Ann Microbiol
DOI 10.1007/s13213-011-0201-1

ORIGINAL ARTICLE

Evaluation of phenotypic and genotypic detection methods for biofilm-forming methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* clinical isolates

Salman S. Atshan · Mariana N. Shamsudin

Received: 29 January 2010 / Accepted: 26 July 2010

Abstract Three hundred Staphylococci (MRSA150, MSSA150) isolates from different clinical samples were tested for the production of slime. Phenotypic characterization was performed using the tube method, Congo red agar (CRA), modified Congo red agar (MCRA), and microtiter plate assay (MPA), while the presence of *icaADBC* genes required for biofilm were assessed using PCR. In all the isolates tested, there was a 44.66 and 100% correlation between the lack of production of slime (red colonies) on CRA and the presence of the *icaADBC* genes, and between slime production (black colonies) on MCRA and the tube method established in this study with the presence of *icaADBC* genes, respectively. The standard test method (MPA) used to quantify production of biofilm revealed that all isolates in the present study were positive using a spectrophotometric assay. In conclusion, this study led to the development and the establishment of a method for identifying and detecting *Staphylococcus aureus* biofilm production, whereby the phenotypic characterization developed was molecular-based. In addition, the modified Congo Red agar was optimized to confirm slime production in contrast to previous published selective media.

S. S. Atshan (✉) · M. N. Shamsudin
Laboratory of Medical Microbiology and Parasitology,
Faculty of Medicine and Health Science,
Universiti Putra Malaysia,
43400 Serdang,
Selangor, Malaysia
e-mail: salmanatshan@yahoo.com

M. N. Shamsudin
Laboratory of Marine Science and Aquaculture,
Institute of Bioscience, Universiti Putra Malaysia,
43400 Serdang,
Selangor, Malaysia

Keywords MSSA and MRSA · Biofilm · *icaADBC* genes · MCRA

Introduction

A biofilm is defined as a surface-adherent, aggregated bacterial consortium embedded in a self-synthesized hydrated polymeric matrix (Holger et al. 2010). The first observations of biofilm were obtained through scanning electron microscopy which showed primary attachment of monolayer bacterial consortia, embedded in an amorphous mucous structure on the surfaces of medical devices. This phenotype was initially referred to as slime formation. In retrospect, in most cases, the 'slime' was very likely polysaccharide intercellular adhesin (PIA), and so PIA and 'slime' are considered to be the same. Today, this special mode of thick extracellular matrix (maturation phase) is generally termed biofilm formation (Hall-Stoodley et al. 2004). In particular, Staphylococci are able to colonize surfaces of medical devices by bacterial production of polysaccharide slime (Francois et al. 1996). Knowledge on the *icaADBC* protein product is limited. All encoding genes have been cloned individually or in combination in *S. carnosus*, and in vitro biosynthesis of PIA has been analyzed using either the membrane fraction, cellular, or extracellular extracts (Gerke et al. 1998). *icaA, C* and *D* are located in the membrane fraction, *icaB* is mainly present in the culture supernatant, and PIA is localized mainly on the cell surface. The coexpression of *icaA* and *icaD* increases N-acetylglucosaminyl transfersse activity and slime production (Arciola et al. 2006). *icaB* codes for an extracellular protein whose function is still unknown, while *icaC* codes for a membrane protein that is thought to have a receptor function for polysaccharidic

Published online: 22 January 2011

Table 1 The composition of both published and modified Congo red agar per liter of distilled water used in this study

Composition/litter	Published Congo red agar	Modified Congo red agar
Congo red dye	0.8 g	0.4 g
Sucrose	36 g	—
Glucose	—	10 g
BHIA	52 g	—
BAB-2	—	42 g
Water	1,000 ml	1,000 ml

BHIA Brain Heart Infusion Agar, *BAB-2* Blood Agar Base-2

antigens. Simultaneous expression of all gene products is necessary for PIA synthesis and biofilm formation on polymeric surfaces and autoaggregation in liquid culture (Ziebuhr et al. 1997).

