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Biofilm production in *Staphylococcus epidermidis* strains isolated from the skin of hospitalized patients: genetic and phenotypic characteristics

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SUMMARY

A major virulence factor of *Staphylococcus epidermidis* is its ability to form biofilms, permitting it to adhere to a surface and, in turn, to form a mucoid layer on polymer surfaces. Multiple factors have been found to influence bacterial attachment. Currently, this bacterium is commonly associated with hospital infections as a consequence of its ability to colonize, albeit accidentally, medical devices. This study investigated the genetic and phenotypic formation of biofilm in 105 *S. epidermidis* strains isolated from the skin of hospitalized patients. Fifty-eight of these patients were positive for the *mecA* gene (MRSE) and 47 were found to be negative (MSSE). Genetic characterizations were performed for the detection of the *mecA*, *icaADBC*, *atlE*, *aap*, *bhp*, *IS256* and *agr* groups by PCR. Biofilm production was examined by culturing the strains in TBS medium and TBS with 0.5 and 1% respectively of glucose, and a semiquantitative assay on tissue culture plates was used. Although a molecular analysis estimate of detailed biofilm formation is costly in terms of time and complexity, a semiquantitative assay can be proposed as a rapid and cheap diagnostic method for initial screening to discover virulent strains. We confirmed a close correlation between genetic and phenotypic characteristics, highlighting the fact that, when *S. epidermidis* isolates were cultured in TSB with 1% of glucose, an increase in biofilm production was observed, as confirmed by positivity for the *ica locus* by molecular analysis.

KEY WORDS: S. epidermidis, Biofilm, Skin, Methicillin resistance.

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INTRODUCTION

Staphylococcus epidermidis is the most frequently isolated species from human epithelia, colonizing predominantly axillae, head, and nares (Otto, 2009). In recent decades, it has emerged as the cause of hospital-acquired infections in immune-compromised patients, and the increasing antibiotic resistance of nosocomial isolates of *S. epidermidis* aggravates this problem (Du *et al.*, 2013). Moreover, gram-positive and coagulase-negative staphylococci (CoNS) are important agents of hospital-acquired infections, especially regarding their ability to grow as biofilms on various metals and materials (Speziale *et al.,* 2008).

Many medical devices implanted in the human body can be colonized by microbial species which subsequently develop well-organized sessile communities (Fancolini *et al.,* 2014). Specifically, *S. epidermidis* is one of the major biofilm-producing bacteria (Carvalhais *et al.,* 2014) and it works by attaching itself to several surfaces (El Farran *et al.,* 2013). It is the most common cause of infection in in-dwelling medical devices such as catheters, heart valves, vascular bypass grafts, nervous shunts and prosthetic implants. Cells in a biofilm are embedded in an extracellular polymeric matrix constituent, proving resistant to conventional

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therapeutic doses of antimicrobial agents and clearance by the host response (Sanchez *et al.*, 2013). Biofilm formation proceeds via initial adhesion to the surface and subsequent aggregation into multicellular structures. Thus, the development of a biofilm requires adhesive forces for the colonization of surfaces and cell interaction (Otto, 2009).

The principal component of biofilm is a polysaccharide intercellular adhesin (PIA) or polymeric *N*-acetyl-glucosamine (PNAG), produced by *ica* operon-encoded enzymes including four genes (*A,B,C,* and *D*), a regulatory gene (*icaR*) and a transposable element, *IS256* (Diemond-Hernández *et al.*, 2010). PIA plays an essential role in initial bacterial adherence to surfaces and intercellular adhesion for the cells to aggregate (O'Gara, 2007; El Farran *et al.*, 2013). The products of the *ica* gene locus comprise an N-acetylglucosamine transferase (*icaA* and *icaD*), a PIA deacetylase (*icaB*), and a putative PIA exporter (*icaC*) (Otto, 2008). Expression of the *icaADBC* genes is controlled by a complex variety of conditions and factors: one of these is the excision or insertion of *IS256* at various locations on the operon (Diemond-Hernández *et al.*, 2010).

