RESEARCH LETTER

A comparative analysis of phenotypic and genotypic methods for the determination of the biofilm-forming abilities of *Staphylococcus epidermidis*

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Abstract

The collection of 146 *Staphylococcus epidermidis* strains isolated from the nasopharynx of lung cancer patients has been studied for the ability of slime secretion and biofilm formation using the Congo red agar (CRA) test and the microtiter plate (MtP) method, respectively. The prevalence of the *icaAD* and the *aap* genes was also analyzed. Some isolates (35.6%) were biofilm positive by the MtP method, while 58.9% of isolates exhibited a slime-positive phenotype by the CRA test. The sensitivities of the CRA test evaluated using the MtP method as a gold standard of biofilm production were 73.1%, 97.3% and 13.3% for all the strains screened, *ica*-positive and *ica*-negative strains, respectively. The genotype $ica^{+}aap^{+}$ was correlated with a strong biofilm-producer phenotype. Interestingly, some of the $ica^{-}aap^{-}$ isolates could also form a biofilm. The correlation between the presence of *icaAD* genes and the biofilm-positive phenotype by the MtP method as well as slime production by the CRA test was statistically significant ($P < 0.0001$). However, some *S. epidermidis* strains possess the potential ability of *ica*-independent biofilm formation; thus, further studies are needed to determine reliable, short-time criteria for an *in vitro* assessment of biofilm production by staphylococci.

Introduction

The coagulase-negative staphylococci (CoNS), including *Staphylococcus epidermidis*, are a major component of the normal microbial communities of the human body, colonizing preferably the upper airways and skin. These opportunistic pathogens rarely cause infections in a normal host; however, in recent years, CoNS have generally been accepted as important nosocomial pathogens, especially in patients with predisposing factors such as an indwelling or an implanted foreign body. The biomaterial-associated staphylococcal infections are more resistant to the host immune response and antimicrobial chemotherapy at a standard dosage. For these reasons, such infections are very difficult to eradicate (O'Gara & Humphreys, 2001; Hamilton, 2002; Cerca *et al.*, 2005).

The ability of *S. epidermidis* to form a biofilm represents the most important virulence determinant (Götz, 2002; Vuong & Otto, 2002). Several studies have been undertaken in order to determine the genetic and/or the environmental factors responsible for *in vitro* biofilm formation by *S. epidermidis*, the leading opportunistic pathogen involved in infections associated with biomaterials. A number of reports (Ziebuhr *et al.*, 1997; Galdbart *et al.*, 2000; McKenney *et al.*, 2000; Mack *et al.*, 2004, 2007; Maira-Litran *et al.*, 2004; Rohde *et al.*, 2005; Stevens *et al.*, 2008) have highlighted that the *ica* and *aap* genes, known determinants of polysaccharide- and protein-mediated biofilm production, are widespread among clinically significant *S. epidermidis* isolates. Therefore, polysaccharide intercellular adhesin, also called the slime exopolysaccharide component, and accumulation-associated protein have been described as factors playing an essential role in biofilm formation (Cramton *et al.*, 1999; Mack, 1999; O'Gara & Humphreys, 2001; Götz, 2002).

A number of methods are available to detect the capability of staphylococci to colonize the biomedical devices. The Congo red agar (CRA) assay described by Freeman *et al.* (1989) and/or the microtiter plate (MtP) test devised by

Christensen *et al.* (1985) were most commonly used as the phenotypical methods for slime and/or biofilm production. We would like to point out that the slime-positive strain (measured by the CRA test) does not necessarily indicate the ability of this strain to form a biofilm (by the MtP test). Therefore, 'slime' and 'biofilm' terms cannot be used alternatively. In this study, a collection of 146 nasopharyngeal *S. epidermidis* strains was screened for the presence of genetical biofilm markers (*icaAD* and *aap* genes), the ability of slime secretion using the CRA test and biofilm formation by the MtP method. The aim of our work was to evaluate the relationship between these phenotypic data and the genotypic pattern of the screened strains.