The main problem concerning biofilm production arises from the ambiguity in the results obtained from available detection techniques. The main shortcoming of the ambiguity is inaccuracy of the predictive value as a result of false negatives. Hence, accurate identification of biofilm in vitro is required. The ability of *S. aureus* to form biofilm is a long-known fact (Baselga et al. 1993), but the problem involving the issue of biofilm identification has remained since the availability of the phenotypic approach of growth on highly selective or differential media. This particular approach can provide identification of biofilm formation but with a high margin of error through many false negative outcomes. In line with these shortcomings, the present study embarked on two main strategies, whereby the first aims to overcome the issue of inaccurate biofilm identification through the development of modified selective media that can provide positive identification and establishment of a genotypic system through detection of multiple genes confirming the biofilm-forming ability. The other strategy aims to provide phenotypic and genotypic approaches as complementary methodologies for accurate and rapid identification. In addition, approaches providing accurate predictive value techniques for use in diagnostic microbiology laboratories will improve management of patients with biofilm-associated infections. In order to exploit the genotypic approach, the genetics underlying biofilm formation are considered.

Materials and methods

Bacterial strains

Three hundred clinical isolates of *S. aureus* (MSSA 150 and MRSA 150) and 4 reference strains were used. The reference strains used were of the positive biofilm producer, ATCC 35556, negative biofilm producer, ATCC 12228, MRSA ATCC 700698, and MSSA ATCC 29278. These strains were obtained from the Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. All isolates were initially identified by standard microbiological techniques and then confirmed by targeting of the species-specific *Sa442* gene (Shrestha et al. 2002) and the antibiotic resistant gene, *mecA* (Oliveira and Lencatre, 2002).Total cellular DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis Sdn. Bhd., Malaysia).

Slime-phenotypic characterization

Tube test assay

Tube test assay is performed to evaluate production of slime formation in liquid media (Christensen et al. 1982). From an overnight plate culture, a single colony of bacteria was taken and inoculated into 10 ml sterile Tryptic Soy Broth (TSB) containing 0.04% Congo red dye supplemented with 1% glucose in 125-ml conical flasks. These cells were cultured at 37°C overnight in an incubator-shaker (approximately 100 rpm), and were observed for black colored pigment formation after 24 h.

Table 2 The sequences of the primers used with respective product sizes

Gene	Sequence	Product size
icaA	Forward 5'- ACACTTGCTGGCGCAGTCAA -3' Reverse 5'- TCTGGAACCAACATCCAACA -3'	188 bp
icaD	Forward 5'- ATGGTCAAGCCCAGACAGAG -3' Reverse 5'- AGTATTTTCAATGTTTAAAGCAA -3'	198 bp
icaB	Forward 5'- AGAATCGTGAAGTATAGAAAATT -3' Reverse 5'- TCTAATCTTTTTCATGGAATCCGT -3'	900 bp
icaC	Forward 5'- ATGGGACGGATTCCATGAAAAAGA -3' Reverse 5'- TAATAAGCATTAATGTTCAATT -3'	1,100 bp

Table 3 The optimized thermocycler program for each primer used

Thermocycle	*icaA*	*icaD*	*icaB*	*icaC*
Initial denaturation	94°C	94°C	94°C	94°C
	4 min	4 min	2 min	2 min
Denaturation	94°C	94°C	94°C	94°C
	1 min	1 min	1 min	1 min
Annealing	52°C	52°C	52°C	51°C
	1 min	1 min	1 min	1 min
Elongation	72°C	72°C	72°C	72°C
	2 min	2 min	2 min	2 min
Final extension	72°C	72°C	72°C	72°C
	4 min	4 min	4 min	4 min
Cycle	26	26	26	26

Modified Congo red agar (MCRA)

A modified Congo red agar (MCRA) was performed following some modifications of the methodology described by Freeman et al. (1989). The modifications include changing the concentration of Congo red dye, sucrose substitution with glucose (Baker, UK) and replacement of BHIA with an alternative agar, Blood Base Agar-2 (BAB-2) (Oxoid, Basingstoke, UK) as shown in Table 1. Bacteria were plated on both published and MCRA, incubated for 48 h at 37°C, and subsequently stored at room temperature for 2–4 days. Slime-producing Staphylococci grow as black colonies, while non slime-producing isolates grow as red colonies.

