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Isolation of a Strictly Anaerobic Strain of *Staphylococcus epidermidis*

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Staphylococcus epidermidis **is a well-characterized, nonfastidious, aerobic gram-positive coccus commonly isolated in the clinical microbiology laboratory. Although coagulase-negative staphylococci, including *Staphylococcus epidermidis*, are often considered a contaminant in the clinical laboratory, an increasing number of reports describe their pathogenesis, in particular in infections of prosthetic devices. This article describes the isolation of a strictly anaerobic strain of *Staphylococcus epidermidis* in pure culture from the site of an infected prosthetic hip. This isolate was unique in that it grew only under strictly anaerobic conditions. Initially, the isolate was thought to be a known anaerobic gram-positive coccus. However, certain key biochemical and antimicrobial tests performed as part of the standard laboratory identification procedure were not consistent with results expected for any known anaerobic gram-positive coccus; the isolate was catalase positive and metronidazole and penicillin resistant. This isolate was characterized by further biochemical analysis, antimicrobial testing, and nucleic acid sequencing. This paper presents the first documented isolation of a strictly anaerobic *Staphylococcus epidermidis* strain, confirmed by *rpoB* gene sequencing.**

Staphylococcus species are aerobically growing gram-positive cocci. Clinical isolation of *Staphylococcus* spp. is usually not difficult since staphylococci are not fastidious organisms and will grow well on commonly used media and under a variety of conditions (10). They can usually be easily identified in the laboratory by standard microscopy, colony morphology, and biochemical and antimicrobial testing. This report investigates an unusual isolate of *Staphylococcus epidermidis* cultured from hip tissue that grew anaerobically, but would not grow under aerobic or microaerophilic conditions despite repeated attempts.

Staphylococcus epidermidis is classed in the group coagulase-negative staphylococci; these staphylococci are well known as colonizers of the skin and mucosal surfaces. Coagulase-negative staphylococci were considered harmless for many years and when isolated clinically were thought to be contaminants. However, their significance as pathogens is ever increasing, particularly with regard to their affinity for foreign materials (e.g., catheters and in-dwelling prosthetic devices such as heart valves and joints) and their involvement in nosocomial infections (10, 15).

Coagulase-negative staphylococci are most often isolated from the bloodstream but are also commonly isolated from prosthetic sites, wounds, and soft tissue, causing infections such as sepsis, bacteremia, endocarditis, and meningitis. The reason these infections are significant in the hospital setting is that they usually involve immunocompromised or immunosuppressed patients (a population that is constantly increasing), and coagulase-negative staphylococci are among the rising number of resistant pathogens (8). At greatest risk are premature babies, patients with malignancies, and those undergoing chemotherapy and organ transplantation.

Staphylococcus epidermidis is the predominant coagulase-negative staphylococcus and a common pathogen in indwelling or implanted foreign devices. For example, in the average hospital patient, *Staphylococcus epidermidis* is responsible for 50 to 70% of intravenous catheter-related infections (20). Various virulence factors in these bacteria allow them to attach to the polymer material used in these devices to form biofilms (20, 22). In our case, *Staphylococcus epidermidis* was isolated from a prosthetic hip site.

CASE REPORT

The patient was a 72-year-old male with a history of osteoarthritis who had undergone a left hip replacement in 2000. The patient had suffered intermittent draining of the left hip and in 2004 the hip caused the patient some discomfort, although X-rays showed no sign of septic or aseptic loosening or dysfunction of the hip. In addition, an aspirate taken a few months prior to the arthroplasty and discovery of the infection for which he was ultimately treated in late 2004 did not yield any organisms on either aerobic or anaerobic bacteriology cultures from direct inoculation of joint aspirate (collected by injection of saline into the left hip joint and reaspiration). However, due to persisting symptoms and elevated sedimentation rate and C-reactive protein (consistent with infected total hip arthroplasty), it was determined hip arthroplasty was required with reimplantation after intravenous antibiotics. There were no complications during this operation in late 2004.