Materials and methods

Bacterial strains

The collection of 146 *S. epidermidis* strains isolated from the nasopharynx of lung cancer patients was included in the present study. These strains were collected during patients' hospitalization at The Department of Thoracic Surgery of Medical University of Lublin. The patients with resectable lung cancer received a preoperative antimicrobial prophylaxis according to hospital policy (piperacillin, cefuroxime alone or in combination with amikacin). At the time of sampling, none of the patients had clinical symptoms of airway infections. The study has been approved by the Ethical Committee of the Medical University of Lublin. Informed consent was obtained from all patients.

Clumping factor detection using the Slidex Staph Kit (BioMerieux, France), a coagulase-test tube and the ID32-Staph system (BioMerieux) was performed for species identification of staphylococcal isolates. *Staphylococcus epidermidis* strain ATCC 12228 and *S. epidermidis* ATCC 35984 were used in biofilm assays as a negative and a positive control, respectively.

MtP method

Biofilm formation *in vitro* was carried out as described by Christensen *et al.* (1985) and Mack *et al.* (1992), with a slight modification. All strains were grown overnight at 35 °C in Trypticase soy broth (TSB; Biocorp, Poland) as well as in a medium supplemented with 0.5% glucose and 4% NaCl. The cultures were diluted 1 : 200 in the appropriate medium (TSB as standard conditions and TSB supplemented with 0.5% glucose plus 4% NaCl as inducing conditions), and 200 μL of cell suspensions per well were used to inoculate sterile 96-well polystyrene microtitrate plates (Nunc, Denmark). After a 24-h incubation at 35 °C, the wells were carefully emptied and gently washed three times with 200 μL of sterile phosphate-buffered saline (Biomed, Lublin, Poland), and then air-dried in an inverted position. Adherent cells were stained with 0.25% safranin for 5 min. The wells were rinsed again with distilled water. After drying, 200 μL of a 0.9% NaCl solution was added to each well and the $A_{490\,nm}$ was determined using an enzyme-linked immunosorbent assay plate reader (BioTek ELx800, Vermont). The OD values were corrected by subtracting values from noninoculated negative controls. A strain was considered as biofilm positive if the average OD value obtained by safranin staining was higher than the average OD value of the negative control (*S. epidermidis* ATCC 12228; OD values > 0.125). Strains with OD values between 0.126 and 0.9 were regarded as weak biofilm producers, whereas an $OD \geq 1$ indicated strong biofilm producers (Jain & Agarwal, 2009). Each assay was performed in quadruple in two separate experiments.

CRA test

Slime production was assessed on the basis of the color of staphylococcal colonies cultured on CRA according to the criteria reported by Freeman *et al.* (1989). Briefly, *S. epidermidis* isolates were inoculated onto nutrient agar plates supplemented with sucrose (50 g L^{-1}) and Congo red (0.8 g L^{-1}), and then cultured for 20 h at 35 °C. Strains intensively producing slime formed black colonies with a metallic sheen, strains moderately producing slime formed dark-pink colonies and nonproducing slime strains formed light-pink colonies.

Isolation of DNA

DNA was isolated from 2 mL of overnight bacterial culture. Extraction was performed using the Genomic Mini Kit (A&A Biotechnology, Poland) according to a protocol for Gram-positive bacteria. After isolation, DNA was measured using a BioPhotometer (Eppendorf, Germany) to determine the concentration and purity.

