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A comparative study of antibodies against proteins extracted from staphylococcal biofilm for the diagnosis of orthopedic prosthesis–related infections in an animal model and in humans ☆

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ABSTRACT

Staphylococcus aureus and *Staphylococcus epidermidis* are the microorganisms most frequently seen in periprosthetic infections (PPI) with the capacity of forming biofilm. To find potential antigens for the diagnosis of PPI, the immunogenicity of protein components in biofilm from a model biofilm-positive strain (*S. epidermidis* RP62A) was investigated. A guinea pig animal model of PPI was developed and sera were obtained. Sera of patients with PPI and those of controls were also collected. Data generated with an enzyme-linked immunosorbent assay showed that there were significantly higher levels of anti-extracellular protein IgG in sera of infected animals than in controls. We also found significantly higher anti-extracellular protein IgG levels in infected patients, compared to the controls; however, receiver operating characteristic curves did not aid in diagnosing PPI.

1. Introduction

Staphylococcus epidermidis (*S. epidermidis*) is a normal inhabitant of healthy human skin and mucosal microflora and, as a commensal bacterium, has low pathogenic potential. In recent decades, however, *S. epidermidis* and other coagulase-negative staphylococci have emerged as common causes of numerous nosocomial infections, mostly in association with the use of medical devices, such as pacemaker electrodes, synthetic vascular drafts, urinary tract catheters, and orthopedic implants (Ziebuhr et al., 2006). Infections caused by *S. epidermidis* are often subacute, persistent, and relapsing. Moreover, *S. epidermidis* has become the most common causes of periprosthetic infections (PPI) (Hope, et al., 1989). The length of hospital stays was significantly longer for surgeries that resulted in infection compared to uninfected procedures.

Early and accurate diagnosis of infection is a major problem, and it is particularly important to distinguish between infection and mechanical (aseptic) loosening (Kamme and Lindberg, 1981). However, the early diagnosis of PPI using the classical tools of microbiological analysis is difficult. The diagnosis is usually made at advanced stages of infection when severe complications often occur, such as the formation of abscesses, pain, and unsealing of the prosthetic devices. Specific and noninvasive laboratory tests to diagnose these infections are not yet available.

Current tests available to aid the diagnosis of prosthesis-related infections include the erythrocyte sedimentation rate, the level of C-reactive protein, plain radiography, radioisotope scans, aspiration, biopsy, and histology (Fitzgerald, 1992; Garvin and Hanssen, 1995). Serologic methods based on the detection of elevated levels of antibody to microbial antigens offer rapid, noninvasive detection of infection. Inclusion of enzyme-linked immunosorbent assay (ELISA) for diagnostic purposes is desirable because the assay is simple, reproducible, quick to perform, and does not require the withdrawal of biomaterial. However, identification of a suitable

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antigen for the accurate diagnosis of staphylococcal sepsis remains unresolved. The reason is complicated by the likelihood that many individuals may have a significant level of antibody against the common cellular antigens of staphylococci, such as the wall peptidoglycan and teichoic acids, derived from exposure to their own commensal skin flora (Wergeland, et al., 1989). Studies conducted in the last years revealed that detection of staphylococcal infections by ELISA is feasible, but larger and well-organized clinical studies are necessary. Detection of antibodies against whole bacterial cells is considered a sensitive way for serodiagnosis, but previous work demonstrated that the specificity of this assay was low (Ryding, et al., 2002). Instead of using whole cells, mixtures of slime antigens have been used as probes for the detection of *S. epidermidis*–specific antibodies (Kjerulf, et al., 1994). Extracellular carbohydrate antigens of *S. epidermidis* were prepared by gel filtration chromatography from concentrated brain heart infusion culture supernatants, but these antigens were not specific for *S. epidermidis* bone infection (Lambert et al., 1996). Antibodies against discrete slime antigens such as lipid S (Elliott et al., 2000; Rafiq et al., 2000; Worthington, et al., 2002) and poly-*N*-acetyl-B-(1,6)-glucosamine (PNAG; also referred to as polysaccharide intercellular adhesion) (Kelly-Quintos et al., 2005) have been investigated by many researchers, but the production f purified antigens is often labor intensive (Itoh et al., 2002)

In light of these data, no single antigen of *S. epidermidis* has been proven to be an effective assay in all patients with *S. epidermidis* infection. In order to know what antigens are "seen" by the immune system in the case of biofilm-mediated infections, we attempted to assess different components of *S. epidermidis* biofilms, such as extracellular proteins, cell wall proteins, cytosolic proteins, and total cell proteins as antigens for the detection of prosthesis-associated infections of animal model and clinical patients.

