy persons.

Keywords: *S. epidermidis*, Biofilm, Planktonic Cell, *clf, icaA, IgG*

Introduction

Methicillin-resistant staphylococci-caused urinary tract infections (UTIs) are a rising issue for many healthcare facilities, particularly when they are linked to the development of biofilm by these isolates on both living and non-living surfaces[1]. A coagulase-negative nosocomial bacterium has recently been discovered to have a role in subclinical prosthetic joint infections (PJIs). One of the main reasons for the failure of prosthetic joints is infection. The most significant of these microbes are *S. epidermidis* species, which are opportunistic microorganisms and are thought to be the root cause of nosocomial infections, bloodstream infections (particularly those related to catheter use and neonatal sepsis), urinary tract infections, mastitis, wound infections, and ear/ eye infections[2]. *S. epidermidis* antibiotic resistance poses a serious threat to public health[3]. Due to

the lack of immediate clinical symptoms brought on by the host immune system response, the diagnosis of PJIs caused by *S. epidermidis* is frequently complicated and challenging. The evaluation of the molecular basis of biofilm formation is made possible by protein pattern analysis in *S. epidermidis*, a biofilm-producing organism.

Infections in hospitals, a serious problem everywhere in the world, are most commonly caused by bacteria[4,5]. *Staphylococcus* spp. are commensal skin bacteria that have been identified from a variety of clinical sources, including endocarditis, soft tissue infections, blood infections, urinary tract infections, and respiratory tract infections.[6-8] Its ability to function as a pathogen or commensal suggests that virulence gene regulation is dynamic and flexible.[9,10] This opportunistic bacteria's pathogenic lifestyle has been the focus of research, and it is understood that its capacity to build biofilm is a key virulence element.[11–13] On the other hand, little is known about how the bacterium changes during colonisation and how the environment affects its commensal lifestyle. According to previous studies by Grice et al. and Grice and Segre,[14,15] the skin at different anatomical sites generates substantially differing microenvironments that differ in temperature, pH, moisture, and sebum concentration. The composition of a person's skin's microbial communities is generally consistent over time, despite changing external stimuli.[16] Invasive *S. epidermidis* are characterised by infection-supporting characteristics (such as increased biofilm formation, growth in nutrient-poor conditions, and antibiotic resistance), as well as particular genetic properties.[17]

The majority of *S. epidermidis* infections involve biofilm production, and *S. epidermidis*, like many other Coagulase negative *Staphylococcus* (CoNS), is an excellent biofilm maker. Biofilms are bacterial aggregations that stick to surfaces and are entrenched in an extracellular matrix. They offer resistance to antibiotics and host defence mechanisms.[18,19] When compared to those against planktonic (free-floating) bacteria, the Minimum inhibition concentration (MIC) values for many antibiotics can be several logs higher against bacteria in biofilms.

The staphylococcal cassette chromosome mec (SCCmec), the IS256 insertion sequence (associated with regulation of biofilm formation and genes encoding aminoglycoside resistance),[20,21] and the ica locus (associated with biofilm formation) have all been linked to invasive *S. epidermidis* isolates in previous studies, but the discriminatory power of these associations has been questioned.[22]

Methodology

Ethical Statement

The College of Science Research Ethics Committee accepted this research (ref. CSEC/1220/0081). Each participant gave their consent for the specimens to be given to the researcher. According to the Declaration of Helsinki, informed consent was acquired from each participant for their participation in the study.

Sample Collection

In this study, 30 *S. epidermidis* isolates were collected from Hospitals in Baghdad, Iraq, from July 2021 to January 2022. All isolates were identified by biochemical tests Vitek 2 system. All these samples were tested by PCR to analyse virulence genes. Of these, 11 isolates that had biofilm formation, virulence factors, and linezolid resistance genes were selected; 5 among them were commensals and 6 were from clinical samples. The identification of *S. epidermidis* isolates was confirmed by *16S rRNA* sequencing.

