Original Article

Slime layer formation and the prevalence of *mecA* and *aap* genes in *Staphylococcus epidermidis* isolates

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Abstract

Introduction: *Staphylococcus epidermidis* strains are frequently associated with catheter-related infection, acute bacteremia, and hospital-acquired infection. Some isolates produce an extracellular matrix called slime that may make them more resistant to antibiotics. The aim of this study was to determine antimicrobial resistance patterns, the prevalence of slime production, and the distribution of genes (*mecA* and *aap*, respectively) associated with these phenotypes in *S. epidermidis* nasal isolates from health-care personnel.

Methodology: A descriptive cross-sectional study was performed on 163 nasal swabs from health-care staff (one swab per subject). *S. epidermidis* isolates were tested for slime production on congo red agar and antibiotic resistance. PCR-based screening for *mecA* and *aap* genes was performed upon the extracted DNA of *S. epidermidis* isolates.

Results: A total of 99 *S. epidermidis* strains were cultured from 58.9% of the study participants (n = 96). Of these strains, 34 (34.3%) isolates produced slime. A significant relation between slime production and resistance to penicillin 32(94%) , oxacillin 30(88%),, tetracycline 20(59%), erythromycin 27(79%), and clindamycin 26(77%) was found. Respectively, 95.8% and 94.8% of all isolates were PCR-positive for *mecA* and *aap*, but only 59.8% of *mecA*+ strains were oxacillin-resistant and 37.3% of *aap*+ strains were slime producers.

Conclusions: The surveillance of nasal colonization with slime-forming oxacillin-resistant *S. epidermidis* in health-care workers might be helpful in breaking the epidemiological chain of hospital-acquired infections.

Key words: *Staphylococcus epidermidis*; staphylococci nasal carriage; gene frequency

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Introduction

Staphylococcus epidermidis and other coagulase-negative staphylococci (CNS) are normal microflora of the skin and mucosal surfaces [1-3]. *S. epidermidis* is one of the most important species because it is a frequent contaminant of blood cultures but may also act as an opportunist pathogen in immune deficient patients. Cases of acute bacteremia, catheter-related infections, infections following the utilization of artificial instruments and hospital-acquired infections have been reported [4-6]. One subgroup of *S. epidermidis* produces an extracellular matrix, called slime layer that is made of carbohydrate and protein molecules. A major part of the layer is called the polysaccharide intercellular adhesion. The over-production of component exo-polysaccharides leads to a decrease in metabolic activities of the cells as it can be a barrier to the penetration and diffusion of the nutrients and oxygen [7]. Studies show that the eradication of slime-producing bacteria is more difficult than for bacteria that do not produce slime [8-10]. This glycocalixic layer contributes to antibiotic resistance by limiting or preventing antibiotic diffusion [11]. Surface proteins have an important role in biofilm formation as well [12,13].

A critical problem facing infectious disease medicine is microbial antibiotic resistance. Up to 90% of the staphylococcal isolates found in hospitals have been reported as methicillin (oxacillin)-resistant [14]. Moreover, the resistance in *S. epidermidis* can be transferred to *Staphylococcus aureus* (15). *mecA* gene detection through PCR is the gold-standard method for detecting resistance to oxacillin in staphylococci [16]. Since hospital health-care staff are one of the major sources of infection transmission, the aim of this study was to evaluate

the prevalence of slime formation, to detect the *aap* (as one of the major factors in biofilm formation) and *mecA* genes by molecular methods, as well as to characterize the relationship between slime production and antibiotic resistance in *S. epidermidis* from hospital workers.

Methodology

Sample collection and S. epidermidis *identification*

Nasal swab samples were collected from the hospital staff of the clinical wards (including nurses and physicians) of three hospitals (Sina, Shariati, and Children's Medical Center) between July and December 2008 (n = 163 swabs, one swab per person). The subjects were 65% female and 35% male. The cotton swabs were pre-sterilized and samples were collected aseptically. Swabs were directly inoculated onto blood agar and subsequently cultured at 37°C for 18-24 hrs. *S. epidermidis* isolates were identified by biochemical and microbiological standard methods such as catalase, Dnase, and coagulase tests, aerobic acid production from trehalose, mannitol, mannose, maltose, and sucrose (Merck, Hohenbrunn, Germany), as well as novobiocin and polymixin B (MAST, Merseyside, England) resistance tests.

