CHAPTER

*Staphylococcus epidermidis* is cleared from biomaterial implants but persists in peri-implant tissue in mice despite rifampicin / vancomycin treatment
Staphylococcus epidermidis is cleared from biomaterial implants but persists in peri-implant tissue in mice despite rifampicin / vancomycin treatment

Corine A.N. Broekhuizen 1, Leonie de Boer 1, Kim Schipper 1, Christopher D. Jones 2, Shan Quadir 2, Christina M.J.E. Vandenbroucke-Grauls 1, Sebastian A.J. Zaat 1

1 Department of Medical Microbiology, Center of Infection and Inflammation Amsterdam (CINIMA), Academic Medical Center, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands
2 Emergent Product Development UK Ltd. (Formerly Microscience Ltd.), 540-545 Eskdale Road, Winnersh Triangle, Wokingham, RG41 5TU, Berkshire, United Kingdom

ABSTRACT

Infections associated with implanted biomedical devices (BAI) are predominantly caused by *Staphylococcus epidermidis*. We previously observed in murine experimental BAI that *S. epidermidis* persists in peri-implant tissue rather than on the implanted biomaterial itself (Boelens et al., J Infect Dis 2000; 181: 1337–49; Broekhuizen et al., Infect Immun 2007; 75: 1129–36). To investigate the efficacy of rifampicin / vancomycin to clear *S. epidermidis* from implants and peri-implant tissues, mice with two implants were challenged with $1 \times 10^7$ cfu *S. epidermidis* per implant and received daily injections of rifampicin (25 mg/kg) and vancomycin (50 mg/kg). On the day of termination, implants and peri-implant tissue were collected and processed for culture and histology. After 1 and 8 days, implants of control mice were culture positive in 14/18 and 5/16 cases, respectively, and tissue biopsies were all culture positive. In the antibiotic-treated mice, bacteria were recovered from only 1/18 and 1/16 implants after 1 and 8 days, respectively, whereas the tissues were culture positive in 14/18 and 7/16 biopsies, respectively. In microscopy, bacteria were seen in the tissue at a distance of several cell layers from the tissue-implant interface, colocalized with host cells. Thus, although a regimen of rifampicin / vancomycin sterilized the implants, *S. epidermidis* persisted in peri-implant tissue, which might be an as yet unrecognized reservoir in the pathogenesis of BAI.

INTRODUCTION

Infections associated with inserted or implanted biomaterial devices are a frequently occurring problem in modern medicine. Extensive antibiotic treatment is usually required, and the biomedical device often needs to be removed, increasing hospital stay and costs. *Staphylococcus epidermidis*, commensal bacteria of the skin, are the predominant cause of these biomaterial-associated infections (BAI). The bacteria adhere to the biomaterial surface and produce extracellular polysaccharides (formerly designated as slime) and form, together with host proteins, a biofilm on the implanted device. Bacteria in such a biofilm have a reduced susceptibility to antimicrobial agents. BAI are usually treated with vancomycin, often in combination with rifampicin. Vancomycin is known to penetrate biofilms and effectively reduce...
the numbers of bacteria\textsuperscript{12,13}. Despite this, vancomycin treatment still may have a relatively high rate of failure\textsuperscript{14}. This might be explained in part by low metabolic activity of bacteria in the biofilm\textsuperscript{15}.

In previous studies, we showed that \textit{S. epidermidis} is also found in the tissues surrounding the implant both in an experimental animal model\textsuperscript{16-19} and in humans\textsuperscript{20,21}. Thus, peri-implant tissue may well be a niche for \textit{S. epidermidis}, where the bacteria are not effectively killed by routine antibiotic regimens designed to act on bacteria within biofilms. To test this hypothesis, we investigated the efficacy of rifampicin combined with vancomycin to clear \textit{S. epidermidis} from implants as well as from the peri-implant tissue in our experimental BAI mouse model.

**MATERIALS AND METHODS**

**Animals.** All animal experiments were approved by the Animal Ethical Committee of the University of Amsterdam. Specified pathogen-free C57Bl/6 female mice, 6–8 weeks old and weighing 15–20 g, were used (Harlan, Horst, The Netherlands). All animals were housed in a pathogen-free environment and were provided with sterile food and water. After the surgical procedure, the animals were housed singly, in individually ventilated cages.

**Biomaterials.** Segments of polyvinylpyrrolidone-grafted Silicon Elastomer (SEpvp) catheters (Medtronic PS Medical, Goleta, CA) with a diameter of 2.5 mm, a wall thickness of 0.6 mm, and 1 cm in length were used\textsuperscript{16-19}. Catheter segments were sectioned in a laminar flow cabinet and sterilized for 30 min under UV-light. Sterile gloves were used throughout the procedure.