2. Materials and methods

2.1. Strain and growth conditions

As controls, 2 staphylococci reference strains were used: the biofilm-producing strain *S. epidermidis* RP62A (ATCC 32984), which was kindly provided by Prof. Gerald Pier (Harvard Medical School, Boston, MA, USA), and the non–biofilm-producing strain 388, which was collected from patients who were hospitalized at the Mignot Hospital of Versailles, France, with infected implant devices. Clinical strains 444 and 5, which are biofilm-producing strains used in this study, were collected from patients who were hospitalized at the Mignot Hospital of Versailles, France, with infected implant devices. The bacterial strains used in this study are summarized in Table 1(Chokr et al., 2006). Stock cultures of bacteria were maintained in Tryptic Soy Broth (TSB) (Becton Dickinson, Le Pont de Claix, France) supplemented with 20% glycerol at −70 °C.

Table 1
Staphylococcus epidermidis strains used in this study (Chokr et al., 2006).

	444	5	388	RP62A
Biofilm	+	+	−	+
PIA	+	+	−	+
Ica locus	+	+	−	+

444, 5, and 388 are clinical strains collected from patients with infected implant devices (described in Materials and Methods).
PIA = Polysaccharide intercellular adhesion.
Ica locus = The gene that encodes the biosynthetic proteins for production of PIA.
+ or − = positive or negative.

2.2. Growth of S. epidermidis biofilm in vitro

An overnight culture grown in TSB was diluted 1:100 in pre-warmed TSB and incubated at 37 °C with shaking (130 rpm) until exponential growth phase was reached according to the growth curves of bacteria. Cultures were then transferred to a 3-L flask containing 750 mL TBS supplemented with glucose (0.25%, w/v) and cultured in static condition at 37 °C for 24 h.

2.3. Extraction and fractionation of biofilm proteins

The TSB was removed and the adherent biofilm was gently rinsed with 0.9% saline 3 times. The biofilm was scraped, suspended in saline, transferred to a plastic centrifuge container, and sonicated on ice (IKASONIC sonicator, IKA Labotechnick, Staufen, Germany, 4 × 30 s, 50% amplitude, intensity 0.5). Cells were removed by centrifugation (5000 rpm, 15 min, Sigma 3K15, Germany). The supernatant was further clarified by centrifugation at 10,000 rpm for 10 min (Sigma 3K15). To this clear extracellular extract, phenylmethanesulfonyl fluoride (PMSF) was added (final concentration: 1 mmol/L) to inhibit proteases. The extract was dialyzed overnight at 4 °C and concentrated by lyophilization to obtain a small volume. Proteins were precipitated by adding 100% trichloroacetic acid (TCA) to a final concentration of 10% after centrifugation (10 min at 12,000 rpm; Sigma 3K15). The precipitate was solubilized in 0.1 mol/L KOH and dialyzed. The preparation of biofilm extracellular proteins was freeze-dried and stored at −80 °C until use.

Our laboratory uses the well-documented lysostaphin digestion protocol to isolate cell wall–associated proteins (Brady, et al., 2006). Briefly, harvested biofilm was centrifuged to collect the bacteria, and the pellet was resuspended in 5 mL of lysis buffer (50 mmol/L Tris-Cl, 20 mmol/L $MgCl_2$, pH 7.5) supplemented with 30% raffinose (Sigma, St. Louis, MO, USA). PMSF and lysostaphin (40 μg; Sigma, St. Louis, MO, USA) were added. The suspension was incubated at 37 °C without shaking for 35 min and then centrifuged at 10,000 × *g* at 4 °C for 20 min. The supernatant, containing the cell wall protein fraction, was collected. The pellet was resuspended in 1.0 mL of lysis buffer, and 0.1-mm silica beads (0.7 g) were added to the cell suspension. Cells were disrupted violently by vortexing. Disrupted cells were then centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant, containing the cytosolic protein fraction, was isolated. For the preparation of total protein, glass beads were added directly after the lysostaphin digestion. All protein fractions were precipitated by TCA as described above.

2.4. Guinea pig serum samples

We studied humoral immune responses to a staphylococcal biofilm–related infection by using a tissue cage (TC) animal model developed earlier by our group (Chokr, et al., 2007). Briefly, 2 small multi-perforated Teflon tubes (tissue cage, TC) filled with polymethylmethocrylate and titanium beads were implanted subcutaneously in the flanks of guinea pigs. A blood sample was taken from the ears of animals prior to inoculation (control serum sample). Seven days after TC implantation, the animals were inoculated with a minimum infectious dose of *S. epidermidis* strain (10^3 CFU). The animals were divided into 4 groups ($n = 8$) and infected by *S. epidermidis* RP62A (ATCC35984), 5 (CIP 109562), 444 (CIP 109563), and 388, respectively. Two weeks after inoculation, the TCs were aseptically removed from the sacrificed animals, washed with saline, and sonicated to detach bacteria from the biofilms of the implants. A blood sample was taken from each of the animals before euthanasia. Serum was obtained after centrifugation at 6000 × *g* for 10 min at 20 °C. Serum samples were aliquoted and stored at −80 °C.