Microtiter plate assay (MPA)

A microtiter plate assay (MPA) has been described by Stepanovic et al. (2007) as a control method standardized for determining the quantity of slime adherence to the wells. The optical density (OD) of each well was measured using a Microplate ELISA auto reader (E max, USA) at 570 nm. Standard deviations were defined as the cut-off OD (ODc) for the microtiter plate test above the mean OD of the negative control. The adherence ability of the tested isolates was classified into four categories based on the OD suggested by Christensen et al. (1985): non-adherent (OD < ODc), and weakly (ODc < OD < 2×ODc), moderately (2×ODc < OD < 4×ODc), and strongly (4×ODc < OD) adherent.

Slime-genotypic characterization

Bacterial DNA extraction

Total DNA from the *S. aureus* isolates were extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis). The *icaADBC* gene sequences were taken from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) GenBank sequence database. The primers were derived from the *icaADBC* sequence data of *S. aureus* ATCC 35556 (GenBank accession no. AF086783). The primers were commercially synthesized by EUROGENTEC AIT/SINGAPORE139552. The sequences and the thermo cycler conditions for each primer used in this study were previously described by Rohde et al. (2001) and Kiem et al. (2004) and are as shown in Tables 2 and 3, with minor modifications in cycling conditions. Samples of 25 µl of the master reaction mixtures were used for each primer set, and amplified products were checked by gel electrophoresis using 1.2% agarose in Tris-borate-EDTA, using a 100-bp molecular weight marker. The gel was viewed under UV light in an Alphalmager® Imaging System and the gel image obtained was evaluated for biofilm production through the presence or absence of *icaA, D, B,* and *C* genes in the MRSA and MSSA clinical isolates.

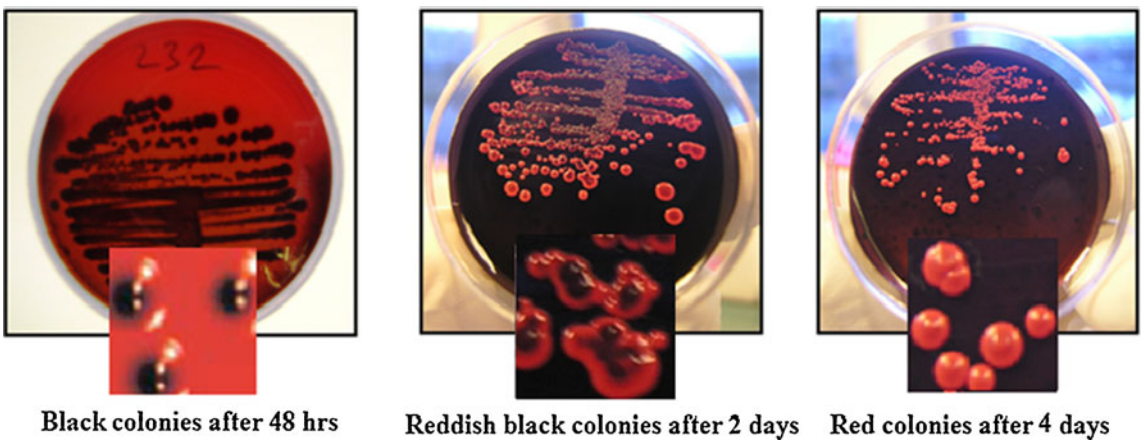

Fig. 1 Colony morphologies of clinical *S. aureus* isolates on the published Congo red agar medium