Detection by *16S rRNA*

Total DNA extraction was done by culturing each isolate overnight at 35°C by shaking in Brian Haret infusion (BHI) broth (BD, San Jose, CA, USA). The Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) was used to extract the genomic DNA from the bacterial isolates, and the AccuPower® PCR PreMix and Gradient Master Cycle (Eppendorf, Germany) were used for all amplifications. Promega Kit was used for PCR.[23]

Sequencing

PCR amplicons were sent to Macrogen Corporation, Korea, for Sanger sequencing utilizing the automated DNA sequencer ABI3730XL. Using Genius software, the *16S rRNA* sequences were generated from forward and reverce ordering data. The NCBI GenBank's Basic Local Alignment Search Tool (BLAST) was applied to investigate DNA sequencing data (http://www.ncbi.nlm.nih.gov).To generate the phylogenetic tree, MEGA7 sequence analysis software with 500 bootstrap values was employed.

Real-time PCR (RT-PCR)

It was conducted to compare *S. epidermidis* gene expression among clinical and healthy isolates in planktonic cell and biofilm form to detect higher gene expression levels for three virulence genes (*icaA, clf and IgG*) and higher biofilm formation.

Biofilm Formation for *16S rRNA*

For biofilm formation, *S. epidermidis* isolates in 200µl of tryptic soy broth (TSB) with 1% glucose as biofilm formation inducer (bacteria concentration was adjusted in equivalency to McFarland standard no. 0.5) were added to each well in sterile 96-well polystyrene microplates before the plates were enclosed and incubated aerobically at 37 °C for 24 hours. Each microbe was examined three times.[24]

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Free Cell Suspension

The isolated strains were incubated for 48 hs at 37°C in Mannitol salt broth (MSB) medium culture fluid. To extract the cell-free supernatant (CFS), the culture fluids were centrifuged for 10 minutes at 10,000 rpm.[25]

Isolated *Staphylococcus epidermidis* RNA Extraction

GoTaq® 1-Step RT-qPCR System, $MgCl_2$, nuclease-free water, and Quantifluor RNA System were used in this study. The kit from GeneAid in Thailand was used to separate the RNA from *S. epidermidis. S. epidermidis* isolates were grown in TSB with 1% glucose as biofilm inducer overnight and were then transported to microtiter plates in order to produce biofilm cells. The plates were thoroughly washed in distilled water to eliminate any trace of methanol which was added to remove any cells that didn't adhere to the wells. As soon as there was no visible biofilm on the glass surface, the biofilm cells were re-suspended in cold, sterile normal saline using a pipette. Bacterial cells were then transferred to 1.5 ml micro-centrifuge tubes. They were centrifuged at 14000 g for two minutes, and the supernatant was completely discarded[26]. The GENEzol TriRNA Pure Kit manufacturer's instructions were followed in order to isolate the RNA from this lysed sample.

Synthesis of Complementary DNA (cDNA)

The HiSenScript™ RH(–) RT Premix Kits and RT Master Mix were used to quantify the mRNA levels of the biofilm, clumping factor and IgG binding region (*icaA, clf and IgG*) encoding genes using the cDNA primers as shown in Table 1. 15 µl of nuclease-free water and 5 µl of total RNA were added to the kit's designated tube. Vortexing was used to combine the ingredients, and a brief centrifugation followed. Reverse transcription took place for an hour at 50 °C, and then RTase inactivation was done for 10 minutes at 85 °C as part of the cycling procedure. The cDNA-containing samples were kept at -20 °C until use.

Quantitative PCR Protocol

Relative quantification of *S. epidermidis* transcripts was preformed using quantitative PCR (Qpcr)[27]. Briefly, qPCR was carried out using the 7300 Real-time PCR instrument (Applied Bio-systems) in combination with the KAPA SYBR® FAST qPCR Master Mix (2×) ABI Prism (Merck) (Table 2). Master mixes were created as shown in Table 3. Relative quantities of transcripts were calculated by a standard curve for each gene generated using a 6-fold serial dilution of *S. epidermidis* ATCC 12228 and RP62A wild-type cDNA mixture.

