Adherence of *Staphylococcus aureus* slime-producing strain variants to biomaterials used in orthopaedic surgery

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Summary. *The adherence of Staphylococcus aureus to biomaterials used in orthopaedic surgery (polymethylmethacrylate, fresh bone, steel and titanium alloys) and to glass was studied in vitro at 1, 2, 6, 24 and 48 h of incubation. Nonslime-producing strains (72, 80 and 510) and slime-producing variants of these strains were used. An automated and fast method of ATP-bioluminescence was applied to determine bacterial viability. The lowest adherence corresponded to polymethylmethacrylate and bone, and the highest to metals. Significant adherence was detected in all cases after 6 h and was strain dependent, being lowest for strain 72. In most cases, adherence of nonslime-producing variants was not significant compared with controls, and slime-producing were more adherent than nonslime-producing variants. These differences were maximal at 6 h or 48 h, depending on the strain and the material. The findings suggest that the appearance of slime-producing cells within a given nonslime-producing bacterial population may jeopardise postoperative immune systems and antibiotic efficacy as a consequence of biofilm formation on implants and prostheses.*

Résumé. *L'adhérence de Staphylococcus aureus à des biomatériels utilisés dans la chirurgie orthopédique (polyméthylmétacrilate, PMM; os frais; alliages d'acier et de titane) et du verre a été étudiée après 1, 2, 6, 24 et 48 h d'incubation, en utilisant des souches (72, 80 et 510) non productrices (NSP) d'exopolysaccharide (slime) et les variantes productrices d'exopolysaccharide (SP) de ces souches. Une méthode automatisée et rapide d'ATP-bioluminescence a été appliquée pour déterminer la viabilité bactérienne. L'adhérence a été la plus basse dans le cas du PMM et de l'os et la plus haute dans le cas des métaux (les deux alliages se sont comportés de façon similaire). Après 6 h, l'adhérence s'est montrée significative, en dépendant aussi de la souche utilisée: la souche 72 a eu la moindre adhérence. Dans la majorité de cas, le degré d'adhérence des variantes NSP a été similaire à celle des contrôles et les variantes SP ont été plus adhérentes que les variantes NSP. Ces différences sont devenues maximales après 6 h ou 48 h, selon la souche et le matériel utilisés. La signification de ces observations peut se trouver dans le risque d'apparition des cellules SP dans une population NSP en présence de biomatériels particuliers utilisés dans la chirurgie orthopédique, en considérant que les cellules bactériennes SP peuvent avoir un haut degré d'adhérence et qu'elles peuvent mettre en danger la surveillance immunitaire post chirurgie et l'efficacité antibiotique à cause de la formation de biofilm sur les implants et les prothèses.*

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Introduction

Bacterial infections associated with implanted biomaterials represent a serious problem in orthopaedic and cardiovascular surgery. *Staphylococcus aureus* is the most frequent pathogen associated with metal surfaces [8], acute and chronic osteomyelitis [19]. The increasing difficulty of eliminating chronic *S. aureus* infections may be partly attributed to the fact that microorganisms which produce exopolysaccharides (slime) may have a special capacity to form large microcolonies and adhere to different surfaces [8, 14, 17]. These films are less affected by antibiotics and, because of their size, they cannot be eliminated by phagocytes [3, 6]. A surgical approach is therefore needed to remove the implant and clean the affected tissue.

A particular strain of *S. aureus* may present two variants, slime-producing (SP) and nonslime-producing (NSP), both of which can be obtained from each other in the laboratory [5]. Many other bacterial species, including coagulase-negative staphylococci, have this property [16]. SP strains usually form biofilms which adhere easily to tissues and biomaterials [2, 19, 22]. Adherence does not only involve exopolysaccharide, but also receptors on the bacterial cell surface for different molecules, including specific proteins such as osteonectin, fibrinogen and collagen [14]. These all promote adherence. Differences between bacterial species and strains with regard to exopolysaccharide production have been reported [1, 17].

The most commonly used biomaterials in orthopaedic surgery are metals (stainless steel, chrome-cobalt and titanium alloys), polymers (polymethylmethacrylate, PMM), high density polyethylene and bioglass [14]. Adherence of *S. aureus* has been studied using different strains, but comparisons within strains of different variants, for example SP vs. NSP, have not been made.