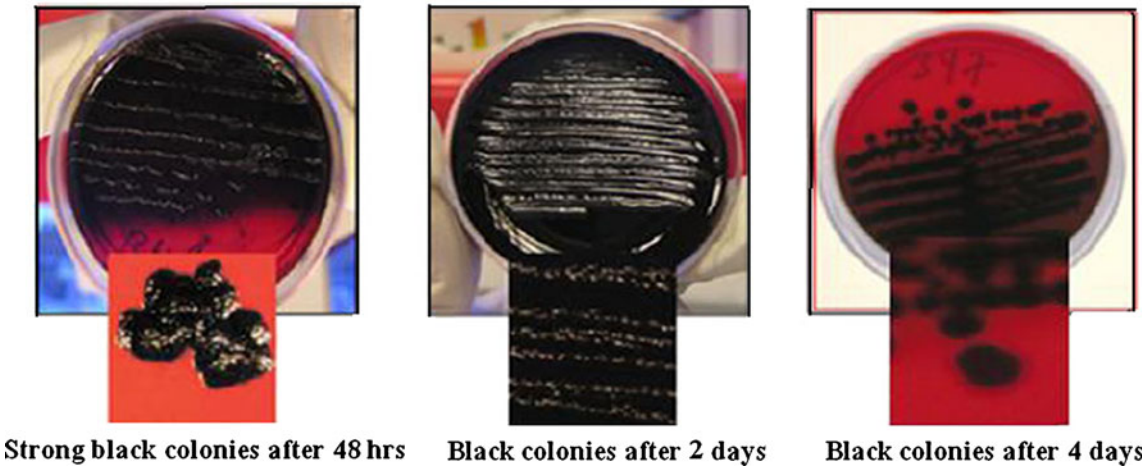

Fig. 2 Colony morphologies of clinical *S. aureus* isolates on the modified Congo red agar (MCRA) medium

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS, Chicago, IL, USA). The Fisher exact test was used for screening significant association for every variable growth from published and MCRA, MPA, and tube method for detection of biofilm formation. A *P* value of ≤ 0.05 indicates statistical significance. Data obtained from the MPA is considered a gold standard and is compared with data obtained from Freeman CRA, MCRA, and the tube method using parameters like sensitivity: a / (a + c) × 100, and specificity: d / (b + d) × 100. The gold standard method was evaluated on the basis of its ability to determine biofilm quantity in accordance to presence of the *ica* biofilm required genes determined by molecular methods.

Results

Slime-phenotypic characterization

Fig. 1 shows data related to phenotypic characteristics of the 300 isolates tested on the Freeman CRA. A total of 166 isolates (55.33%) were positive after 48 h incubation at 37°C after which the black colonies became reddish and turned into a red color after 2 and 4 days, respectively, at room temperature, whereas all 300 isolates (100%) formed stable strong black colonies after 48 h incubation at 37°C and remained constant even after 2–4 days incubation at room temperature when tested with the MCRA (Fig. 2), and the tube method test (Fig. 3). The MPA standardized test demonstrated the 300 isolates' ability to adhere to surfaces and form biofilm.

Slime-genotypic characterization

As shown in Fig. 4, all 300 *S. aureus* isolates were PCR-positive for the *icaA, icaD, icaB,* and *icaC* biofilm required genes giving 188-bp, 198-bp, 900-bp, and 1,100-bp bands, respectively. Among these, 134 (44.66%) *S. aureus* isolates were Freeman CRA plate test negative, but all 134 isolates tested positive for presence of the *icaADBC* genes. There was a correlation between slime production on the MCRA,

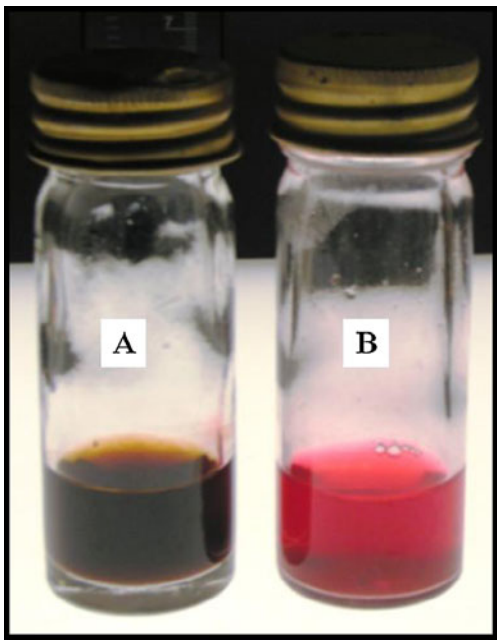

Fig. 3 Screening of biofilm formation producers by the tube method. **a** Positive biofilm producing reference strain ATCC 35556 (*black* pigments), **b** negative biofilm producing reference strain ATCC 12228 (*pink* pigments)