**\textit{S. epidermidis} strain.** The study was performed with \textit{S. epidermidis} strain AMC5, a clinical isolate causing persistent infection in the mouse model\textsuperscript{19}. AMC5 produces slime, as determined by the method of Christensen \textit{et al.}\textsuperscript{22}, $\delta$-toxin as determined according to Hebert and Hancock\textsuperscript{23} and is positive for the biofilm-associated \textit{icaA}, \textit{atlE}, \textit{sarA}, and \textit{aap} genes by PCR. Primers for \textit{icaA}, \textit{atlE}, and \textit{aap} were according to Vandecasteele \textit{et al.}\textsuperscript{24}. \textit{SarA} was detected using primers \textit{sarA\_d} (AGCAAATGCTACATTGCTAATTC) and \textit{sarA\_r} (ATTTGCTTCTGTGATACGGTGTTG). MIC values (\(\mu g/ml\)) according to standard E-test (AB Biodisk N.A. Inc, Solna,
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Sweden) of strain AMC5 were: rifampicin < 0.016, teicoplanin < 1.5, gentamicin 0.125, and vancomycin 4. Antibiograms were made to identify the reisolated bacteria and to determine possible subtle changes in antibiotic susceptibility. The following antibiotic tablets were used for antibiograms of challenge bacteria and colony-forming units (cfu) recovered from the mice: spectinomycin (200 µg), penicillin low (5 µg), chloramphenicol (60 µg), vancomycin (5 µg), erythromycin (78 µg), rifampicin (30 µg), minocyclin (80 µg), and gentamicin (40 µg). The challenge strain was susceptible to all antibiotics tested.

Inoculum preparation. Fifty milliliter of a logarithmic culture of S. epidermidis AMC5 in Tryptic Soy Broth (TSB) was centrifuged at 2200xg for 10 min. The pelleted bacteria were washed twice with 30 ml of pyrogen-free NaCl 0.9% (saline) (Fresenius Kabi, Sévres, France) and resuspended in 5 ml saline. The optical density at 620 nm was measured and an inoculum suspension containing 4x10⁸ cfu/ml saline was prepared based on this OD620.

Antibiotic regimen. An antibiotic regimen of 25 mg/kg rifampicin (Rifadin, Aventis, Hoevelaken, The Netherlands) and 50 mg/kg vancomycin (Vancomycin 500, Abbott bv, Hoofddorp, The Netherlands) daily was chosen 25-27. On the basis of an average body weight of the mice of 20 g, 0.5 ml of an antibiotic solution of 2 mg/ml vancomycin + 1 mg/ml rifampicin in 0.9% NaCl was injected intraperitoneally (i.p.). Control mice received injections of 0.5 ml of 0.9% NaCl.

Mouse BAI model. Mouse BAI studies were performed as described earlier 17,19,28. In brief, 36 mice were anesthetized with FFM-mix (1 ml of Hypnorm [fentanyl citrate {Fluanisone}], 1 ml of Midazalam, and 2 ml of distilled water) (0.07 ml 10 g of body weight) and given an intraperitoneal injection of rifampicin / vancomycin, or saline. Thirty minutes later, an SEpvp catheter segment was inserted subcutaneously at each side of the back and the incisions were closed with a single stitch. Subsequently, a bacterial inoculum of 10⁷ cfu in 25 µl saline was injected along each implant, and mice received daily injections of antibiotics or saline (control mice) until the day of termination.

Two groups of nine mice (one control and one antibiotic- treated group) were sacrificed after 1 day, and 2 groups at 8 days after challenge. Mice were anesthetized
with FFM-mix, and a standardized biopsy of 12 mm in diameter was taken from each implantation site comprising the implant with surrounding tissue \(^7\). The right side biopsy was used for quantitative culture; the left side biopsy was cut in two halves, one for quantitative culture, and one for histology. Cardiac puncture was performed to collect blood and terminate the mice.