Hip tissue specimens taken during the operation grew gram-positive cocci on anaerobic media only. The patient was initially given cefazolin and was then transitioned to vancomycin plus metronidazole. The culture was reported as an anaerobic

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gram-positive coccus (previously grouped together as "*Peptostreptococcus* species") and the antibiotic regimen was changed to ampicillin-sulbactam. At the time of discharge there was no evidence of wound breakdown or infection. There was no follow-up with this patient at the University of California—Los Angeles Medical Center after discharge and therefore no amendment to the antibiotic regimen once the isolate was determined to be *Staphylococcus epidermidis*.

MATERIALS AND METHODS

Specimen collection and bacterial culture. Three tissue specimens were collected from the left hip during the hip arthroplasty: intramedullary tissue, tissue from the acetabulum, and unspecified tissues. Aerobic cultures (5% sheep blood agar and chocolate agar plates and [supplemented thioglycollate medium] [broth with hemin and vitamin K_1; BBL prepared culture media; Becton Dickinson Diagnostic Systems, Cockeysville, Md.]) were incubated at 37°C in 5% CO_2, and primary anaerobic cultures (Brucella 5% sheep blood agar, LKV [laked blood with kanamycin and vancomycin] agar, and PEA [phenylethyl alcohol] agar plates [Anaerobe Systems, Morgan Hill, Calif.] and supplemented thioglycollate broth) were incubated at 37°C in an anaerobic chamber. Subsequent isolation of anaerobes was on BBL Brucella blood agar plates. Aerotolerance tests were carried out in 5% CO_2 on chocolate agar plates (for aerobic growth) and in both a reduced oxygen chamber, 2% O_2, and anaerobic chamber on Brucella blood agar plates, all at 37°C.

Phenotypic identification and susceptibility testing. The strains cultured anaerobically were characterized by growth on agar plates (Gram stain and colony morphology), a combination of classical biochemical tests with a short biochemical scheme, a sodium polyanethol sulfonate disk (SPS disk, an anticoagulant that has antibacterial properties), and three antibiotic disks, vancomycin (5 μg), kanamycin (1,000 μg), and colistin (10 μg) (Anaerobe Systems), as described in the Wadsworth anaerobe manual (14). A RapID ANA II ID system (Remel, Inc., Lenexa, Kans.) and another commercial system, the Becton Dickinson BBL Crystal Anaerobe ID system (BD Diagnostic Systems), were also set up, according to the manufacturers' instructions. In addition to the commercial biochemical systems, prereduced anaerobically sterilized tubed biochemical test media (PRAS) biochemical analysis and gas-liquid chromatography (GLC) analysis of metabolic end products and cellular fatty acids were carried out. PRAS biochemical and GLC analyses were carried out at the Wadsworth Anaerobic Bacteriology Laboratory, VA Medical Center, West Los Angeles, and resulting patterns were compared to those of *Staphylococcus saccharolyticus* and other known anaerobes and facultative anaerobes.

Antibiotic susceptibilities were carried out using the E-test (AB Biodisk North America, Inc.). The isolate was tested for β-lactamase production, with a nitrocefin disk (BBL Cefinase paper disk, Becton Dickinson Diagnostic Systems). MICs were determined following the CLSI-approved Wadsworth agar dilution technique at the Wadsworth Anaerobic Bacteriology Laboratory (17).

Nucleic acid sequencing. The isolate was sent to two separate laboratories, the Wadsworth Anaerobic Bacteriology Laboratory and the R. M. Alden Research Laboratory, Santa Monica, Calif., to confirm the identification by 16S rRNA gene sequencing. Genomic DNA was extracted and purified from cells in the mid-logarithmic-growth phase with the QIAamp DNA minikit (QIAGEN Inc., Chatsworth, Calif.). The 16S rRNA gene fragments were amplified as previously described (2). The amplified product was purified using the QIAamp PCR purification kit (QIAGEN Inc.) and directly sequenced with the BigDye Terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, Calif.) on an ABI PRISM 3100-Avant genetic analyzer (Applied Biosystems) at the Wadsworth Anaerobic Bacteriology Laboratory. Extraction and amplification of the isolate followed the same method at the R. M. Alden Research Laboratory but direct sequencing was carried out with an ABI PRISM 3730 genetic analyzer.

