Effects of Vancomycin, Daptomycin, Fosfomycin, Tigecycline, and Ceftriaxone on *Staphylococcus epidermidis* Biofilms

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ABSTRACT: Infection of medical implanted material is associated with considerable morbidity and costs. In the following work, we investigated the effects of vancomycin, daptomycin, fosfomycin, tigecycline, and ceftriaxone on biofilms formed by *Staphylococcus epidermidis* isolates causative for implant infection and catheter-associated bacteremia. Biofilms were studied using the static microtiter plate model and incubated with the antibiotics increasing the concentration from 1× to 128× the minimal inhibitory concentration (MIC) of the respective isolate tested. To quantify the reduction of the biomass, the optical density ratio (ODr) of stained biofilms and the number of growing bacteria were determined. Incubation of the staphylococcal biofilms with the antibiotics decreased the biofilm ODr (at baseline = 1) for ceftriaxone (0.83 ± 0.48) but minimally only for fosfomycin (0.96 ± 0.64), daptomycin (1.05 ± 0.59), tigecycline (1.18 ± 0.66), and vancomycin (0.98 ± 0.44) at exceedingly high concentrations of 128 × MIC. The significant reduction of the bacterial growth was not achieved for all antibiotics, not even at the highest concentrations tested. Using higher doses of the antibiotics may be of some value in the treatment of biofilm-associated infections, although effects are seen only at clinically unachievable doses. However, to eradicate the staphylococcal biofilm, additional measures like debridement and/or removal of the implant are needed. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:1361–1365, 2009

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Infection of medical implanted material is associated with considerable morbidity and costs.[1–3] The use of orthopedic implants, cardiac devices, percutaneous intravascular catheters, invasive methods to sustain life at intensive care units and other implants is increasing. Dependent on the site of implantation, the infection rates range from 0.2% to 5% in orthopedic and trauma surgery, up to 40% in artificial hearts.[3,4] Given the high incidence of fracture stabilization devices of 2 million per annum, the number of implant infections amounts up to 100,000 per year.[3] The major pathogens of implant-related infections are coagulase-negative staphylococci, *Staphylococcus aureus*, and primarily *Staphylococcus epidermidis*.[1,5]

Staphylococcus epidermidis may grow in a biofilm on implants and prosthetic devices thus causing persistent or recurrent infections.[6,7] A biofilm consists of a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to a surface. Biofilm-associated infections are frequently resistant to conventional antimicrobial therapy because the bacterial biofilm on the surface serves as a reservoir where bacteria are quasi inaccessible to antibiotics and the host defences. In the clinical routine, antibiotic susceptibility is tested by determining the minimal inhibitory concentration (MIC) of the antibiotic on free-floating bacteria in the growth phase. A low concentration of the MIC indicates the susceptibility of the microorganism and a rough approximation on the efficacy of the treatment.

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There is evidence that high antibiotic concentrations reduce biofilms and bacterial growth.[8] Thus, we hypothesized that increasing the antibiotic concentration may reduce the biofilm thickness and eradicate the bacterial growth. The following antibiotics were chosen for the experiments: ceftriaxone, a beta-lactam antibiotic widely used to treat staphylococcal infections; vancomycin, used in case of beta-lactam resistance,[9,10] and three alternative agents: fosfomycin, a small molecule antibiotic with excellent tissue penetration, and two newer antibiotics, the glycylglycine tigecycline and lipopeptide daptomycin.[11,12] In the following work, we investigated the effects of these five antibiotics in increasing concentrations on biofilms of *Staphylococcus epidermidis* isolates causative for implant infection and catheter-associated bacteremia.