Our aim has been to study the degree of adherence of SP vs. NSP *S. aureus* to materials used in orthopaedic surgery for a 48-h period, during which biofilms are formed [3].

Materials and methods

Test organisms

Staphylococcus aureus strains 72, 80 and 510 were stored in TSB with 15% glycerol at -20°C . Before the adherence assay, the strains were cultured in TSB for 18–24 h at 37°C reaching a concentration of 2×10^9 bacteria/ml.

Two variants were available from each of the strains: 72-, 80- and 510- (the NSP variant of the corresponding strain) and 72+, 80+ and 510+ (the SP variant of each strain). These were

produced in our laboratory from the original NSP isolates as described in our previous studies [5]. SP strain variants were distinguished from NSP by their colony morphology in Congo Red agar, as previously described for coagulase-negative staphylococci [12] and for *S. aureus* [5].

Biomaterials

Those tested were stainless steel and titanium alloys obtained from Kirschner wires and Landos rods, respectively (Synthes and Landos, Barcelona, Spain); glass because of its similarity to bioglass, PMM (Cement Sulfix 6, Escumed Whinterthur, Germany); and fresh bone from adult New Zealand male rabbits (limb segments without marrow). Each material was cut into pieces of a particular size because of difficulties of standardisation and data were presented relative to 1 cm^2 for statistical comparison.

Before the assay, each piece was flamed and incubated overnight in TSB at 37°C for sterility testing. Controls containing a piece of material without bacteria were included.

Adherent biofilm formation

Fifty microliters of an overnight, stationary growth phase, trypticase-soybroth bacterial culture were added under sterile conditions to glass tubes, each of which contained a piece of one of the materials and TSB (2 ml). Samples were subsequently incubated at 37°C for 1, 2, 6, 24 or 48 h, the growth medium being renewed every 12 h. Under aseptic conditions, each piece was recovered with forceps, washed 3 times to eliminate bacteria, and transferred to a tube with DMSO (2 ml; Pancreac, Barcelona, Spain) used as ATP extractant (diluted to 1/9 vol:vol [20]). The tubes were placed in an ultrasonic bath (P-Selecta, Barcelona, Spain) for 30 minutes at 40 Hz, and $22-24^{\circ}\text{C}$, to disintegrate bacterial clumps. Viable bacteria were not detected from these pieces; when growth on trypticase-soy agar was attempted after this treatment. The number of adherent bacteria (CFU/cm²) was determined by ATP-bioluminescence.

ATP-bioluminescence method

A luminometer (Luminoskan RS 1.0, Labystems, Helsinki, Finland) was used for measurement of light emission in the ATP-bioluminescence (luciferin-luciferase) reaction.

ATP standard, ATP monitoring reagent (the enzymatic system, luciferin-luciferase, and tris-acetate buffer (0.1 M tris-acetate and 1 mM EDTA pH 7.75 – Bio-Orbit, Turku, Finland) were used for assay following the makers' instructions.

For the assay, a total of 150 μl of Tris-acetate buffer, 25 μl of ATP monitoring reagent and 10 μl of ATP standard, were distributed in that order in each well, which contained 40 μl of the mixture sample-extractant. Light emission was determined after the addition of each of these compounds. Counts were recorded as relative light units (RLU) per well. The amount of ATP was recorded at the last reading. Tests were done in duplicate and on two different dates. Results were expressed in CFU/cm² for each material tested. A calibration curve (bacterial ATP vs. CFU) was produced [23] to convert ATP moles to conventional CFU using *S. aureus* and DMSO as extractant. A linear relationship was found between the detected bacterial ATP and the number of bacteria (CFU in plate counts/ml) in the interval between 3.5×10^5 CFU/ml and 3.5×10^9 CFU/ml. The observed correlation was high ($r = 0.99$).

Statistical analysis

A three-factorial analysis of variance (strain, variant, hour) to study the dynamics of adherence at different times, for different strains and different variants (SP vs. NSP) was applied with the help of the programme SUPERANOVA for Macintosh, with the following tests: type III sums of squares; least squares means, and Dunnett two-tailed.

