Occurrence of *ica* genes for slime synthesis in a collection of *Staphylococcus epidermidis* strains from orthopedic prosthesis infections

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ABSTRACT ***Staphylococcus epidermidis* is a frequent pathogen in infections associated with orthopedic implants. We studied 123 *S. epidermidis* strains from infections related to orthopedic implants, as regards their ability to express a factor of virulence, namely the slime, an extracellular polysaccharide, which mediates adherence to implants and bacterial colonization. The slime-producing ability was determined by PCR detection of *icaA* and *icaD* genes responsible for slime synthesis, and by culture on Congo red agar plates in which slime-producing strains form black colonies, while nonslime-forming ones develop red colonies. 56% of the *S. epidermidis* isolates were *icaA*- *icaD*-positive and grew to become black colonies. In the evaluation of the distribution of slime-forming strains in different sites and types of implants, we found a slight, but not statistically significant, increase in slime-forming strains in total joint prostheses, where tissue compression near the articular faces can form niches in which bacteria crowd, sheltered by the slime. Our findings confirm the role of *ica* genes as a virulence marker in the pathogenesis of implant-associated orthopedic infections. However, they do not show the existence of a higher frequency of slime-positive strains in a specific type of implant.**

Staphylococcus epidermidis is an important pathogen in orthopedic implant infections (Maderazo et al. 1988, Steckelberg and Osmon 1994). A recent review showed a roughly equal incidence of *Staphylococcus epidermidis* and *Staphylococcus aureus* in infected total hip replacements, accounting for 50–60% of all orthopedic infections (An and Friedmann 1996).

Several studies have clarified the mechanisms by which staphylococci cause infections associated with biomaterials (Foster and McDevitt 1994, An and Friedman 1998, Mack et al. 2000, Montanaro and Arciola 2000). Staphylococcus epidermidis can colonize the artificial surfaces in a self-generated viscous biofilm composed of polysaccharides, called slime (Gristina 1987).

Recently, the genetic control of the slime production has been determined (Gerke et al. 1998, Cramton et al. 1999, McKenney et al. 1999). Synthesis of the capsular polysaccharide is mediated by the ica operon. On activation of this operon, a polysaccharide intercellular adhesin (PIA) is synthesized. This supports cell-to-cell bacterial contacts by means of a multilayered biofilm. The PIA is composed of linear ß-1,6-linked glucosaminylglycans. It is synthesized in vitro from UDP-N-acetylglucosamine by the enzyme N-acetylglucosaminyltransferase, which is encoded by the intercellular adhesion (*ica*) locus and, in particular, by the *icaA* gene. Sole expression of *icaA* induces only low enzymatic activity, but co-expression of *icaA* with *icaD* significantly increases the activity and is related to the phenotypic expression of the capsular polysaccharide (Gerke et al. 1998).

Using an *ica*-specific gene probe for Southern blot hybridization, Ziebuhr et al. (1997) reported

Table 1. Origin of the 123 isolates

	n (%)
Total joint replacements (hip or knee)	46 (37)
Plates, pins, screws (for fixation of tibia, femur, ulna, etc.) [a]	41 (33)
Ligaments or tendon reconstruction (comprising suture threads)	26 (21)
Others: associations of 2 or more prosthesis types or particular cases of prosthetic surgery (e.g., osteosynthesis of cotyloid cavity)	10 (8)
Total	123 (100)

n Number of isolated strains
[a] External fixation devices were also included in this category

striking differences between blood culture isolates and saprophytic staphylococci isolated from the skin and mucosa of healthy volunteers. An intercellular adhesion gene cluster was detected in 44 of 52 blood culture isolates, as compared to only 2 of 36 saprophytic strains.

We determined the occurrence of *icaA* and *icaD* genes for slime production in a collection of *S. epidermidis* clinical isolates by a simple, rapid and reliable polymerase chain reaction (PCR) method developed in our laboratory (Arciola et al. 2001). The search for ica genes was done in 2 *S. epidermidis* reference strains and in 123 *S. epidermidis* isolates from orthopedic implant infections. The slime-forming ability was evaluated in the Congo red agar plate test (Freeman et al. 1989).

Material and methods

Bacterial strains

We used 2 *S. epidermidis* reference strains, the slime producer ATCC 35984 (RP62A) and the slime-negative ATCC 12228 strain. In the present study, 123 *S. epidermidis* isolates from orthopedic implant infections were evaluated (Table 1). The isolates were obtained from 123 consecutive Italian patients, who had infections of undoubted monomicrobial origin, and had undergone implant revision procedures in the Rizzoli Orthopaedic Institute. The isolates were characterized using classical microbiological methods. In particular, the staphylococcal species were identified by the Api-Staph test (Biomérieux, Lyon, France), a biochemical identification kit, and by negativity in the coagulase test.