icaA, *icaD*, *aap* amplification

All PCR reactions were performed on a Mastercycler ep gradient (Eppendorf). For the detection of *icaA*, the primers were as follows: 5′-AACAAGTTGAAGGCATCTCC and 5′-GA TGCTTGTTTGATTCCCT (Tormo *et al.*, 2005). The two primers for the detection of *icaD* were, respectively, 5′-CC GGAGTATTTTGGATGTATTG (forward primer) and 5′-TT GAAACGCGAGACTAAATGTA (reverse primer). According to Vandecasteele *et al.* (2003), for the detection of the *aap* gene, following primers were used: 5′-ATACAACTGGTGCAGAT GGTTG (forward primer) and 5′-GTAGCCGTCCAAGTTTT ACCAG (reverse primer). The cycling conditions were as follows: preheating for 4 min at 96 °C, followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 60 °C for 30 s, primer extension at 70 °C for 30 s and final extension at 70 °C

for 4 min. DNA of the reference biofilm-negative *S. epidermidis* strain ATCC 12228 was used as a positive control for *aap* and as a negative control for the *icaADBC* operon. Amplified products were analyzed by agarose gel electrophoresis.

Statistical analysis

Fisher's exact test was used for statistical analyses of data using GRAPHPAD INSTAT Software (La Jolla, CA). The differences with P lower than 0.05 were considered as statistically significant. The sensitivity, positive predictive value and negative predictive value of the CRA test were also calculated using this software. The MtP method was used as the gold standard in these calculations.

Results

The PCR technique was performed for *icaAD* and *aap* genes in all the 146 staphylococcal strains. As shown in Table 1, the majority of tested isolates (106/146; 72.6%) were *ica* negative, among which $ica^{-}aap^{+}$ was the dominant genotype (76/106; 71.7%). Among the *ica*-positive isolates (40/146, 27.4%), the $ica^{+}aap^{+}$ genotype was the most common (34/40; 85.0%). Out of the total 146 *S. epidermidis* nasopharyngeal isolates, 52 (35.62%) were biofilm positive by the MtP method, while 86 (58.9%) isolates exhibited a slime-positive phenotype by the CRA test (Table 1). The prevalence of the *icaAD* and the *aap* genes in relation to biofilm-positive (by the MtP method) and slime-positive (by the CRA test) phenotypes of nasopharyngeal *S. epidermidis* isolates was analyzed (Table 1). Thirty-one (59.6%) of 52 biofilm-positive isolates by the MtP method were positive for *icaAD* and *aap* genes, whereas six (11.5%) strains were *ica* positive and *aap* negative. However, among the biofilm-negative isolates by the MtP method, three isolates with the $ica^{+}aap^{+}$ genotype were found. Most of the *ica*-positive isolates were found to be strong biofilm producers. Most of the *ica*-negative strains (91/106; 85.8%) did not produce a detectable amount of biofilm *in vitro*, including 68 isolates harboring the *aap* gene. Fifteen (28.8%) isolates produced an *ica*-independent biofilm, including eight (15.4%) *aap*-positive and seven (13.5%) *aap*-negative strains. Interestingly, two out of $ica^{-}aap^{-}$ isolates were strong biofilm producers. Among 40 of the *ica*-positive strains, 39 were classified as slime producers by the CRA test (Table 1). However, out of 106 *ica*-negative isolates, 47 were slime positive. The concordance between the occurrence of *icaAD* genes and the ability of biofilm formation determined by the MtP method as well as slime production examined by the CRA test was statistically significant ($P < 0.0001$). There was no relationship between *aap* occurrence and biofilm formation ($P = 1$) or slime production ($P = 0.56$) (Table 1).

The data obtained using the CRA and MtP methods among *ica*-positive and *ica*-negative staphylococci are presented in Table 2. The strains that yielded matching results using both the CRA and the MtP methods were 84 (57.5%) of all the strains screened. For all the strains tested, the sensitivity of the CRA test evaluated using the MtP method as a gold standard of biofilm production was 73.1%. The differentiation of the sensitivity of the CRA test was observed when *ica*-positive and *ica*-negative staphylococcal strains were analyzed separately (97.3% and 13.3%, respectively).