Indeed, its reversible integration into the *ica* locus causes a biofilm-positive to biofilm-negative phenotypic switching (O'Gara, 2007; Koskela *et al.*, 2009). Despite the role of the *ica* locus in staphylococcal biofilm development, scientific evidence has demonstrated the existence of PIA/PNAG-independent biofilm mechanisms (O'Gara, 2007).

Different protein factors in *ica*-independent biofilm mechanisms have been identified, including the major cell-wall autolysin (AtlE) which promotes the early stages of *S. epidermidis* adherence to hydrophobic surfaces (Stevens *et al.*, 2009).

Another mechanism includes specific surface proteins such as the accumulation-associated protein Aap and Bhp. The latter is the Bap homologue, which is a biofilm-associated protein in *S. aureus* (O'Neill *et al.*, 2008; Mekni *et al.*, 2012; Büttner *et al.* 2015).

An important component of bacterial biofilms is their capacity to communicate using quorum-sensing systems. The most important and best-characterized quorum-sensing system in staphylococci is the accessory gene regulator (*agr*) system, which does not seem to affect *ica* expression or the production of PIA/PNAG (O'Gara, 2007).

The *agr* locus in *S. epidermidis* has been shown to be polymorphic, being divided into three distinct genetic groups. It seems to affect biofilm formation via regulation of the expression of autolysin (AtlE) (Li *et al.*, 2004). However, in addition to several genetic bases of biofilm, environmental factors (such as the presence of glucose and ethanol, osmolarity, and temperature) have all been reported to affect biofilm formation (Lim *et al.*, 2004; Zhang *et al.*, 2011). Nevertheless, much research remains to be undertaken in order to understand specific molecular mechanisms.

The purpose of this study was to confirm the close correlation between genotypic and phenotypic markers involved in biofilm production by examining 105 *S. epidermidis* strains. These had been isolated from the skin of patients hospitalized at the University Hospital of Palermo, Italy. The study deployed a semiquantitative assay as an economical method in clinical routine practice.

MATERIALS AND METHODS

Bacterial strains

One hundred and five *S. epidermidis* isolates were recovered from the skin of hospitalized patients at the "Paolo Giaccone" University Hospital in Palermo, Italy from January 2011 to December 2012. Skin swabs were sent to the microbiology laboratory of the Department of Sciences for Health Promotion and Mother-Child Care, where species identification was determined by a BD Phoenix™ system.

PCR for the detection of mecA, icaADBC, atlE, aap, bhp, IS256 and agr groups

Isolates were screened for the presence of the *mecA* gene by PCR amplification using the primers described by Zhang *et al.* (2005). The *ica* locus segments (RADBC) were detected as described by Arciola *et al.* (2005) with the modification of two separate multiplex PCRs instead of one. Three pairs of primers in a multiplex PCR for *icaB, icaC* and *icaR* were used where-

as twopairs for *icaA* and *icaD* were used in the other multiplex PCR. The presence of the virulence-associated genes *atlE, aap, bhp* and *IS256* was confirmed by amplification with primers, as adopted by Rohde *et al*. (2004). The *agr* groups (I, II and III) were defined according to Li *et al.* (2004).

In brief, DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN) and all amplifications were carried out on a GeneAmp-9700 (Applied Biosystems) in the following conditions: an initial 5 min denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at the corresponding temperature of the specific pair of primers used and a 1 min extension at 72°C, with a final extension at 72°C for 7 min. PCR products were analyzed by agarose gel electrophoresis (2.5% agarose in Tris-borateEDTA) in the presence of ethidium bromide (0.3 μg ml^{-1}) and the gel images were captured on a gel documentation system (Gel-Doc, BioRad). The primers used in this study are listed in Table 1.