Phenotypic detection of slime production

All the isolates were cultured onto congo red agar (CRA). CRA plates were prepared by adding 0.8 g of congo red agar and 36 g of saccharose to 1 L of brain heart infusion agar [17]. The plates were incubated at 37°C for 24 hours and subsequently at room temperature overnight. The color scale ranged from "very black" to "very red". "Very black" and "black" colonies were considered normal slime producer strains; "almost black" colonies were considered indicative of weak slime production activity. Conversely, "very red" to "Bordeaux"-colored colonies were considered grades of red and were classified as strains unable to produce slime.

Antibiotic susceptibility

Disk diffusion testing was conducted as recommended by the clinical and laboratory standards institute (CLSI) [18]. The following antimicrobial agents were assayed: penicillin 10 U; oxacillin 1 µg/disk; vancomycin 30 µg/disk; mupirocin 5 µg/disk; tetracycline 30 µg/disk; erythromycin 15 µg/disk; clindamycin 2 µg/disk; ciprofloxacin 5 µg/disk; rifampin 5 µg/disk; trimethoprim-sulfamethoxazole 1.25/23.75 µg/disk; and tobramycin 10µg/disk (MAST, Merseyside, England). *S. aureus* (ATCC 25923) was used as the susceptible control.

Genotype analysis

DNA extraction: Genomic DNA was isolated by using a genomic DNA extraction kit (Bioneer Inc, Seoul, South Korea) as recommended by the manufacturer.

PCR analysis: The collection of isolates was screened for the presence of *mecA* and *aap* genes by PCR, using previously described primers as follows [19,20].

F: mecA1 (5'-GAA ATG ACT GAA CGT CCG AT-3')

R: mecA2 (5'-GCG ATC AAT GTT ACC GTA GT-3')

F: aap1 (5'-ATA CAA CTG GTG CAG ATG GTT G-3')

R: aap2 (5'-GTA GCC GTC CAA GTT TTA CCA G-3')

These primers amplify fragments corresponding to 150 bp and 399 bp of the *mecA* gene and *aap* genes, respectively. DNA amplification was performed in a thermocycler (Eppendorf, Hamburg, Germany) in a final volume of 50 µl: containing 10 µl of 10X PCR buffer; 3.5 µl of Mgcl2 (10mM); 0.2mM dNTPmix; 20 ρM of each primer;1U of Taq polymerase (Fermentas, Opelstrasse, Germany); and 4 µl of template DNA. Amplification was performed by denaturation at 97°C for 6 minutes (first denaturation), followed by 35 cycles according to the following program: denaturation at 92°C for 30 seconds, annealing at 55°C for 30 seconds, and an extension at 72°C for 45 seconds plus a final extension at 72°C for 10 minutes [19,20].

Detection of PCR products

PCR products were resolved by electrophoresis through a 1% agarose gel containing ethidium bromide [20]. DNA molecular weight standard 100 bp (Fermentas, Opelstrasse, Germany) was used as the marker (Figure 1).

Statistical analyses

Statistical analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) software (Cairo, Egypt) version 16, and the Chi-squared and Fisher's exact tests. A $P \leq 0.05$ was considered significant.

Results

From the nasal swabs collected from the 163 health-care workers at three hospitals, 96 swabs/study participants were culture positive for *S. epidermidis*. The colonization of more than one *S. epidermidis* isolate was observed in three samples (*n* = 99 strains in total). *S. epidermidis* carriers were co-colonized with *S. aureus* (*n* = 39), *Streptococcus intermedius* (*n* = 1), and other CNS (*n* = 18). On CRA medium, the 99 *S. epidermidis* isolates were characterized as either "very black" (*n* = 10; 10.1%), "black" (*n* = 11; 11.1%), "almost black" (*n* = 14; 10.1%), "Bordeaux" (*n* = 44; 44.1%), "red" (*n* = 19; 19.2%), or "very red" (*n* = 1; 1%).. Overall, 35 (35.1%) of *S. epidermidis* strains were slime producers.