Table 1. Relationship between genotype, biofilm formation using the MtP method and slime production using the CRA test in nasopharyngeal *Staphylococcus epidermidis* isolates ($n = 146$)

		No. of strains positive	
Genotype	Total	By MtP method	By CRA test
ica^{+}			
aap^{+}	34	31*	33
aap^{-}	6	6†	6
ica^{-}			
aap^{+}	76	8‡	35
aap^{-}	30	7§	12
Total	146	52	86

*27 and 4 strains with strong and weak biofilm producer phenotypes, respectively.
†5 and 1 strains with strong and weak biofilm producer phenotypes, respectively.
‡1 and 7 strains with strong and weak biofilm producer phenotypes, respectively.
§2 and 5 strains with strong and weak biofilm producer phenotypes, respectively.

Table 2. Comparison of the CRA test and the MtP method for the detection of slime and biofilm production in nasopharyngeal *Staphylococcus epidermidis* isolates

	MtP method		
	Positive	Negative	Total
ica-positive isolates ($n = 40$)			
CRA test			
Positive	36 (90%)	3 (7.5%)	39 (97.5%)
Negative	1 (2.5%)	0 (0%)	1 (2.5%)
Total	37 (92.5%)	3 (7.5%)	40 (100%)
ica-negative isolates ($n = 106$)			
CRA test			
Positive	2 (1.9%)	45 (42.4%)	47 (44.3%)
Negative	13 (12.3%)	46 (43.4%)	59 (55.7%)
Total	15 (14.2%)	91 (85.8%)	106 (100%)

For *ica*-positive isolates: sensitivity – 97.3%, positive predictive value (PPV) – 92.3%, negative predictive value (NPV) – 0%, percent of matching results – 90% (36/40).
For *ica*-negative isolates: sensitivity – 13.3%, positive predictive value (PPV) – 4.2%, negative predictive value (NPV) – 78.0%, percent of matching results – 45.3% (48/106).

In our study, the ability of biofilm formation *in vitro* by 146 nasopharyngeal *S. epidermidis* isolates was assessed using two variations of medium: TSB (standard conditions) and TSB supplemented with 0.5% glucose plus 4% NaCl (inducing conditions). Fifty-two (35.6%) tested isolates were classified as biofilm positive. Strains biofilm positive by the MtP method in correlation to the genotype and the medium used are listed in Table 3. Thirty-one out of the *ica*-positive isolates produced biofilms irrespective of the conditions used – standard or inducing. Among these *ica*-positive strains, one was able to produce biofilms only in TSB and five only on TSB-supplemented medium. In contrast, most of the *ica*-negative isolates (11/15) formed biofilms only in TSB. The difference in the ability of *S. epidermidis* isolates to form biofilms under optimal conditions was statistically significant ($P < 0.0001$).

Discussion

MtP, CRA and/or PCR methods have been used by many researchers to determine the crucial virulence factors of CoNS, i.e. the ability of biofilm formation (Christensen *et al.*, 1985; Freeman *et al.*, 1989; Arciola *et al.*, 2002, 2006; Bozkurt *et al.*, 2009; El-Mahallawy *et al.*, 2009). Some reports (Frebourg *et al.*, 2000; Galdbart *et al.*, 2000; Vandecasteele *et al.*, 2003; Chokr *et al.*, 2006; Satorres & Alcaráz, 2007; Mateo *et al.*, 2008; Jain & Agarwal, 2009) indicate that these methods, alone or in combination, can be useful to discriminate between colonizing or commensal and invasive staphylococcal strains and can lead to the early detection and management of potentially pathogenic isolates responsible for device-associated nosocomial infections. In this study, using three *in vitro* screening procedures (the MtP method, the CRA test and the PCR technique), we tested 146 nasopharyngeal *S. epidermidis* strains. Only 57.5% of all the strains tested exhibited a positive phenotype (biofilm and slime positive) in both MtP and CRA methods. As found by Arciola *et al.* (2006), 80% of *S. epidermidis* strains isolated from orthopedic implant infections yielded matching results using both these methods. Moreover, several studies have reported a significant difference between sensitivity, 7.6% (Mathur *et al.*, 2006) and 75.86% (Jain & Agarwal, 2009), of the CRA test evaluated using the MtP method as a gold standard of biofilm production. In our study, the sensitivity of the CRA test was 73.1% for all the strains tested.