Semiquantitative biofilm assay on polystyrene

The biofilm assay was performed approximately as described by O'Neill *et al*. (2008) using tissue culture-treated and serum (Sero Well Sterilin Hounslow, UK) 96-well polystyrene plates. In brief, each strain was grown overnight in trypticase soy broth (TSB). Thereafter, the bacterial suspensions were diluted 1:100 in fresh TSB and in TSB with glucose (0.5% and 1% respectively). Two hundred microliter aliquots then were placed in sterile, well-shaped, flat-bottom micro-titer plates. TSB and glucose-enriched TSB served as background controls. After a 24 h incubation period at 37°C, the content of each well was gently removed and the wells were washed twice with 200 μl of phosphate-buffered saline (PBS) to remove non-adherent bacteria. The plates were dried for 1 hour at 60°C prior to staining with a 0.4% (w/v) crystal violet solution. After 10 minutes, the plates were washed under running tap water to remove any excess stain. Biofilm formation was determined by the solubilization of the crystal violet stain in 200μl

TABLE 1 - *Oligonucleotide primers used in this study.*

Target gene	*Primer*	*Primer sequence (5'-3')*	*Product size (bp)*
mecA	MecA147-F	GTGAAGATATACCAAGTGATT	147
	MecA147-R	ATGCGCTATAGATTGAAAGGAT	
icaR	IcaR-F	TAATCCCGAATTTTTGTGAA	469
	IcaR-R	AACGCAATAACCTTATTTTCC	
icaA	IcaA-F	ACAGTCGCTACGAAAAGAAA	103
	IcaA-R	GGAAATGCCATAATGACAAC	
icaD	IcaD-F	ATGGTCAAGCCCAGACAGAG	198
	IcaD-R	CGTGTTTTCAACATTTAATGCAA	
icaB	IcaB-F	CTGATCAAGAATTTAAATCACAAA	302
		AAAGTCCCATAAGCCTGTTT	
icaC	IcaC-F	TAACTTTAGGCGCATATGTTTT	400
	Icac-R	TTCCAGTTAGGCTGGTATTG	
agr	A1	GCTGCAACCAAGAAACAACC	1022
	A2	CGTGTATTCATAATATGCTTCGATT	
	B1	TATGCAAGCCAAGCACTTGT	453
	B2	GTGCGAAAGCCGATAACAAT	
	C1	CCTTGGCTAGTACTACACCTTC	615
	C2	GTGCTTGGCTTGCATAAACA	
atlE	AtlE-F	CAACTGCTCAACCGAGAACA	
	AtlE-R	TTTGTAGATGTTGTGCCCCA	
aap	Aap-F	AAACGGTGGTATCTTACGTGAA	
	Aap-R	CAATGTTGCACCATCTAAATCAGCT	
bhp	Bhp-F	ATGGTATTAGCAAGCTCTCAGCTGG	
	Bhp-R	AGGGTTTCCATCTGGATCCG	
IS256	IS256-F	TGAAAAGCGAAGAGATTCAAAGC	
	IS256-R	ATGTAGGTCCATAAGAACGGC	

of 33% glacial acid for 10 min, shaking and measuring the absorbance with a microtiter plate reader (Labsystems Multiskan® MCC/340) at 492 nm. The absorbance obtained from the medium control well was deducted from the absorbance tests and the values were then averaged. Assays were performed in triplicate and repeated at least three times. A biofilm-positive phenotype was defined as an A_{492} of ≥0.17. The well-characterized biofilm-producing strain *S. epidermidis* RP62A (ATCC 35984) and the biofilm-negative *Staphylococcus carnous* TM 300 strain were used as positive and negative controls respectively.

Pulsed field gel electrophoresis (PFGE)
To exclude the clonality of strains, PFGE was used for molecular characterization. PFGE was carried out according to Mulvey *et al*. (2001). Restriction fragments were separated using a BioRad CHEF DR III apparatus. PFGE profiles obtained were analyzed with Bio-Numerics software.