The implants were rinsed and subsequently sonicated for 30 s in 500 µl saline. The sonicate was cultured quantitatively by plating 6 droplets of 10 µl of the neat sonicate (detection limit of 8 cfu) and of 10-fold serial dilutions. The implant itself was cultured in 80 ml of liquid Brewer Tween broth (BT), containing 3% (w/v) thioglycolate, 0.03% (w/v) polyanetholesulfonic acid, and 0.5% Tween 80, adjusted to pH 7.6 with 1M NaOH, for up to 14 days at 37°C. When the plate cultures were negative and the liquid culture was positive, the segments for statistical purposes were defined to have had 5 adherent cfu. The tissue samples were homogenized and quantitatively cultured. In addition, 1/10 volume of the homogenate was cultured in 80 ml BT for up to 14 days at 37°C. If the BT culture was positive and the plate cultures were negative, the total homogenate was considered to have contained 10 cfu, since one tenth of the homogenate had been cultured. Positive BT cultures of any of the implants or tissue samples were streaked on blood agar plates, which were incubated overnight at 37°C.

Assessment of staphylocidal activity of antibiotics in tissue homogenates. To test whether the tissue homogenates contained staphylocidal antibiotic levels, 10 µl drops of undiluted and 10-fold diluted homogenates of the tissue of antibiotic-treated and control mice sacrificed at day 1 were spotted on plates inoculated with the challenge strain \(S. \text{epidermidis} \) AMC5. Plates were inspected for growth inhibition after incubation for 24 h at 37°C.

Blood culture. Twenty-five microliters of blood were added to 80 ml BT broth and cultured for up to 14 days at 37°C.

Histological examination. Biopsies were fixed in formaldehyde, embedded in plastic (methylmethacrylate / buthymethacrylate; Merck Schuchart, Hohenbrunn, Germany), and 3–5 µm sections were cut from different levels in the biopsies. These sections were hematoxylin-eosin (HE) or Gram stained and examined by light
microscopy.

Immunohistochemical staining to specifically detect *S. epidermidis* was performed on selected slides using monoclonal antibodies raised against *S. epidermidis* lipoteichoic acid (anti-LTA; QED, Bioscience, San Diego, CA). Sections were deplastified and cooked, incubated with 3.5 µg/ml anti-LTA for 60 min, rinsed three times with PBS, and incubated for 30 min with an IgG1-specific horse radish peroxidase-conjugated secondary antibody (Immuno-Vision Technologies, Brisbane, CA). Peroxidase activity was detected using 3,3-DiAmino Benzidine tetrachloridestaining. The slides were counterstained with hematoxylin.

**Statistics.** The Fisher's exact test was used to assess significances in differences in the frequencies of positive cultures of the biopsies and biomaterial implants from different groups of mice. Differences in numbers of cfu were analyzed with the Mann-Whitney test. For both tests, p < 0.05 was considered significant.

**RESULTS**

**Effect of rifampicin / vancomycin on biomaterial and tissue colonization by *S. epidermidis* AMC5.** After 1 day, 14/18 (78%) of biomaterial implant cultures, and all cultures of peri-implant tissues in the control group, were positive (Figure 1). In the antibiotic treated mice, all except one biomaterial segment yielded negative cultures. Although the antibiotic treatment had significantly decreased the numbers of cfu in peri-implant tissues when compared to the control group (p ≤ 0.0001), still 78% of these tissues were positive.

After 8 days, 31% of the biomaterial implants and all of the tissue biopsies in the control group yielded positive cultures (Figure 1). Compared to day 1, only the culture positive implants had decreased in numbers (p < 0.05), whereas all the tissues remained positive. Most of the implants of the antibiotic treated mice were sterile (6% culture positive), but still 44% of the tissue biopsies yielded positive cultures. Thus, as in the control mice, *S. epidermidis* persisted in the peri-implant tissue of the antibiotic-treated mice, although the numbers of cfu had decreased when compared to day 1. In both the control and antibiotic treated groups, the tissues contained higher numbers of cfu than the corresponding biomaterial implants at 1 as well as 8 days (p < 0.05). Blood cultures were negative for all samples.
Figure 1: Effect of rifampicin / vancomycin on biomaterial and tissue colonization by S. epidermidis AMC5. Frequencies of positive cultures and numbers of cfu recovered from tissue biopsies (T) and biomaterial implants (BM) of C57Bl/6 mice, at 1 and 8 days after challenge with 1x10⁷ cfu of S. epidermidis AMC5 are indicated. Frequencies of positive cultures are given above the lanes. Antibiotic injections (25 mg/kg rifampicin and 50 mg/kg vancomycin) were given daily until the day of sacrifice. An asterisk indicates p < 0.05. Samples with positive liquid broth cultures, but negative plate cultures are represented by open dots.