Phenotypic characterization of slime-producing ability

Production of slime from all strains was studied by culture of the strains, using the Congo red agar (CRA) plate test (Freeman et al. 1989). CRA plates (0.8 g of Congo red (Sigma-Aldrich, Milano, Italy) and 36 g of saccharose (Sigma-Aldrich) to 1 L of brain heart infusion agar (Oxoid, Basingstoke, Hampshire, England)) were incubated for 24 h at 37 °C, and then overnight at room temperature. On CRA, slime-producing strains form black colonies, while non-producing strains develop red colonies.

Strain storage

A single colony of each bacterial strain was seeded in 8 mL of trypticase soy broth (TSB). After incubation for 24 h at 37 °C, the broth culture was fractionated in 1-mL aliquots, which were stored at –80 °C.

Bacterial DNA extraction

Bacteria were harvested by centrifuging 100 µL of each broth culture. Cells were resuspended in 45 µL of H_20, to which 5 µL of lysostaphin (Sigma-Aldrich) solution (100 µg/mL) were added and the samples were incubated at 37 °C. After 10 min, 5 µL of proteinase K (Sigma-Aldrich) solution (100 µg/mL) and 150 µL of 0.1 M Tris/HCl, pH 7.5, were added and the incubation continued for a further 10 min. The samples were then heated for 5 min at 100° C.

PCR method for amplification of icaA and icaD sequences

The sequences of icaA and icaD were taken from the GenBank Sequence Database of the National Center for Biotechnology Information (http://

www.ncbi.nlm.nih.gov) (accession number for the ica operon: U43366). Primers specific for icaA and icaD were selected from the gene sequences in the Primer3 program (National Institutes of Health, National Human Genome Research Institute. (http://www.genome.wi.mit.edu/genome_software/other/primer3.html].

The primers were synthesized by M-Medical Genenco (Florence, Italy). The following primers were used to detect *icaD*: 5'-ACAGTCGCTACGAAAAGAAA as the forward primer (primer 1, corresponding to nucleotides 1824 to 1843) and 5'-GGAAATGCCATAATGACAAC as the reverse primer (primer 2, corresponding to nucleotides 1907 to 1926). These primers include a 103-bp region. The following primers were used to detect *icaD*: 5'-ATGGTCAAGCCCAGACAGAG as the forward primer (primer 1, corresponding to nucleotides 1963 to 1982) and 5'-CGTGTTTTCAACATTTAATGCAA as the reverse primer (primer 2, corresponding to nucleotides 2138–2160). These primers include a 198-bp region. The PCR was done in a DNA thermal cycler (UNO II Thermocycler Biometra GmbH, Göttingen, Germany), as described in Arciola et al. (2001), and the PCR mixture was then analyzed by agarose gel electrophoresis.

Statistics

All data were evaluated with the Chi square test, comparing the prevalence of slime production in the various types of infected prostheses, in order to determine whehter there were any conspicuous differences. A p-value of less than 0.05 was considered significant.

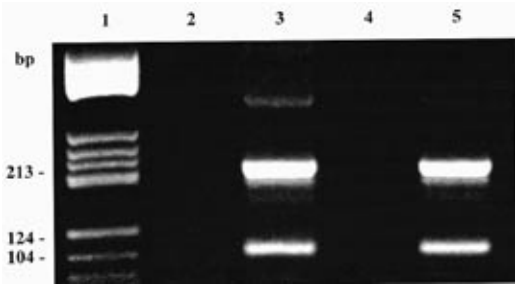
Results

Detection of slime-producing phenotype of S. epidermidis strains by the CRA plate test

69/123 (56.1%) of the *S. epidermidis* clinical isolates proved to be slime-producing, as assessed by cultures on CRA.

PCR detection of icaA and icaD

The PCR technique was applied to the two reference strains and to the 123 *S. epidermidis* clinical isolates. Typical results are shown in the Figure.



PCR detection of *icaA* and *icaD* genes in the same amplification process. Lane 1, Molecular weight markers; lane 2, negative control (DNA template absent); lane 3, 103-bp band for *icaA* and 198-bp band for *icaD* obtained with DNA from a slime-forming clinical isolate; lane 4, absence of bands obtained with DNA from a nonslime-forming clinical isolate; lane 5, 103-bp band for *icaA* and 198-bp band for *icaD* obtained with DNA from the slime-forming ATCC 35984 (RP62A).