Table 1.Primers used for Real-time PCR

Primers	Sequence 5'→3'	Target Genes	Reference
IcaA-F[25]	TCTCTTGCAGGAGCAATCAA	*icaA*	41
IcaA-R[25]	TCAGGCACTAACATCCAGCA	*icaA*	
Clf-F[26]	GGCTTCAGTGCTTGTAGG	*clf*	28
Clf-R[26]	TTTTCAGGGTCAATATAAGC	*clf*	
IgG-F[26]	CACCTGCTGCAAATGCTGCG	*IgG*	
IgG-R[26]	GGCTTGTTGTTGTCTTCCTC	*IgG*	
16S- F	TGTCGTGAGATGTTGGG	*16S rRNA*	40
16S- R	CGATTCCAGCTTCATGT	*16SrRNA*	

Table 2.Real-time Quantification PCR for *icaA, clf* and *IgG* Genes according to the Promega Programme

Steps	Temperature (°C)	Duration	Cycle
RT enzyme activation	37	15:00	1
Initial denaturation	95	05:00	40
Denaturation	95	00:20	
Annealing	58	00:20	
Extension	72	00:20	

Table 3.Quantitative PCR Component

Master Mix Components	Volume (µl)
qPCR Master Mix	5
RT mix	0.25
$MgCl_2$	0.25

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Forward primer	0.5
Reverse primer	0.5
Nuclease-free water	2.5
RNA	1
Total volume	10
Aliquot per single rxn	9 µl of Master mix per tube and 1 µl of template

Results and Discussion

Staphylococcus epidermidis isolates were confirmed using 16SrRNA sequencing technique; 4commensal isolates from (SA, DI, HDS and SP73) and 2 clinical isolates were identified as *S. epidermidis* isolates(21 and SP137).[28]

Staphylococcus epidermidis Isolates Detected by 16S rRNA Sequencing

The *16S rRNA* gene serves as the first key for phylogeny-based identification among the many genes found in a bacterial genome[29,30]. The *S. epidermidis* sequences have just recently been accessible at the NCBI GenBank. The strongly implies that gene function and protein structure are the same . Using BLAST on the NCBI website, the forward sequences of 11 *16S rRNA* gene PCR products were compared with the GenBank database to identify similar sequences (Figure 1). As shown in Table 4, the six examined sequences shared a high degree of similarity with the sequences recorded in GenBank. *S. epidermidis* 3620 showed 99.93% similarity to SA and DI strains, while *S. epidermidis* as (table 4) strain showed 100% and 99.93% strain similarity to SA and HSD strains, respectively. The taxonomists came to the conclusion that 97% or higher *16S rRNA* sequence similarity, together with 70% or higher DNA-DNA relatedness with 5% or less divergence within related sequences, is the best way to define a species.[31] The first three isolates (SA, HDS, and DI) were found to be grouped together in a sister group, however, the SP73, SP137and 21 isolates diverged from them, as per the phylogenetic analysis of the 16S rRNA (Figure 2) of the examined isolates from urine, wound, blood and fingerprint in the GenBank database.

Expression of *icaA*, *clf* and *IgG* Genes in *Staphylococcus epidermidis* Isolates

Staphylococcus epidermidis isolates were identified using 16S rRNA isolated from clinical samples (wound, urine, and blood). The expression levels of the *icaA, clf* and *IgG genes*, which are implicated in biofilm formation, clumping factor and IgG binding protein, respectively were examined using qPCR. The isolates were chosen based on the presence of these genes in *S. epidermidis* in clinical and healthy individuals to study the difference in gene expression in planktonic cells (without 1% glucose) and in biofilm (grown in 1% glucose) form. The isolate SP137 with robust biofilm formation exhibited significantly higher expression of *icaA*, *clf*, and *IgG* genes (27, 3783.3, and 247.6, respectively) compared to the other isolates ($p < 0.01$) (Table 5).

Table 4.Bacteria Isolated from Clinical and Healthy Individuals Identified according to the Results of BLAST on the GenBank Database in NCBI

Isolates	Accession Number in GenBank	Closest Species in GenBank Databasce	Similarity Index			
			Score (bit)	E-value (%)	Identity (%)	Gap (%)
SA13	MT225635.1	*S. epidermidis* 1910ICU248	2603	0	100.0	0
DI5	MT613456.1	*S. epidermidis 3039*	2590	0	100.0	0
HDS30	KY194740.1	*S. epidermidis SA144*	2604	0	100.0	0
SP73	OR534287.1	*S. epidermidis* BOF876_10458762	1958	0	98.8	0
SP137	ON000570.1	*S. epidermidis* 5TM-4	618	0	95.0	0
21	MT604781.1	*S. epidermidis* 2322	669	0	93.0	0