Statistical analysis
The statistical analysis was performed with the R statistical software package [R Development Core Team. R statistical software package, version 2.13.0, 2011]. Two-sided tests were used and *p*-values of <0.05 were considered statistically significant. Absolute and relative frequencies were calculated for qualitative variables. Quantitative variables were evaluated for normality of distribution (Shapiro-Wilk test p >0.05) and non-normally distributed variables were summarized as median (an interquartile range). The Kruskal-Wallis test was used for comparisons between groups. Absorbance at 492 nm was log-transformed due to the skewed distributions. Multiple linear regression was performed to assess the relationship between gene presence and log-transformed absorbance at 492 nm. Goodness of fit was calculated for each model, and the model with the lowest Akaike information criterion was considered to possess the best fit. Finally, receiver-operating-characteristic (ROC) curves were plotted and the area under the curves was calculated for absorbance at 492 nm in different TSB media in order to identify *ica* positive isolates.

TABLE 2 - *Results of molecular characteristics of 105 S. epidermidis included in the study.*

Gene	*PCR results*	*No. of strains*	*(%)*
mecA	+	58	(55.2)
	-	47	(44.8)
agr	*I*	74	(70.5)
	II	30	(28.6)
	III	1	(0.9)
ica	+	45	(42.9)
	-	60	(57.1)
atlE	+	90	(85.7)
	-	15	(14.3)
aap	+	71	(67.6)
	-	34	(32.4)
bhp	+	28	(26.7)
	-	77	(73.3)
IS256	+	51	(48.6)
	-	54	(51.4)
Total		105	100.0

RESULTS

As shown in Table 2, this study involved a total of 105 *S. epidermidis* isolates, of which 58 (55.2%) were positive for the *mecA* gene (MRSE) and 47 (44.8%) were negative (MSSE). The *agr* groups I, II and III were found in 74 (70.5%), 30 (28.6%) and 1 (0.9%) strains respectively while the *ica* locus was found in 45 (42.9%) strains, which expressed some or all genes.
Moreover, *atlE*, *aap* and *bhp* were present in 90 (85.7%), 71 (67.6%) and 28 (26.7%) isolates respectively. *IS256* was detected in approximately half (n=51; 48.6%) of the analyzed bacteria. Table 3 summarizes the univariate analysis of the genetic factors and biofilm production in TSB and TBS, as supplemented with 0.5% and 1% glucose concentrations.
Elevated biofilm production was observed when *S. epidermidis* isolates were cultured in TSB with 1% of glucose (absorbance at 492 nm =0.19 vs. 0.14 in TSB with 0.5% glucose and 0.07 in TSB respectively). No statistically significant differences were found between MSSE and MRSE isolates.
Isolates positive for *agr* group I demonstrated a significantly higher biofilm production at TSB with glucose concentrations of 0.5% and 1% (p-value <0.01 in both cases). *S. epidermidis*, which was positive for *ica* locus, produced significantly more biofilm in a TSB medium

TABLE 3 - *Univariate analysis of factors associated with biofilm production in different TSB solutions.*

	PCR results	*TSB median absorbance at 492 nm (interquartile range)*	*TSB with 0.5% glucose median absorbance at 492 nm (interquartile range)*	*TSB with 1% glucose median absorbance at 492 nm (interquartile range)*
Total		0.07 (0.11)	0.14 (0.23)	0.19 (0.36)
mecA	+	0.07 (0.09)	0.16 (0.25)	0.19 (0.33)
	-	0.08 (0.18)	0.13 (0.30)	0.19 (0.35)
agr	*I*	0.08 (0.12)	0.16 (0.38)**	0.25 (0.45)***
	II	0.06 (0.06)	0.09 (010)**	0.13 (0.12)***
	III	0.10 (0)	0.13 (0)**	0.92 (0)***
ica	+	0.15 (0.22)***	0.38 (0.59)***	0.46 (0.64)***
	-	0.05 (0.06)***	0.10 (0.9)***	0.12 (0.14)***
atlE	+	0.07 (0.12)	0.15 (0.29)	0.21 (0.38)
	-	0.09 (0.10)	0.12 (0.20)	0.16 (0.19)
aap	+	0.08 (0.17)	0.15 (0.38)	0.25 (0.41)*
	-	0.07 (0.06)	0.13 (0.18)	0.16 (0.18)*
bhp	+	0.05 (0.10)	0.15 (0.42)	0.21 (0.37)
	-	0.08 (0.10)	0.14 (0.20)	0.19 (0.34)
IS256	+	0.07 (0.10)	0.15 (0.39)	0.24 (0.37)
	-	0.07 (0.11)	0.14 (0.23)	0.19 (0.33)