Antibiotics in peri-implant tissue. To assess staphylocidal activity present in peri-implant tissue after intraperitoneal injection of rifampicin / vancomycin, the tissue homogenates of antibiotic-treated and control mice were spotted on plates inoculated with the challenge strain S. epidermidis AMC5. The undiluted tissue homogenates of the antibiotic-treated mice produced inhibition zones of 16–19 mm in diameter, indicating the presence of bactericidal levels of antibiotics in the tissue. The tissue homogenates of the control mice did not inhibit, but rather stimulated the growth of the bacteria (Figure 2).
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Antibiotic susceptibility of S. epidermidis. Bacteria retrieved from biomaterial implants and tissues were tested for antibiotic resistance. In all cases single colonies of the reisolated bacteria had the same antibiogram as the challenge strain. No resistance to rifampicin or vancomycin was observed for S. epidermidis reisolated after 1 or 8 days. Moreover, as the zones of inhibition around the antibiotic disks for isolates from control and antibiotic-treated mice (range 46–52 and 18–22 mm zone diameter for rifampicin and vancomycin, respectively) were almost identical to those of the inoculum suspension (48–50 and 21 mm zone diameter for rifampicin and vancomycin, respectively), the antibiotic regimen apparently had not selected for isolates with decreased susceptibility.

Histopathology. S. epidermidis were predominantly cultured from the tissue biopsies surrounding the biomaterial implant rather than from the implants themselves, even after 8 days of antibiotic treatment. To investigate the localization of these bacteria, we analyzed biopsies from the various groups of mice by microscopy, on sections

Figure 2: Influence of peri-implant tissue homogenates of antibiotic-treated (upper panels) or control mice (lower panels) on growth of S. epidermidis AMC5. Aliquots of 10 µl of undiluted (1) or 10-fold diluted homogenates (0.1) were spotted on BA plates inoculated with a lawn of S. epidermidis AMC5. Dark zones indicate growth inhibition due to the presence of antibiotics in the homogenates of the antibiotic-treated mice (upper panels). The undiluted homogenates of the control mice did not inhibit, but stimulated growth (lower panels). Results for homogenates of two mice representative for all homogenates from each group are shown.
collected from different levels in the biopsies. In samples that had yielded only low numbers of cfu, no bacteria were observed neither in the tissue nor at the tissue-implant interface (not shown). In samples that had yielded higher numbers of cfu (control mice sacrificed at 8 days; Figure 3), gram-positive bacteria were seen in the tissue but not at the interface between tissue and biomaterial (Figure 3C, D). These samples were further analyzed by immunohistochemistry using anti-LTA, confirming the presence of bacteria in co-localization with a dense infiltrate of phagocytic and other inflammatory host cells (Figure 3E, F). Since high numbers of cfu were cultured from these tissue biopsies, we assume that at least a considerable number of these tissue-residing bacteria were viable.

**DISCUSSION**

Despite rifampicin / vancomycin treatment, *S. epidermidis* survived in peri-implant tissue in our experimental BAI mouse model. After 1 day, the rifampicin / vancomycin regimen had cleared the bacteria from the biomaterial implants but not from the peri-implant tissue. After 8 days none but one implant yielded positive cultures, but still 44% of the peri-implant tissue specimens were positive, with 25% of all samples yielding high numbers of *S. epidermidis* cfu. Histology showed high numbers of bacteria colocalized with cells, especially in the control group after 8 days. Hardly any bacteria were seen at the interface between tissue and biomaterial implants.

The survival of *S. epidermidis* in peri-implant tissue was not due to low tissue levels of antibiotics, since tissue homogenates of 1 day antibiotic-treated mice strongly inhibited the growth of the test strain on plates inoculated with a lawn of these bacteria. Resistance to the antibiotics used might have been another explanation for the observed survival of *S. epidermidis* in the peri-implant tissue. Rifampicin resistance occurs rapidly because of point mutations in the gene encoding the β-subunit of the bacterial RNA polymerase which alter the target site and decrease the binding affinity for rifampicin. Resistance can develop even when high concentrations of rifampicin are used. However, development of resistance is reduced when this antibiotic is used in combination with vancomycin. Our results are in agreement with the latter studies, as all bacteria recovered from the mice were still fully susceptible to both antibiotics. This indicates that the bacteria most likely survived in the tissue since they were not reached by the antibiotics,
Figure 3: Implant-tissue interface sections and peri-implant tissue of control mice sacrificed at 8 days. Hematoxylin eosin (A, B), Gram (C, D), anti-LTA (E), and anti-LTA control (F)-stained sections of implant-tissue interface (arrow) (A, C, E, F) and peri-implant tissue (B, D, E, F) of C57Bl/6 mice with implanted SEpvp, challenged with $1 \times 10^7$ cfu of S. epidermidis AMC5 and sacrificed 8 days later. The anti-LTA control staining (F) consisted of the anti-LTA staining procedure without the anti-LTA antibody step. The figures are representative for sections in different levels in the biopsies examined. Magnification x150. (A) and (B) reveal dense inflammatory infiltrates composed of neutrophils, plasmacells, lymphocytes, and histiocytes. In (A), close to the implant (left) multinucleated giant cells can also be seen, as a reaction to the foreign material. Gram staining shows accumulation of gram-positive bacteria in macrophages not bordering the implant, especially in (D), as confirmed by immunohistochemistry (E); negative staining in control (F).
or that they were present in a dormant state such as the so-called small colony variants, which are less responsive to antibiotic treatment.