PCR was performed on the *S. epidermidis* strain DNA (*n* = 99). The results showed that 95.8% of all isolates had *mecA* and 94.8% of them had *aap* genes. An example of *aap* PCR amplification products run on an agarose gel is illustrated in Figure 1.

The frequency distributions of *S. epidermidis* and the related factors (slime layer production, resistance to oxacillin, presence of *mecA* and *aap*) among the health-care staff of the hospitals studied are shown in Table 1. Of note, a higher percentage of colonized health-care workers was observed at the Children's Medical Center (71.1%) as compared to Shariati and Sina hospitals (55.6% and 57.3%, respectively). Oxacillin resistance was lowest among the Sina hospital isolates (48.9%) as compared to the Children's Medical Center and Shariati hospital

Figure 1: PCR – product of *aap* gene from *S. epidermidis* isolates (1% gel agarose)
M-molecular size marker 100 bp(Fermentas) , lane 1 – negative control , lane 2 – positive control of *aap* gene (*S. epidermidis* ATCC12228) , lane 3 – negative isolate for *aap* gene, lane 4 - positive isolate for *aap* gene (399 bp).

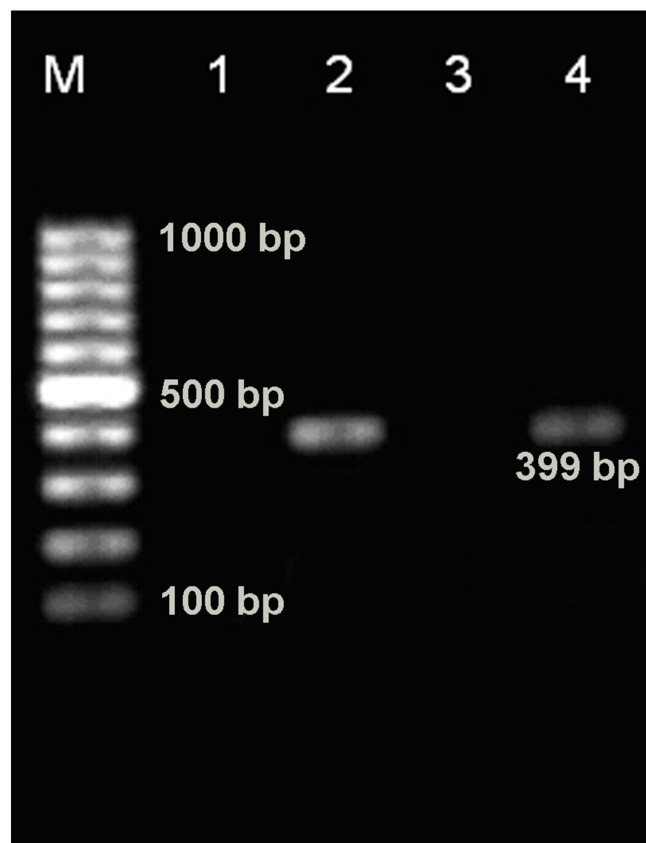

Table 1. Frequency distribution of *S. epidermidis* and related factors in health-care staff of three hospitals studied

Hospital	*S. epidermidis Isolates*		Slime Production		Oxacillin resistance		*mecA* (+)		*aap* (+)	
	No.	(%) Carriers per hospital	No.	(%) Per hospital	No.	(%) Per hospital	No.	(%) Per hospital	No.	(%) Per hospital
Sina	47	57.3	13	27.6	23	48.9	44	93.6	42	89.4
Shariati	20	55.6	8	40	12	60	19	95	20	100
Children's Medical Center	32	71.1	13	40.6	21	62.5	29	90.6	29	90.6
Total	99		34		56		92		91	