Results

The three-factorial analysis for each tested material (Fig. 1) revealed significant differences ($P < 0.001$) in adherence for comparison between variants, strains and biofilm ages, as well as for the paired interactions between these variables ($P < 0.05$).

The lowest overall adherence was found with PMM and bone, and the highest with metals. No differences were found between adherence to titanium and steel alloys, so data for both metals were pooled as a unit – ‘metals’. Under the conditions applied, significant adherence was found in all cases only after 6 h (Table 1). Comparison of overall adherence between strains showed that for strain 72 it was lowest, even when comparing SP variants of different strains. ATP values reflecting the adherence of NSP variants only differed from the controls in a few cases: adherence to PMM (strain 80- at 24 h and strain 510- at 6 h), to glass (strain 510- at 6 h) and metals (strain 510- at 6 and 48 h).

SP variants were more adherent than NSP, and differences between the two became significant after 6 h and reached a maximum at 6 h in strains 72 and 510, and at 48 h for the adherence of strain 80+ to fresh bone.

Concerning the overall evolution of adherence with time, there was a variation between strains and between materials. Adherence reached a maximum at 6 h, with a tendency ($P < 0.05$) to decrease thereafter (strain 510), to be maintained (strain 72 with PMM) or to vary (strain 80 with various materials).

The progress of adherence was studied step by step from pair-wise combinations at different periods: 1 vs. 2 h; 2 vs. 6 h; 6 vs. 24 h, and 24 vs. 48 h (Table 2). NSP showed fewer differences than SP strains, none being found for strain 72- at any period; strain 80- only showed differences with PMM for the 2 vs. 6 h and 6 vs. 24 h comparisons,

Fig. 1. Differences between strains of *S. aureus* with regard to adherence to bone, polymethylmethacrylate, glass and metals (stainless steel and titanium alloys). Means and standard errors are provided

Table 1. Significance of differences in adherence to biomaterials when comparing different strains and variants (slime producing, SP = “+”; and non slime producing, NSP = “–”) in each of the studied incubation periods (biofilm ages)

Material	Strain	Time (h)				
		1	2	6	24	48
Bone	72+	NS/	NS/	NS/c	NS/x	*/bx
	72–	NS/	NS/	NS/	NS/	*/
	80+	NS/	NS/	*/cx	*/cx	***/*cx
	80–	NS/	NS/	*/	*/	***/*
	510+	NS/	NS/	***/*abx	NS/b	NS/b
	510–	NS/	NS/	***/*	NS/	NS/
Glass	72+	NS/	NS/	NS/b	NS/c	NS/bc
	72–	NS/	NS/	NS/c	NS/	NS/
	80+	NS/	NS/	***/*acx	NS/	***/*acx
	80–	NS/	NS/	***/*	NS/c	***/*
	510+	NS/	NS/	***/*abx	***/*ax	NS/*abx
	510–	NS/	NS/	***/*ax	***/*b	NS/
PMM	72+	NS/	NS/	***/*bcx	***/*bcx	NS/bx
	72–	NS/	NS/	***/*b	***/*	NS/
	80+	NS/	NS/	***/*acx	***/*acx	***/*ax
	80–	NS/	NS/	***/*ac	***/*x	***/*
	510+	NS/	NS/	***/*abx	***/*abx	***/*x
	510–	NS/	NS/	***/*bx	***/*b	***/*
Metals	72+	NS/	NS/	NS/bc	NS/bc	NS/bc
	72–	NS/	NS/	NS/c	NS/	NS/c
	80+	NS/	NS/	***/*acx	***/*acx	***/*ax
	80–	NS/	NS/	***/*c	***/*	***/*c
	510+	NS/	NS/	***/*abx	***/*abx	***/*ax
	510–	NS/	NS/	***/*abx	***/*	***/*abx

Numbers to the left of the slash indicate differences between variants of the same strain. NS = non significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Numbers to the right of the slash indicate differences between strains of the same type of variant (e. g., SP type). Characters a, b, c and x represent significance ($P < 0.05$) found in comparisons with strains 72, 80, 510 and control, respectively

and differences were found for strain 510– for glass and metal. SP showed differences in most of the comparisons.