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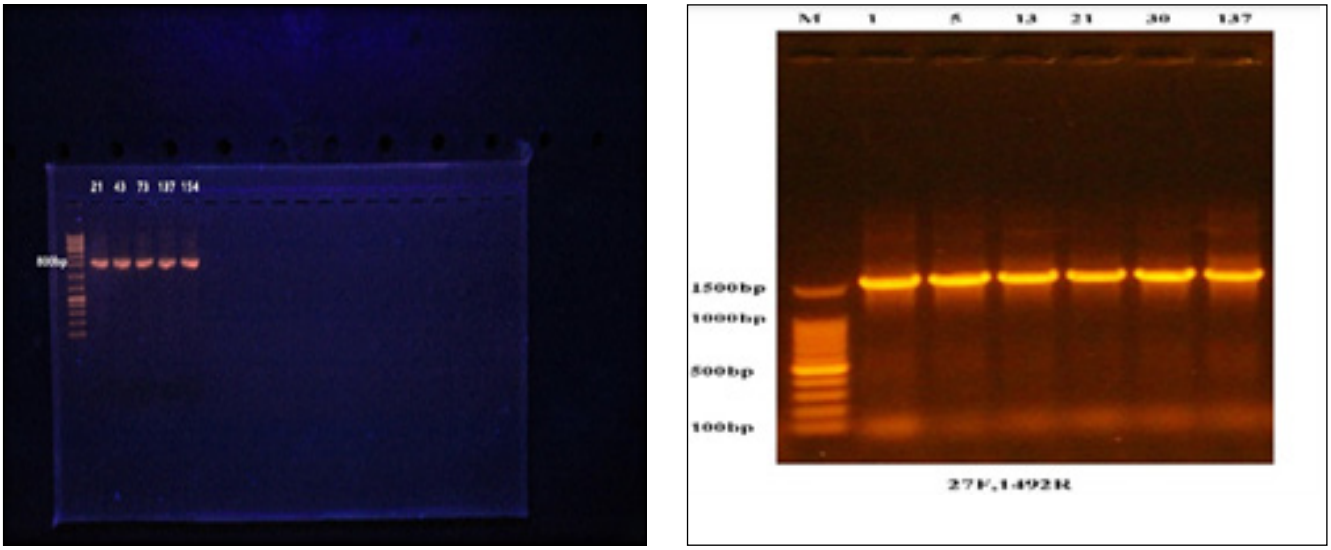

Figure 1. PCR Products of the *16S rRNA* Gene (1500 bp)