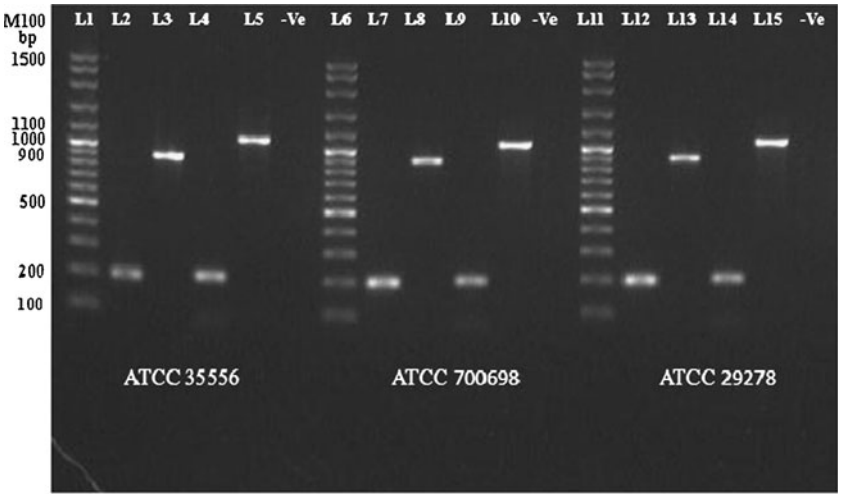

Fig. 4 PCR amplification of *icaADBC* genes of biofilm-positive producing reference strains ATCC 35556, ATCC 700698 and ATCC 29278. *Lanes 1, 6,11* the molecular weight marker 100 bp; *lanes 2, 7,12* the 188-bp band obtained with primers for *icaA*; *lanes 3, 8,13* the 900-bp band obtained with primers for *icaB*; *lanes 4, 9, 14* the 198-bp band obtained with primers for *icaD*; *lanes 5, 10, 15* the 1,100-bp band obtained with primers for *icaC*; *−Ve* the negative control (deoxyribonucleic acid template absent)

the tube test, the MPA, and presence of *icaADBC* genes, compared with the questionable result of slime production absence on the Freeman CRA but with presence of *icaADBC* genes.

Discussion

The indeterminate identity of biofilm agents contributes to a significant problem worldwide and is responsible for major medical and economic consequences. Rapid identification with accurate precision and reliable detection methods for successful microbiological surveillance and for investigating biofilm infections will ensure effective patient management. In this study, the published Congo red agar previously described by Freeman et al. (1989) used as a phenotypic reference test for slime production did not always correlate with the presence of *icaADBC* genes. This is consistent with observations from other studies, in which only few or no slime-producing isolates could be detected using Freeman CRA (Kaufmann et al. 1999; McKenney et al. 1999; Mathur et al. 2006). Thus, optimization was done with respect to a reduction in the concentration of Congo red dye and replacements of sucrose with glucose, and of Brain Heart Infusion Agar with the alternative Blood Base Agar-2 (Table 1). Composition modification resulted in the promising phenotypic property of intense black pigment production without change in pigmentation over time (Fig. 2). Results obtained from modified Congo red agar (MCRA) was significantly different from that of Freeman CRA upon statistical analysis using the Fisher exact test, $P \leq 0.05$ (Table 4).

The influence of the basic medium and the respective sugar supplementation on increased biofilm or slime formation production is speculative, as some of the bacterial isolates have been found to appear red in color on Freeman CRA containing sucrose, indicating negative slime production. Despite this indication, addition of 1% glucose and replacement of Brain Heart Infusion Agar with Blood Base Agar-2 triggered the bacterial isolates to form black pigments on MCRA. These results suggest that different regulatory mechanisms could activate the expression of slime formation and also indicate a strong dependence of biofilm formation in *S. aureus* on growth environmental conditions. Glucose was the only compound that consistently produced strong reactions from very low concentrations in contrast to sucrose concentration. This finding is in agreement with Rohde et al. (2001) who reported that the ability of *S. aureus* to produce biofilm is dramatically affected by the presence of an additional carbohydrate source in the medium. The addition of 1% glucose increased the percentage of biofilm-producing *S. aureus* from 34.4 to 83.3%, and the carbohydrate effect was never detected for other staphylococcal species. Mercier et al. (2009) also reported the presence of glucose and UDP-N-acetylglucosamine to stimulate PIA (slime) production and black colored biofilm formation.