Table 2. Antibiotic susceptibility pattern of all *S. epidermidis* isolates taken from nasal carriers

Antimicrobial Agent	Susceptible (%)	Intermediate (%)	Resistant (%)
Penicillin	29 (29.3)	0	70 (70.7)
Oxacillin	43 (43.4)	0	56 (56.6)
Vancomycin	99 (100)	0	0
Mupirocin	93 (93.9)	1 (1)	5 (5.1)
Tetracycline	58 (58.6)	2 (2)	39 (39.4)
Erythromycin	39 (39.4)	3 (3)	57(57.6)
Clindamycin	42 (42.4)	2 (2)	55 (55.6)
Tobramycin	88 (88.9)	0	11 (11.1)
Ciprofloxacin	85 (85.9)	1 (1)	13 (13.1)
Trimethoprim-sulfamethoxazole	63 (63.6)	2 (2)	34 (34.3)
Rifampicin	97 (98)	0	2 (2)

(62.5% and 60.0%, respectively). Likewise, slime production was lowest among the Sina hospital isolates (27.6%) as compared to the Children's Medical Center and Shariati hospital (40.6% and 40.0%, respectively). However, the presence of *mecA* in 92.9% of strains (92 of 99) was not an absolute predictor of phenotypic oxacillin resistance as only 61% (56 of 92) of *mecA*+ strains were oxacillin resistant. Similarly, 91.9% of strains (91 of 99) possessed *aap* by PCR, but only 37.3% (34 of 91) of *aap*+ strains were slime producers.

The percentage and the antibiotic resistance rates of isolated *S. epidermidis* strains are shown in Table 2. The highest rate of resistance was observed to penicillin (70.7%). High rates of resistance to erythromycin (57.6%), clindamycin (55.6%), oxacillin (56.6%), tetracycline (39.4%), and trimethoprim-sulfamethoxazole (34.3 %) were also noted. No resistance to vancomycin was observed. From among the total of 99 isolates, 66 were resistant to three or more antibiotics.

As mentioned, three personnel were colonized with more than one *S. epidermidis* isolate and differed from one another in slime layer production and antibiotic resistance patterns. In two *S. epidermidis* isolates from one person, one strain was methicillin-resistant and positive for slime layer production and the other was methicillin-susceptible and was negative for slime layer production.

A strong correlation was found between slime production and resistance to penicillin 32 (94%) ($P < 0.0001$) and oxacillin 30 (88%) ($P < 0.0001$). Furthermore, there was a correlation between slime layer production and resistance to tetracycline 20 (59%) ($P = 0.005$), erythromycin 27 (79%) ($P = 0.001$) and clindamycin 26 (77%) ($P = 0.003$). There was no association between slime layer production and resistance to other antibiotics.

Discussion

In the last two decades, *S. epidermidis* has played an important role in the incidence of hospital-acquired infections. The increase in the number of patients with immune deficiency, the use of implants, pacemakers and shunts, as well as the selective pressure of antibiotics and disinfectants on bacterial populations, were important reasons for the rise in CNS, particularly *S. epidermidis* in health-care settings [15,21]. Ecologically, one of the ways that bacteria can escape selective pressure is to produce biofilms and to acquire antimicrobial resistance

genes; this is a significant reason for their success as nosocomial pathogens 15].

Slime layer production, as the major part of biofilm formation, plays a remarkable role in bacterial colonization of exterior surfaces [22]. Several methods have been recognized for examining slime layer production, including the CRA method, the standard tube method, and the Christensen's tube method. Bozkurt *et al.* (2009) determined that there is no significant difference between the three methods; however, they found that the number of the false-negative CRA results was less than those of the other methods [8]. *Ica* operon genes have been also evaluated in many slime layer production studies.