The 2 vs 6 h comparison showed differences in most of the SP strain-material combinations, with few exceptions (strain 72+ to glass and metals). In contrast, the 24 vs 48 h comparisons showed differences in a minority of combinations (adherence of strains 72+ and 510+ to PMM and strain 80+ to bone and glass). The 6 vs 24 h comparison had an intermediate pattern with differences in only some cases, depending on the strain and the material.

Discussion

An explanation for the failure of antibiotic treatment is the biofilm formation, resulting from colonisation of inert surfaces and favoured by slime production [6]. Thus, biofilms are a major problem when implants, prostheses and other devices are used [13].

Bacteria within microcolonies or biofilms resist higher antibiotic concentrations than isolated bacteria. This, together with the observation that in vivo SP variants may promote colonisation of implants and consequent resistance to antibiotics [15], may explain the fact that, in patients with prostheses, only 32% of infections caused by SP coagulase-negative staphylococci are eliminated by antibiotics, reaching 100% successful recovery of the patient in infections by NSP strains [9]. Two hypotheses have been put forward to explain this biofilm-associated phenomenon of resistance [8]: (a) antibiotics do not reach bacteria present in the

Table 2. Significance of differences in adherence to biomaterials between different incubation periods when using different strains and variants (“+” and “–”)

Material	Periods	Strains					
		72+	72–	80+	80–	510+	510–
Bone	1 h vs 2 h	NS	NS	NS	NS	NS	NS
	2 h vs 6 h	**	NS	**	NS	***	NS
	6 h vs 24 h	NS	NS	NS	NS	***	NS
	24 h vs 48 h	NS	NS	***	NS	NS	NS
Glass	1 h vs 2 h	NS	NS	NS	NS	NS	NS
	2 h vs 6 h	NS	NS	***	NS	***	***
	6 h vs 24 h	NS	NS	***	NS	***	**
	24 h vs 48 h	NS	NS	***	NS	NS	NS
PMM	1 h vs 2 h	NS	NS	NS	NS	NS	NS
	2 h vs 6 h	***	NS	***	***	***	NS
	6 h vs 24 h	***	NS	***	***	***	NS
	24 h vs 48 h	***	NS	NS	NS	***	NS
Metals	1 h vs 2 h	NS	NS	NS	NS	NS	NS
	2 h vs 6 h	NS	NS	***	NS	***	*
	6 h vs 24 h	NS	NS	NS	NS	**	*
	24 h vs 48 h	NS	NS	NS	NS	NS	*

See Table 1 for meaning of significances

deeper layers of biofilms because of difficulty in migrating through the exopolysaccharide gel; and (b) bacteria in deeper layers may be in a metabolic state which makes them susceptible to antibiotics. Efficient tests for antibiotic sensitivity against microcolonies and biofilms are essential, but they are not available for routine work at present [10]. An automated test based on the principles applied in this study for bacteria which form microcolonies and biofilms may help to determine which antibiotics would be useful for chronic infections which are difficult to eradicate [18]. Caution is needed in relating the in vitro findings to the in vivo situation, especially when considering differences in bacterial growth and the pharmacokinetics of therapeutic agents.

We have used a simple ATP-bioluminescence method to quantify the number of bacteria adhering to glass and biomaterials [16], and similar methods are being increasingly practised in various assays [4]. These methods are cost and time-efficient, provide objective readings and are automated, which are advantages over older methods [21]. ATP-bioluminescence is therefore a routine way of evaluating the number of bacteria adhering to infective biomaterials.

S. aureus was found to adhere to metals, glass, bone and PMM in that order which confirms previous observations on the preference of this organism for adhering to metals [13], no difference being found between titanium and stainless steel in this regard. A high heterogeneity within this staphylococcal species concerning adherence and biofilm formation was found dependent on strain, variant (SP variants being more adherent than NSP) and biofilmage.

These findings suggest that (a) the use of some biomaterials may increase the risk of colonisation, (b) each bacterial strain may have its own tendency to colonise biomaterials implanted, (c) the number of attached bacteria and the consequent difficulty of controlling infection may vary with time, and (d) an outbreak of SP staphylococcal infection may promote bacterial adherence or colonisation of the biomaterials in the host, consequently difficulting antibiotic therapy.

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