Table 3. Correlation between genotype and medium in biofilm formation in nasopharyngeal *Staphylococcus epidermidis* isolates

	No. of isolates ($n = 52$)		
	Biofilm formation		
Genotype	Irrespective of medium*	TSB†	TSB+G+NaCl‡
ica$^+$			
aap$^+$	26	0	5
aap$^-$	5	1	0
ica$^-$			
aap$^+$	1	7	0
aap$^-$	0	4	3
Total	32	12	8

*Number of isolates forming biofilms in both TSB medium and TSB medium supplemented with 0.5% glucose plus 4% NaCl.
†Number of isolates forming biofilms only in TSB medium.
‡Number of isolates forming biofilms only in TSB medium supplemented with 0.5% glucose plus 4% NaCl.

Molecular techniques, including traditional PCR or real-time PCR, have been proposed recently for the detection of genes playing a crucial role in the pathogenicity of bacteria (Frebourg *et al.*, 2000; Miyamoto *et al.*, 2003; Arciola *et al.*, 2006; Liberto *et al.*, 2007). In *S. epidermidis*, the *ica* operon appears to play an important role in biofilm formation, and consequently, in the pathogenesis of infections associated with indwelling or implanted medical devices (Cafiso *et al.*, 2004; Mack *et al.*, 2004, 2007; Maira-Litran *et al.*, 2004; O'Gara, 2007; Stevens *et al.*, 2008). As found by other authors (Ziebuhr *et al.*, 1997; Frebourg *et al.*, 2000; Miyamoto *et al.*, 2003; Vandecasteele *et al.*, 2003; de Allori *et al.*, 2006; Satorres & Alcaráz, 2007), the majority of *S. epidermidis* clinical isolates (over 80%) possess the *ica* operon. The rates of occurrence of the *ica* operon in isolates, defined as carriage, commensal or contaminant from skin or mucosal membranes of airways, vary significantly from 6% to 80%, depending on the origin of the isolate (hospital or community). Our data indicate that the prevalence of the *ica* operon in nasopharyngeal *S. epidermidis* isolates from hospitalized patients was 27.4%.

As found by other authors (Mack *et al.*, 1992, 2004, 2007; Knobloch *et al.*, 2001; Conlon *et al.*, 2002; Fitzpatrick *et al.*, 2002; Mathur *et al.*, 2006), biofilm formation is influenced by culture conditions, for example medium supplementation with sugars, salts or ethanol, allowing phenotypic biofilm expression. In the present paper, we evaluated the correlation between the presence of *icaAD* genes and the ability of biofilm formation observed using the MtP method as well as of slime production using the CRA test for all staphylococci tested. In terms of the literature data (Mack *et al.*, 1992; Conlon *et al.*, 2002; Fitzpatrick *et al.*, 2002; Mathur *et al.*, 2006) indicating that supplementation of the growth medium by glucose plus NaCl is an environment favoring the activation of *ica* operon transcription, in the MtP method, we used TSB as well as this medium supplemented with glucose plus NaCl. However, the majority of the *ica*-positive *S. epidermidis* isolates described in this paper were able to form biofilm under static conditions using standard or 'inducing' TSB media. In contrast, the *ica*-negative isolates preferably were able to produce biofilms only when the standard medium was used. It is worth

mentioning that biofilm detection using the MtP method is not only dependent on the composition of the medium; additional factors are involved in biofilm development *in vitro* and they can change the results of the test drastically. As described by Dice *et al.* (2009), the time of incubation was also an important parameter for such studies. Some isolates of *S. epidermidis* were found to form a biofilm in a flow cell system only when the time of incubation was increased to 6 days (slower biofilm-forming strains).