The sequences obtained were compared with sequences in the GenBank database by using BLAST software (Pittsboro, NC) and the percent similarity with other sequences was determined (1). Additional nucleic acid analysis carried out at the Wadsworth Anaerobic Bacteriology Laboratory was based on sequencing of the *rpoB* gene, which is a gene that encodes a highly conserved β-subunit of the bacterial RNA polymerase. The *rpoB* gene sequence has been used for nucleic acid studies with staphylococci and a range of other bacteria (e.g., enteric bacteria) (16). The sequence was amplified from the isolate using consensus PCR primers for the *rpoB* gene and purified, sequenced, and analyzed as outlined above (4).

RESULTS

Aerobic cultures had no growth apart from the thioglycollate broth inoculated with the acetabulum tissue, where there was growth in the mid- to lower part of the tube, indicative of anaerobic growth. Gram staining of the thioglycollate broth showed gram-positive cocci in clusters. Anaerobic cultures for the unspecified hip tissue and the acetabulum tissue specimens exhibited growth in the thioglycollate broth and on the primary anaerobic Brucella agar plates. Both specimens grew a few white colonies that, when Gram stained, showed gram-positive cocci in clusters microscopically. The isolate grew solely in anaerobic conditions with no growth under aerobic and microaerophilic (2% O_2) conditions, even after passage three times.

Key biochemical test results were catalase positive, nitrate reductase negative, urease positive, and alkaline phosphatase positive. These biochemical reactions match those of a staphylococcal species with the exception of nitrate reductase reaction being negative. The RapID-ANA and Becton Dickinson BBL Crystal anaerobe ID commercial systems failed to positively identify the isolate. The PRAS biochemicals did not match those of an anaerobe and neither did the GLC pattern, which exhibited large amounts of lactic acid. The isolate was vancomycin sensitive, colistin and kanamycin resistant, and SPS resistant. E-test showed that the isolate was metronidazole resistant (MIC $>$ 256 μg/ml), penicillin resistant (MIC $>$ 256 μg/ml) and vancomycin sensitive (MIC = 1.5 μg/ml). Antibiotic susceptibilities carried out at the Wadsworth Anaerobic Bacteriology Laboratory had the following MICs: penicillin, 16 μg/ml; cefazolin, $\leq$2 μg/ml; metronidazole, 32 μg/ml; vancomycin, 2 μg/ml; ampicillin-sulbactam, $\leq$0.25 μg/ml; erythromycin, $>$128 μg/ml; and imipenem, $\leq$0.06 μg/ml. The isolate was a β-lactamase producer with a positive cefinase disk test.

16S rRNA sequencing confirmed the isolate as *Staphylococcus epidermidis* at both the Wadsworth Anaerobic Bacteriology and R. M. Alden Research laboratories, with a similarity to the *Staphylococcus epidermidis* 16S rRNA gene of $>$99%, although other staphylococcal species also had high percentage identities. The *rpoB* gene sequencing was more discriminatory and had 100% similarity with the sequence for *Staphylococcus epidermidis* but only 86% for a similar staphylococcal species, *Staphylococcus saccharolyticus*, that had a similarly high identity with the 16S rRNA gene.

DISCUSSION

Staphylococcus epidermidis is known as an aerobic gram-positive coccus but here we report on a strain isolated from an infected prosthetic hip site that only grew under obligately anaerobic conditions. The gram-positive cocci in clusters were isolated from the patient's hip specimens in pure culture under anaerobic conditions, but did not fit the criteria of any known anaerobic gram-positive cocci. There are few reported anaerobic gram-positive cocci of clinical significance; these include *Peptostreptococcus anaerobius*, *Peptostreptococcus micros*, *Finegoldia magna*, *Peptoniphilus asaccharolyticus*, *Aerococcus prevotii*, and *Aerococcus tetradius* (these were all previously grouped together as "*Peptostreptococcus* species"). There are also facultative anaerobes that can behave as anaerobes upon initial isolation, such as *Streptococcus intermedius*, *Streptococ-*

cus constellatus, *Gemella morbillorum*, and *Staphylococcus saccharolyticus*.