Comparing different studies, there is a similarity between the CRA results and genotype characterization of the Ica locus detected by PCR [17,20]. Since determining slime layer production can lead to the recognition of the biofilm producing staphylococci [11,23], evaluating slime production was considered a key focus for study [20].

The presence of the *aap* gene in *S. epidermidis* is essential for biofilm development [12]. Some *S. aureus* strains carry the cell wall protein SasG, sharing significant homology with *S. epidermidis* intercellular adhesin Aap [13]. The Aap protein plays an important role in inter-cellular adhesion activities and biofilm formation in *S. epidermidis* strains independent of exo-polysaccharide [13]. In fact, higher expression and transcription levels of *aap* account for a polysaccharide intercellular adhesion-independent biofilm [4]. Additionally, *aap* can take the role of polysaccharide intercellular adhesion if only the 220 kilo Dalton protein encounters proteolytic enzymes and a 140 kDa derivative of the protein can be gained [10]. In the present study, 96.4% of the oxacillin-resistant strains had the *aap* gene and 88% of slime producer strains were also oxacillin-resistant. The combination of antibiotic resistance and the *aap* gene may therefore increase the pathogenic potential of opportunist bacteria; however, substantiating this concept would require further research [24].

In the current study, 66.7% of the *S. epidermidis* isolates were multi-drug resistant and thus represent a source for possible transmission of antibiotic resistance and a challenge for eradication [25,26]. Overall, 56.6% of the *S. epidermidis* isolates were oxacillin-resistant and *mecA*-positive. Recent genetic findings suggest that *S. epidermidis* and the other CNS function act as a gene reservoir for the continuous production of different types of the new staphylococcal cassette chromosome mec (SCCmec) elements. , SCC*mec* elements are genomic islands ubiquitously disseminated among staphylococci and the genetic basis for methicillin (oxacillin)-resistance in *S. aureus* (i.e., MRSA) On the other hand, *S. epidermidis* isolated from patients had a similar type (ST27 type sequence) to that of hospital-acquired isolates (nasal carriers and medical equipment) [15]. Since ST27 and positive biofilm colonies are rarely found outside health-care settings, it is possible that patients were colonized with biofilm producing, multi-resistant *S. epidermidis* isolates and that the newly acquired microflora became the source of later infections [27,28]. Various studies show that the presence of biofilms causes a reduction in antibiotic penetration into the cell [29,30] and an increase in resistance gene expression. Biofilms can provide an appropriate environment for transmitting drug resistance plasmids [31,32]. The findings of the present study show that 94.1% of slime-producing *S. epidermidis* isolates are multi-drug resistant, thus supporting the contribution of slime to the emergence of antibiotic resistance.

This study revealed that the phenotypically characterized oxacillin-resistant isolates also contained the *mecA* gene. However, 40.2% oxacillin-susceptible strains which also possessed the *mecA* gene. A lack of gene expression most likely accounts for the inconsistent phenotypic and genotypic data [33]. For instance, mutations of structural genes can lead to the production of non-functional proteins. The effects of environmental factors such as osmolarity, pH, cation concentration, and incubator temperature should also be considered when explaining this incompatibility [20,34]. MIC values could not be compared because disc diffusion was chosen as the susceptibility method using CLSI guidelines.

Mupirocin ointment is included in nasal declonization strategies for controlling oxacillin-resistant staphylococci, particularly MRSA in patients and health-care workers. Interestingly, our study demonstrated that 5.1% of the *S. epidermidis* isolates were mupirocin-resistant, thus highlighting a potential *S. epidermidis* reservoir for mupirocin resistance genes of *S. aureus*.

In summary, our results indicate that *S. epidermidis* is a frequent constituent in the normal bacterial nasal flora is often polyclonal. However, when the immune system is compromised, true infection with *S. epidermidis* can often occur [35]. Whether or not certain clones of *S. epidermidis* may have increased pathogenicity is currently under study.

Tracking the colonization of multi-drug resistant *S. epidermidis* and their slime producing capability in intensive care unit staff and high-risk patients may be an effective infection control and preventive measure.

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