The majority of biofilm-positive nasopharyngeal *S. epidermidis* isolates obtained using the MtP method had the *ica* operon and/or the *aap* gene. According to the literature data (Arciola *et al.*, 2006; de Araujo *et al.*, 2006), the simultaneous presence of the *ica* operon and the *aap* gene plays an important role in the strong biofilm-producing phenotype, which is in agreement with our data. The dominant genotype of biofilm-positive nasopharyngeal *S. epidermidis* isolates tested in this study was $ica^{+}aap^{+}$. However, it is known that genes other than *ica* and *aap* are also likely to be involved in biofilm formation (Rohde *et al.*, 2005; Chokr *et al.*, 2006; Petrelli *et al.*, 2006; O'Gara, 2007; Qin *et al.*, 2007; Stevens *et al.*, 2008). Our data indicate that $ica^{-}aap^{+}$ as well as $ica^{-}aap^{-}$ isolates of *S. epidermidis* could form a biofilm by the MtP method. In addition, our unpublished data showed that the $ica^{-}aap^{+}$ reference strain of *S. epidermidis* ATCC 12228, considered as biofilm negative, and some clinical staphylococcal isolates without *ica* genes (also aap^{-}) can form biofilms on some polytetrafluroethylene vascular grafts after several days of incubation under static conditions.

The majority of the *ica*-positive nasopharyngeal *S. epidermidis* isolates were also able to produce slime, which was monitored using the CRA test. This is in agreement with the data presented by other authors (Arciola *et al.*, 2002; Stevens *et al.*, 2008; El-Mahallawy *et al.*, 2009); the presence of the *ica* operon was strongly associated with a slime-positive phenotype. However, *ica*-negative and slime-positive isolates in the CRA test were also described in the present paper.

Arciola *et al.* (2006) found a rather good concordance between the occurrence of *ica* genes, monitored using PCR-based analysis, and the CRA test. According to these authors, the MtP method appeared to be less appropriate for an accurate identification of staphylococcal capability of biofilm formation. In our study, there was a relation between the ability of biofilm formation by the MtP method and slime production in the CRA test among the *ica*-positive staphylococcal isolates. In contrast, most of the *ica*-negative strains were positive by the CRA test and possessed a biofilm-negative phenotype determined using the MtP method, especially for isolates harboring the *aap* gene.

The literature data available regarding the CRA test yielded contrasting conclusions. Bozkurt *et al.* (2009) indicate that the CRA test should not be used for a biofilm formation ability assay *in vitro* of *S. epidermidis* because of misleading results. The specificity of this test is limited to the determination of staphylococcal ability to secrete slime rather than for the detection of bacterial adhesion and rapid growth in the form of a biofilm on the material's surface. On the other hand, some authors (Arciola *et al.*, 2006; Jain & Agarwal, 2009) recommended the CRA test as a reliable method to determine biofilm production. In our opinion, CRA and MtP tests are reliable methods to determine the ability of slime/biofilm formation only in *ica*-positive *S. epidermidis* strains.

Although previous studies (Vandecasteele *et al.*, 2003; Cafiso *et al.*, 2004) have suggested that there is no strict association between the presence of the *icaABCD* operon and *in vitro* biofilm formation in invasive, colonizing and contaminant *S. epidermidis*, among the colonizing strains tested in our study, most of the biofilm producers (monitored using the MtP method) were the *ica* positive.

In conclusion, *S. epidermidis* isolates possess the potential ability to form biofilms by *ica*-dependent and/or *ica*-independent mechanisms. In our opinion, further studies are needed to determine reliable, short-time criteria for the assessment *in vitro* of biofilm formation in staphylococci.

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