With regard to this isolate, the standard anaerobic disk susceptibility profile matched that of *Peptostreptococcus anaerobius*, being vancomycin sensitive and colistin and kanamycin resistant. Most anaerobic gram-positive cocci are kanamycin sensitive. However, this isolate was catalase positive and SPS resistant, whereas *Peptostreptococcus anaerobius* is negative (other anaerobic gram-positive cocci have variable catalase activity) and sensitive to SPS (14). The E-test results (confirmed by microdilution results) were another indication that this isolate was not *Peptostreptococcus anaerobius*, which is known to be susceptible to penicillins and metronidazole. We suspected this isolate to be a *Staphylococcus* sp. because of its positive catalase and urease reactions and resistance to metronidazole, an antimicrobial that is effective against all strict anaerobes.

Interestingly, the nitrate reduction reaction was negative whereas for staphylococci it is usually positive. However, there is one report of *Staphylococcus epidermidis* grown anaerobically failing to reduce nitrate, although the nitrate reductase enzyme was found to be present (13). The only known staphylococcus that can behave as an anaerobe is *Staphylococcus saccharolyticus* (14). Because of the unusual phenotype of this organism, nucleic acid analysis was essential in this case for accurate identification of the isolate at the species level.

Nucleic acid analyses have been useful in the species-specific identification of bacteria, including staphylococci, with the 16S rRNA gene being the most widely used and accepted marker for bacterial identification and classification (21). However, this does not always correctly discriminate between staphylococcal species for two reasons. First, in certain species 16S rRNA sequences are highly similar and sequence analysis does not effectively discriminate; for example, similarity among staphylococcal species has been shown to be between 90 and 99%. In comparison, the *rpoB* gene, which has been used for identification of staphylococcal species, has been shown to have a similarity of 71.6 to 93.6% and to be more discriminative than 16S rRNA analyses in 29 staphylococcal species (4). Second, some of the data in the databases is ambiguous in that it is incomplete or there are errors leading to misidentification (9, 18). In this case, the sequencing of the 16S rRNA gene gave high identity for both *Staphylococcus epidermidis* and *Staphylococcus saccharolyticus* (both >99%). However, the *rpoB* sequence from this isolate had 100% identity with that of *Staphylococcus epidermidis* but much lower homology to *Staphylococcus saccharolyticus*, showing the benefit of using such a gene for identification.

Over 500,000 joint arthroplasties are performed each year in the United States with an infection rate that ranges from 1 to 5% (7, 22). A large number of these prosthetic joint infections can be attributed to *Staphylococcus* species, both *Staphylococcus aureus* and coagulase-negative staphylococci. Although these staphylococcal species are normal skin flora, they contribute to a large number of prosthetic joint infections since these bacteria typically grow in biofilms and polymeric matrices and resist antimicrobial and host defenses (22). *Staphylococcus epidermidis* is predominantly isolated in these infections and is well known for its ability to adhere to the surface of prostheses and form biofilms, through virulence factors such as bacterial adhesins and extracellular proteins (5, 11).

In some situations it is thought that these infections are low grade and chronic because reduced accessibility to metabolic substrates in biofilms causes a reduced rate of growth of bacteria; this in turn enables survival as the bacteria go undetected by culture (12, 19). In this case it is quite possible the patient's low-grade infection during the past year was causing the discomfort and the evident inflammatory response, despite no detection of bacteria prior to the arthroplasty and no loosening or displacement of the joint. The isolation of *Staphylococcus epidermidis* in pure culture at the time of the revision arthroplasty is the likely cause of the osteomyelitis seen in the hip but was very unusual because the isolate was anaerobic.

This is the first report of *Staphylococcus epidermidis* as a strict anaerobe. The implications are interesting; for example, in certain strains of *Staphylococcus epidermidis* that are grown anaerobically there is an increase in the production of the *ica* operon, which encodes the polysaccharide intracellular adhesin (3). Galdbart et al. found that there was a significantly higher prevalence of the *ica* operon in *Staphylococcus epidermidis* strains isolated from intravenous catheter-related and joint prosthesis infections (6). Polysaccharide intracellular adhesin has been shown to be associated with *Staphylococcus epidermidis* strains, particularly those that cause prosthetic joint infections and produce biofilms (3, 5). The possibility arises that anaerobic growth of this organism could lead to biofilm formation and contribute to antibiotic tolerance, therefore increasing pathogenicity.

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