*<0.05; **<0.01; ***<0.00

with glucose (absorbance at 492 nm =0.38 vs. 0.10 at TSB with 0.5% glucose; absorbance at 492 nm =0.46 vs. 0.12 at TSB with 1% glucose respectively) and in the absence of glucose (absorbance at 492 nm=0.15 vs. 0.05; p<0.001). Of the strains positive for adensins (*atlE, aap* and *bhp*), only those *aap*$^+$ were found to have produced significantly more biofilm in TSB with 1% of glucose (absorbance at 492 nm =0.25 vs. 0.16, p<0.05).

The multivariate analysis of genetic factors associated with biofilm production is reported in Table 4. The *ica*$^+$ *S. epidermidis* strains were significantly associated with more biofilm production in both TSB without glucose (adj-p<0.001) and TSB with an addition of glucose (adj-p<0.001 in both BT with 0.5% glucose and BT with 1% glucose).

The *agr* locus type III was found to significantly increase biofilm production only when the strains were cultured in TSB-1% glucose (adj-p<0.05).

Finally, both TSB with 0.5% or TSB with 1% glucose produced more accurate results than TSB without glucose in predicting *ica*$^+$ genotype (AUC=0.87 vs. 0.77) (Figure 1). According to the PFGE results and in comparison with the restriction patterns, it is possible to establish that the 105 *S. epidermidis* strains were clonally unrelated.

TABLE 4 - *Multivariable analysis of factors associated with biofilm production (absorbance at 492 nm) in different TSB solutions.*

Gene	*PCR results*	*Log_{10} absorbance at 492 nm in TSB*	*Log_{10} absorbance at 492 nm in TSB with 0.5% glucose*	*Log_{10} absorbance at 492 nm in TSB with 1% glucose*
agr	I	NR	NR	Referent
	II	NR	NR	-0.02
	III	NR	NR	0.92*
ica	+	0.39***	0.58***	0.55***
	-	Referent	Referent	Referent
aap	+	NI	NI	NR
	-	NI	NI	NR

*<0.05; **<0.01; ***<0.001. NI: not included in the model. NR: not retained in the best fitting model.