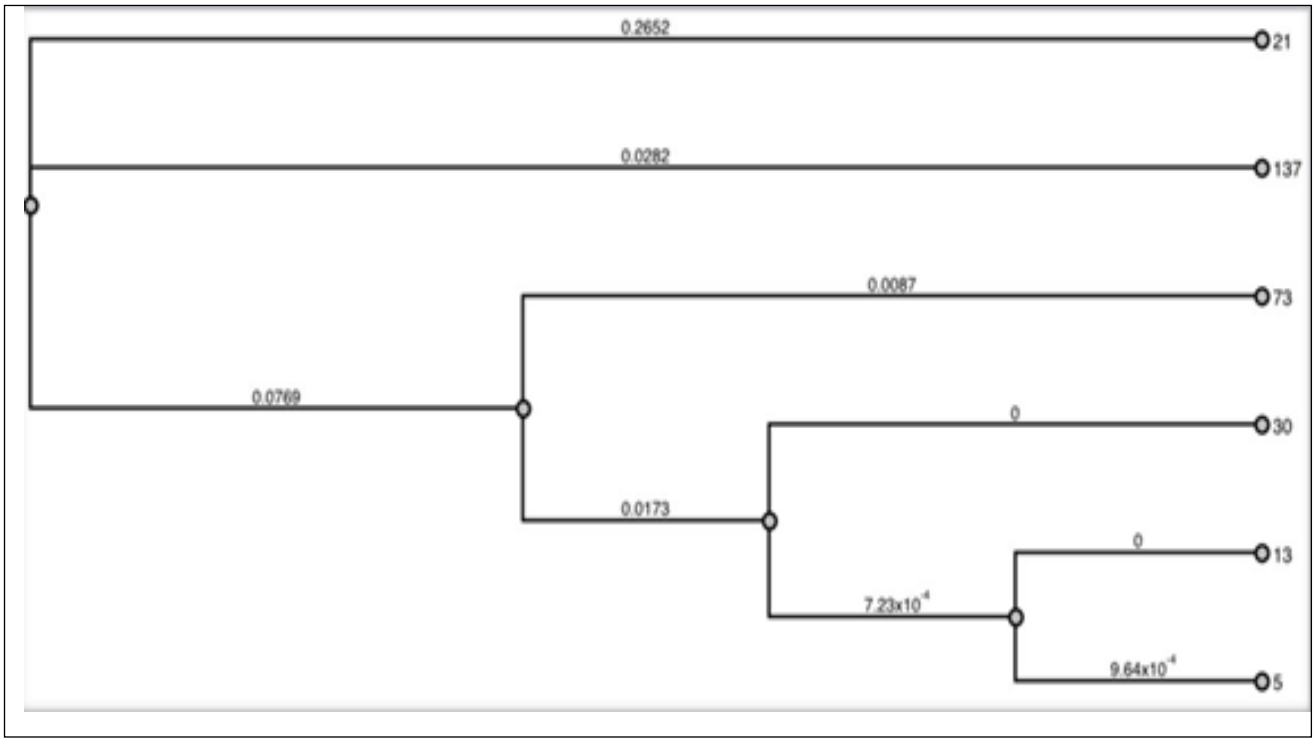

Figure 2.Phylogenic Tree in *S. epidermidis* Isolates from Clinical and Healthy Individuals

Table 5.Gene Expression Values among Clinical and Healthy Individual Strains

Genes	Clinical Isolates			Healthy Isolates		
Sample code	21	SP137	SP73	DI5	SA13	HDS30
icaA	0.862	27.0	7.1	3.26	0.06	1.4
clf	172.800	3783.3	36.7	4.6	50.70	11.0
IgG	137.800	247.6	8.6	3.65	263.40	7.0

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In light of the above-mentioned findings, it has been noted that the capacity to produce biofilms and the degree of *icaA* gene expression are related. Crawford et al.[32] previously examined the expression of *icaA* in two strains of *Staphylococcus pseudintermedius* in relation to biofilm activity as opposed to that during logarithmic and stationary phases. They verified that as compared to the stationary and logarithmic phases, the expression of *icaA* was much greater in the biofilm state. Given the part played by this gene in the development of polymer intercellular adhesion (PIA), these results were not unexpected.

Upon activation, the *ica* operon mostly contributes to the synthesis of capsular polysaccharides. In vitro production of PIA and biofilm formation are both rendered impossible by the deletion of *ica* genes (ABCD).[33] The genes encoding clumping factor (*clfA*) and IgG-binding region were identified as the most significant markers in the occurrence of infectious diseases caused by *S. epidermidis* among all virulence markers found in the *S. epidermidis* strains.[8] According to Eftekhar *et al*.'s study[34] the frequency of the virulence genes *clfB* and *clfA* was 78.60% and 71.40%, respectively. The high frequencies of the *clfA* and *clfB* genes (100%) were noted by Ghasemian et al [35]. The prevalence of the clfA gene was rather high (32.60%) among the bacterial strains used in our study. Brazil and China were shown to have a greater prevalence of this gene.[36,37] The icaADBC and *clfAB* genes were among those connected to biofilm.[38]

We are aware of a few previous investigations on biofilm development in MDR-MRSA isolates. However, we found that there is a connection between the development of biofilms and these isolates. The prevalence of the *clfA* and *clfB* genes was 100%.[39] Similar to this, all MRSA and MSSA strains in Atshans investigation carried the *clfAB* genes.[38] Momtaz et al. however, found that the *clfA* gene was present in over 20% of the *S. aureus* isolates that caused mastitis.[39] In addition to geographical differences, it appeared that the kind of clinical isolates (infection sites) may play a significant role in the prevalence of these genes necessary for colonisation. Additionally, it was noted that the *clfAB* genes were present in every MRSA and MSSA isolate. in general the moleculer techniques were apply in different medicine area.[40,41]

Conclusion

Clinical isolates harbor more genes related to virulence factors than healthy individual isolates. It was discovered that all genes (*icaA, clf,* and *IgG*) are expressed more strongly in clinical isolates than in healthy persons.

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Conflict of Interest: None

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