All the positive strains for *icaA* were also positive for icaD. The slime-producing reference strain ATCC35984 (RP62A) was positive for both genes, giving a 103-bp band for the *icaA* gene and a 198 bp band for the *icaD* gene. The nonslime-producing *S. epidermidis* reference strain ATCC12228 was negative for both genes. Both bands were always obtained in slime-producing clinical isolates of *S. epidermidis*. Conversely, no band was obtained from nonslime-producing clinical isolates. After acquisition of the bp values of the standard bands of molecular weight marker V, the image analyzer system assigned the expected bp lengths to the bands obtained by amplification of the DNA extracted from the slime-producing strains.

Origin of infection

The distribution of ica-positive strains showed no clear link to a particular surgical site or to the type of implant (Chi square, p-value = 0.7) (Table 2). Similarly, the slime production among the clinical isolates, in this study strictly consistent with icaA- and *icaB*-positivity, seemed to show no relation to the surgical procedure. The ica-negative slime-negative strains were found in various surgical sites with different implants.

In this series, only infections associated with joint prostheses had slightly more slime-forming strains, with 29 slime-positive isolates than the expected 26, as estimated considering the mean value of 56% positive strains in the collection of

Table 2. Slime production by 123 strains of *S. epidermidis* isolated from orthopedic prosthesis infections, as regards the specific implant type

	Slime +	Slime –
Total joint replacements	29	17
Plates, pins, screws	22	19
Tendons, ligaments	13	13
Others	5	5
Total n	69	54

Slime + *icaA- icaD*-positive strains and slime-forming on Congo red agar plate. Slime – *icaA- icaD*-negative and nonslime-forming strains on Congo red agar plate

123 clinical isolates. However, this value was not statistically significant.

Discussion

Bacterial adhesion has long been regarded as a virulence factor contributing to infections associated with medical devices and, in particular, with orthopedic prosthesis infections. The interaction of bacteria with biomaterials has been thought to play an essential role in conditioning the development of these severe nosocomial infections (Francois et al. 1996). In orthopedic implants, particulate wear debris may also be an important factor that favors an infection and loosening.

Growth and survival of microbial colonies depend on adaptation to a series of changes in the environment. In the interaction with biomaterials, bacterial cells showed various mechanisms, which varied with the milieu and surface characteristics of the material and bacterium.

As regards the staphylococcal species, two mechanisms may explain how artificial materials are colonized by the secreted exopolysaccharide that permits bacterial proliferation in a well-protected environment (a mechanism shown by *S. epidermidis* and *S. aureus*) and the adhesins, which specifically bind host matrix proteins adsorbed onto the biomaterial surface (a mechanism that is typical of *S. aureus*) (Christensen et al. 1982, Barth et al. 1989, Montanaro et al. 1999a).

Various mechanisms of adherence in the pathogenesis of prostheses-associated infections can be clarified by studying the presence and expression of genes for adhesion molecules. Progress has recently been made in elucidating the genes and molecules needed for bacterial attachment to surfaces and subsequent biofilm formation. PCR methods have also been described to identify rapidly clinical specimens of genes encoding the main microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Patti et al. 1994) involved in prosthesis adhesion, such as the fibronectin-binding protein genes (*fnbA* and *fnbB*) (Montanaro et al. 1999b) and the collagen adhesin gene (*cna*) (Montanaro et al. 1998). Investigation of the presence and expression of cna in a collection of *S. aureus* strains from orthopedic periprosthesis infections has shown that the slime-positive strains predominate over the cna-positive ones, and that a striking association exists between these two mechanisms of adhesion (Montanaro et al. 1999a).

Our findings indicate an important role of ica genes as a virulence marker for clinically significant *S. epidermidis* isolates. Its presence among a high percentage of clinical isolates, and its association with the strains' ability to produce the slime, strongly suggest that expression of *icaA* and *icaD* genes plays a role in the pathogenetic mechanisms of infection associated with orthopedic implants. These findings are consistent with those of other studies, which showed a high incidence of slime-producing staphylococci in isolates from clinically significant infections of different origin (Ziebuhr et al. 1997).

We found a slight excess of slime-forming strains of *S. epidermidis* only in the case of total joint prostheses. Studies on the molecular genetics of bacterial adhesion on biomaterial surfaces may lead to the development of strategies to prevent or reduce biofilm formation (Christensen et al. 1985, Nomura et al. 1997, Kelly et al. 1999, Arciola et al. 1999, An et al. 2000).

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