We carried out further experiments to better explain this phenomenon, using the tube test assay which is a good approach for evaluating biofilm formation in liquid media.

Table 4 Statistical evaluation of published and modified Congo red agar, the tube method and microtiter plate assay for detection of biofilm formation in *S. aureus* (n = 300)

Screening method	Sensitivity (%)	Specificity (%)
Published Congo red agar	55.33	100
Modified Congo red agar	100	100
Tube method	100	100
Microtiter standard method	100	100

This test shows similarity with MCRA, where there is no significant difference between the tube method and MCRA, $P \geq 0.05$ (Table 4). In addition, this is a rapid test for screening black or red color formation after 24 h incubation (Fig. 3). The modified Congo red agar showed very high correlation with microtiter standard methods (Table 4). In the present experiment, the modified composition induced all the clinical isolates of *S. aureus* to produce slime with a consistent black color formation indicating positive biofilm production.

The microtiter plate test was used to evaluate quantity of biofilm production spectrophotometrically. The microtiter plate method remains among the most frequently used assays for investigation of biofilm according to their accuracy and reliability in assessing and appreciating biofilm producing bacteria. The findings from the spectrophotometric assay in the present study revealed that 100% of the MRSA and MSSA isolates produced biofilm. This finding is, however, different from previous reports that stated that only 45% of MRSA and 66% of MSSA isolates were shown to produce biofilm using the spectrophotometric assay (Grinholc et al. 2007).

The molecular approach represents a highly sensitive method for assessing the *ica* global gene, which, in addition (and not as an alternative) to the CRA plate test, can contribute to the clinical impact of both top management of antibiotic therapy and the understanding of the evolution of infections due to slime production and biofilm formation of *S. aureus*. The present study showed a high prevalence of the *icaADBC* genes among *S. aureus* isolates, namely MSSA and MRSA, confirming 100% to be phenotypically slime forming. These data are in accordance with those reported by Fowler et al. (2001) who detected *ica* in all their 61 *S. aureus* isolates by PCR techniques. In contrast, a low percentage of *ica* global genes positive isolates was described by Arciola et al. (2001a, b). The results of the latter are from the detection method, in which primers complementary to the sequence of the *icaADBC* genes were derived from *Staphylococcus epidermidis*, rather than from *S. aureus*. However, since the present study used primers complementary to the sequences of the *icaADBC* from *S. aureus* ATCC 35556, we found that all MSSA and MRSA isolates were *icaADBC*-positive as shown in Fig. 4.

This study shows that modified Congo red agar can offer promising alternatives for assessing the production of slime. The modified agar constituents provided not only stable, 100% formation of black pigments but also a reduction in the cost of preparing the agar. The association of black pigments with the presence of *ica* global genes established in the present study is a complementary approach of both phenotypic and genotypic assays, thus providing exact identity of the agents which will improve biofilm prescription drugs or antibiotics, rendering effective and rapid killing of the pathogen. Further studies will be conducted to determine the real economic performance of developing a modified Congo red agar such as in this study.

In conclusion, stable black pigmentation is an added value to the accuracy of identifying biofilm formation, providing the complementary phenotypic and genotypic approaches for the rapid and accurate identification of the types of bacteria, namely MRSA and MSSA, in a biofilm matrix. We agree with studies that assessed there is correlation between a black colony-forming phenotypes and *S. aureus* biofilm-positive genotypes.

Acknowledgments The authors thank all Medical Microbiology Laboratory members for their assistance in preparing the manuscript and assisting with the bench work. The grant FRGS 02-10-07-319FR is also acknowledged.