Vancomycin is one of the recommended therapies to treat BAIs with or without the addition of rifampicin. Vancomycin penetrates tissues poorly and rifampicin penetrates well in tissues but is reported to be less effective in killing intracellular staphylococci. In accordance with our earlier studies, S. epidermidis was predominantly found in the peri-implant tissue and even localized within host cells, thus likely not effectively targeted by either vancomycin or rifampicin. This would explain the high frequency of positive BT cultures of these tissues, since the host tissue and cells are lysed by the Tween present in the BT broth, liberating the bacteria. Thus, our results suggest that the peri-implant tissue plays an important role in the pathogenesis of BAIs, and that the presence of S. epidermidis in this tissue and within host cells may explain the reduced efficacy of the antibiotic regimen of rifampicin and vancomycin. A possible lowered level of metabolic activity of these bacteria may also contribute to their survival.

Bacterial peri-implant tissue colonization has not yet systematically been investigated in humans, but several studies indicate that this may be an important and as yet generally overlooked phenomenon. In a study on subclinical infection of breast implants, 77% (17/22) of the silicone implants cultured using an extended protocol yielded positive cultures, but also 20% (8/40) of the peri-implant capsules yielded growth. In one case the capsular tissue was culture positive whereas the implant did not yield growth. Similarly, 27/47 (57%) specimens of perivalvular tissue from prosthetic valve endocarditis patients yielded S. epidermidis. It cannot be excluded that the tissue in these studies was infected due to bacterial migration from the implant into the surrounding tissue, or due to the used excision procedure. To more systematically investigate whether peri-implant tissue colonization occurs in humans, we have recently collected central venous catheters and surrounding tissue from deceased patients using a carefully controlled excision procedure. We retrieved bacteria from the tissue in more cases, and at higher numbers than from the corresponding catheters, and also from tissue not directly bordering the catheters, supporting a role of peri-implant tissue as a niche for bacteria causing BAI.

Peri-implant tissue colonization is not unique for the biomaterial SEpvp used in the present study. In mice, tissue survival of S. epidermidis occurs even
more pronounced around pvp-coated polyamide, and somewhat less around non-coated silicon elastomer and polyamide. Vuong et al. reported colonization by *S. epidermidis* of tissue surrounding Tygon silicone tubing implanted subcutaneously in rabbits. In our study on deceased patients (see above), we observed bacterial colonization of tissue around catheters manufactured of different biomaterials. As the physicochemical characteristics of the biomaterial may influence the level of bacterial tissue colonization a novel focus of biomaterials research may be the development of biomaterials, which prevent or reduce bacterial colonization of peri-implant tissue.

Our results imply that an optimal treatment regimen for BAI should include antibiotics that penetrate biofilms as well as peri-implant tissue, which act intracellularly, and also eradicate bacteria in reduced metabolic state such as the small colony variants. Possible new antibiotic combinations such as linezolid, vancomycin, and levofloxacin or quinupristin/dalfopristin combined with levofloxacin or doxycycline significantly reduced staphylococcal infections of subcutaneously implanted Dacron prostheses in rats. Tissue survival of the bacteria was however not investigated in these studies. Linezolid has excellent oral bioavailability and tissue penetration and therefore certainly holds promise for eradication of peri-implant tissue-residing bacteria. Since the presence of a foreign body such as a biomedical device reduces local immunity an additional approach might be immunostimulation by for example interferon-γ either alone or in combination with antibiotics.

**CONCLUSION**

We conclude that *S. epidermidis* in peri-implant tissue are not eradicated by an antibiotic regimen of rifampicin / vancomycin, which does effectively eliminate bacteria from the implant surface. This indicates that the peri-implant tissue is a hitherto unrecognized niche where *S. epidermidis* can persist even in spite of antimicrobial treatment.

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REFERENCES