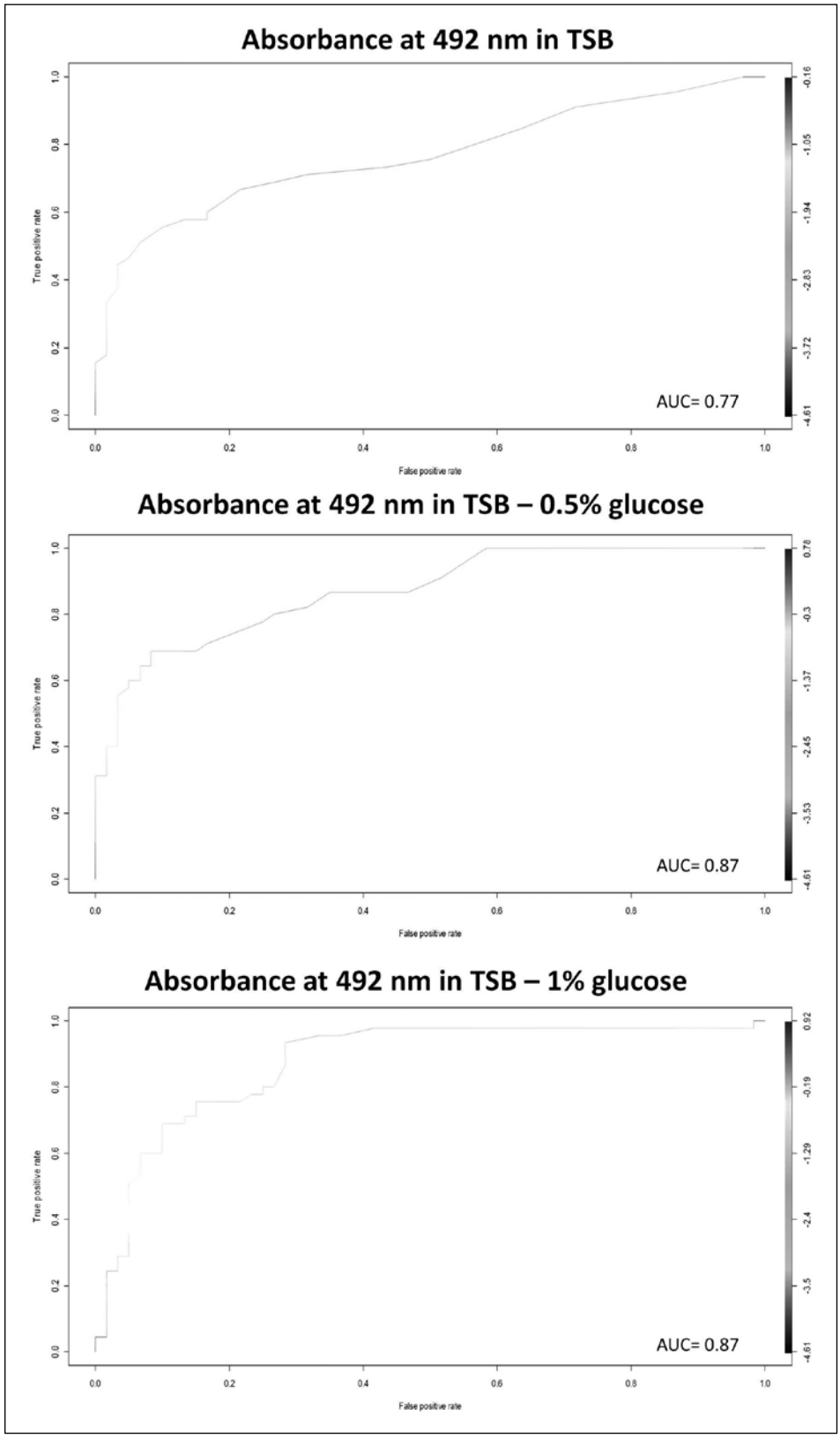

FIGURE 1 - *ROC curves for ica+ genotype in TSB medium (a), TSB medium supplemented with 0.5% (b) and 1% glucose (c).*

DISCUSSION

As a commensal living in human skin, *S.epidermidis* has been found in recent years to play an important role as a pathogen of hospital-acquired infections. This is especially the case after the implantation of medical devices where bacteria are accidentally introduced into the body with the insertion of foreign bodies, adhering and forming biofilm. Indeed, biofilms play a pivotal role in healthcare-associated infections with a risk of antibiotic-resistance phenomena (Francolini *et al*., 2010). The production of biofilms provides a mode of survival for microbes in the presence of unfavorable conditions (El Farran *et al.*, 2013), constituting a complex process of events in which different proteins play a role and several components can influence the organization of biofilms.