References

Arciola CR, Baldassari L, Montanaro L (2001a) Presence of *icaA* and *icaD* genes and slime prodction in a collection of *Staphylococcal* strains from catheter-associated infections. J Clin Microbiol 39:2151–2156

Arciola CR, Campoccia D, Borelli AM, Montanaro ME (2001b) Congo red agar plate method: improved accuracy and new extended application to *Staphylococcus aureus*. New Microbiol 24:355–363

Arciola CR, Campoccia D, Baldassari L, Donati ME, Pirini V, Gamberini S, Montanaro L (2006) Detection of biofilm formation in *Staphylococcus epidermidis* from implant infections. J Biomed Mater Res 76:425–430

Baselga R, Albizu L, De La Cruz M, Del Cacho E, Barberan M, Amorena B (1993) Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. Infect Immun 61:4857–4862

Christensen GD, Simpson WA, Bisno AL, Beachey EH (1982) Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 37:318–326

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH (1985) Adherence of coagulase negative *Staphylococci* to plastic tissue culture plates: a quantitative model for the adherence of *Staphylococci* to medical devices. J Clin Microbiol 22:996–1006

Francois P, Vaudaux P, Foster TJ, Lew DP (1996) Host bacteria interactions in foreign body infections. Infect Control Hosp Epidemiol 17:514–20

Freeman DJ, Falkiner FR, Keane CT (1989) New method for detecting slime production by coagulase negative *Staphylococci*. J Clin Pathol 42:872–874

Fowler VG, Fey PD, Reller LB, Chamis AL, Corey GR, Rupp ME (2001) The intercellular adhesin locus ica is present in clinical isolates of *Staphylococcus aureus* from bacteremic patients with infected and uninfected prosthetic joints. Med Microbiol Immunol 189:127–131

Gerke C, Kraft A, Sussmuth R, Schweitzer O, Gotz F (1998) Characterization of the N- acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. J Biol Chem 273:18586–18593

Grinholc M, Wegrzyn G, Kurlenda J (2007) Evaluation of biofilm production and prevalence of the icaD gene in methicillin-

resistant and methicillin-susceptible *Staphylococcus aureus* strains isolated from patients with nosocomial infections and carriers. Immunol Med Microbiol 50:375–379

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol 2:95–108

Holger R, Stephanie F, Ulrich Z, Dietrich M (2010) Structure, function and contribution of polysaccharide intercellular adhesion (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. Eur J Cell Biol 89:103–111

Kaufmann SHE, Cramton SE, Gerke C, Schnell NF, Nichols WW, Gotz F (1999) The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun 67:5427–5433

Kiem S, Oh WS, Peck KR, Lee NY, Lee JY, Song JH, Hwang ES, Kim EC, Cha CY, Choe KW (2004) Phase variation of biofilm formation in *Staphylococcus aureus* by IS256 insertion and its impact on the capacity adhering to polyurethane surface. J Korean Med Sci 19:779–782

Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A (2006) Detection of biofilm formation among the clinical isolates of *Staphylococci*: and evaluation of three different screening methods. Indian J Med Microbiol 24:25–29

McKenney D, Pouliot KL, Wang Y, Murthy V, Ulrich M, Doring G, Lee JC, Goldmann DA, Pier GB (1999) Broadly protective vaccinefor *Staphylococcus aureus* based on an in vivoexpressed antigen. Science 284:1523–1527

Mercier KA, Cort JR, Kennedy MA, Lockert EE, Ni S, Shortridge MD, Powers R (2009) Structure and function of *Pseudomonas aeruginosa* protein PA1324. Protein Sci 18:606–618

Oliveira DC, Lencatre H (2002) Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 46:2155–2161

Rohde H, Knobloch JKM, Horstkotte MA, Mack D (2001) Correlation of *Staphylococcus aureus icaADBC* genotype and biofilm expression phenotype. J Clin Microbiol 39:4595–4596

Shrestha NK, Tuohy MJ, Hall GS, Isada CM, Procop GW (2002) Rapid Identification of *Staphylococcus aureus* and the *mecA* Gene from bacterial blood culture bottles by using the light cycler system. J Clin Microbiol 40:2659–2661

Stepanovic S, Vukovic D, Hola V, Bonaventura GDL, Djukic S, Cirkovic L, Ruzicka F (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *Staphylococci*. Annu Rev Microbiol 56:187–209

Ziebuhr W, Heilmann C, Gotz F, Meyer P, Wilms K, Straube E, Hacker J (1997) Detection of the intercellular adhesion gene cluster (*ica)* and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. Infect Immun 65:890–896