Given that the recovery of *S. epidermidis* from cultures may be difficult to interpret, the ability to discriminate between invasive and commensal isolates would facilitate clinical decision-making. Furthermore, it remains an important question as to whether all strains of *S. epidermidis*, selected by the hospital environment, have the same ability to cause foreign body infections or if the distribution of virulence-associated genes is crucial as a determinant in biofilm production.

As initial findings of interest, we observed a high colonization rate of MRSE (55.2%) on the skin of hospitalized patients. This result seems to support the concern that bacteria in a hospital environment may acquire pharmacological resistance. For example, most frequently methicillin resistance is conferred by the *mecA* gene carried by a mobile genetic element (Du *et al.*, 2013). Despite this finding, our results revealed no difference in the frequency of biofilm production between the two isolate groups. Membrane proteins play an important role in the organization of biofilms, and gene expression differs between bacteria grown in biofilms and planktonic condictions (Águila-Arcos *et al.*, 2015). A second point of interest concerned our assessment of whether various adhesins or surface proteins can serve in the first step of biofilm formation as receptors for bacterial attachment, as reported by other authors (Fitzpatrick *et al.*, 2005).

Of the genes which correlated with bacterial attachment in this study, a high prevalence of *atlE* (85.7%) and *aap* (67.6%) was found but only the latter was related to higher biofilm production when cultured in TSB with 1% of glucose.

Regarding *IS256* and according to the international literature (Kozitskaya *et al.*, 2004), this genetic element frequently occurs in nosocomial strains and was present in the isolates in this study in approximately half of the bacteria.

Although the presence of *IS256* as a feature of pathogenicity (Kozitskaya *et al.*, 2004) cannot be excluded, no evidence supported this role in the *S. epidermidis* biofilm-formation discussed in this study. However, the prevalence of *ica* locus, which mediates the synthesis of the main exopolysaccharide biofilm component (Arciola *et al*., 2015), was found to be higher when compared with that reported in studies regarding the healthy skin of individuals (Galdbart *et al,* 2000; El Farran *et al.*, 2013).

This fact can be considered the most important genotypic marker in predicting biofilm formation, being significantly associated with higher biofilm production in all environmental conditions, also after adjusting for potential confounding.

Regarding the polymorphic *agr* locus and the elevated percentage of *agrI* (70.5%), the genetic characterization of the isolates was emphasized. As reported by Li *et al.* (2004), the *agr* I group is most frequent in pathogenic *S. epidermidis* isolates acquired in hospital settings when compared to those from healthy subjects. The *agr I* group isolates discussed in this paper produced more biofilm in TBS with 05% and 1% of glucose. As several environmental factors have been shown to affect biofilm production in *S. epidermidis*, we studied biofilm formation in response to glucose. We observed an increased biofilm formation when the isolates were cultured in TSB added to glucose when compared to the broth alone. In particular, TSB added to glucose provides us with an elevated degree of accuracy in identifying strains of *ica*$^{+}$ *S. epidermidis*.

Although discordant data have been reported in different studies regarding the role of glucose in biofilm formation, it should be noted that several molecular mechanisms are still unknown

and this aspect of research merits further study (Dobinsky *et al.*, 2003; Lim *et al.*, 2004). Finally, the skin isolates of *S. epidermidis* seem to be more virulent than biofilm production when positive for the genetic determinant *ica* locus.
In conclusion, given the rich abundance of *S. epidermidis* on the human skin, it can be said to be the most common cause of contamination in clinical specimens (Büttner *et al.* 2015).
Since biofilm formation is regarded as a major patho-mechanism (Schoenfelder *et al.*, 2010), it is a challenge for the medical microbiologist to easily identify distinct and invasive isolates. We consider that the use of a semiquantitative biofilm assay on polystyrene should be introduced in the laboratory as part of clinical practice to highlight more virulent strains, especially those with cultures in TSB with 1% of glucose.
Thus, predicting the characteristics of bacterial virulence would be facilitated prior to using genotypic methods, particularly regarding the strains originating from in-dwelling medical devices in immunocompromised patients.

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