MATERIALS AND METHODS

The University hospital of Vienna is a 2,200-bed primary- and tertiary-care teaching hospital. We collected *Staphylococcus epidermidis* isolates that were identified as pathogens of implant infections (cardiac pacemakers or implanted defibrillators, $n = 7$; implanted vascular catheters, $n = 4$; bone implants, $n = 8$) or of catheter-related bacteremia (21 isolates) from 2004 to 2006. In addition, 15 *Staphylococcus epidermidis* isolates from the skin of non-hospital-associated healthy controls were collected. Susceptibility testing was performed using the routine laboratory methods according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI). Antibiotic susceptibility was determined by the disk diffusion method on cation-adjusted Mueller-Hinton agar (bioMerieux, L-Etoile, France). Resistance to oxacillin was detected by incubating the plates with disks containing 5 μg oxacillin at 30°C and 37°C. The isolates were identified as strains using the pulsed field electrophoresis genotyping method as previously described.[10]

Definitions of Infections

Definitions of the infections were as follows: Implant infection was defined as the presence of an implanted material, signs and symptoms of systemic infection, and the presence of *Staphylococcus epidermidis* in at least two blood cultures and—if explanted—on the implant after removal. Catheter-associated bacteremia was defined as the presence of signs and symptoms of systemic infection and the isolation of *Staphylococcus epidermidis* from at least two blood cultures within 48 h.[10]

Antimicrobial Agents

Vancomycin, fosfomycin, ceftriaxone, and tigecycline were bought from Lilly, Sandoz (Kundl, Austria), and Wyeth-Lederle (Vienna Austria), respectively. Daptomycin (Vienna, Austria) was supplied as a gift by Novartis (Basel, Switzerland).

Determination of the Minimal Inhibitory Concentration

Before treating the biofilms with vancomycin, daptomycin, fosfomycin, tigecycline, or ceftriaxone, the minimal inhibitory concentrations of each isolated to be tested was determined. The determination of MICs was done according to the protocol of CLSI using the microtiter plate method: according to this protocol, cation-adjusted Mueller-Hinton medium and broth contain the amount of calcium (Ca^{++} 50 mg/l) that is recommended by Novartis for in vitro susceptibility testing of daptomycin. For the susceptibility testing of fosfomycin, glucose-6-phosphate (25 μg/ml) was added.

Biofilm Model

Biofilms were studied using the static microtiter plate model established by Christensen et al.[13] The *Staphylococcus epidermidis* isolates were prepared in Muller-Hinton broth (MHB) at a concentration of McFarland 0.5 and diluted 1:100 with MHB. Each well of a 96-well polystyrene flat-bottomed microtiter plate was filled with 50 μl of diluted bacteria and 50 μl supplemented MHB (containing Ca^{++} 50 mg/l) and incubated for 24 h in ambient air at 35°C. Media and planktonic cells are removed. The biofilms in the wells were fixed with formalin (37%, diluted 1:10) plus 2% natriumacetate, and each well was stained with 150–250 μl 1% crystal violet for 5 min. Then the stained biofilms were washed two times with approximately 300 μl distilled water. Wells were then visually checked for the presence or absence of a biofilm based on the presence of staining at the bottom of the well. The mean optical density (OD) was used for quantification of the biomass using a routine microtiter plate reader at 550 nm wavelength. All biofilm experiments were done five times for each isolate to minimize the variability in the OD measurements. To ascertain the biofilm formation, biofilms were grown on cover slides using 24-well plate. After 24 or 48 h, the biofilms were fixed with 2% glutaraldehyde, and biofilm formation is verified by electron microscope scanning.

"Minimal Inhibitory Concentration Testing" on Biofilms

To test the anti-biofilm effects of vancomycin, fosfomycin, tigecycline, daptomycin, or ceftriaxone, biofilms were prepared as described and grown for 24 and for 48 h. After removal of the medium, the biofilms were incubated with 100 μl of either vancomycin, daptomycin, fosfomycin, tigecycline, or ceftriaxone at increasing concentrations of 1×, 2×, 4×, 8×, 16×, 32×, 64×, and 128× the MIC determined for the respective isolate under planktonic conditions (incubation for 20 h at 35°C ambient air). For fosfomycin, glucose-6-phosphate (25 μg/ml) was added. Four wells per isolate were tested for each concentration. To correct for the individual biofilm formation of each isolate, a ratio of the biofilm OD of the isolate incubated with antibiotic to the biofilm OD of the same isolate without antibiotic (native biofilm) was calculated. The baseline of the "untreated" biofilm is set as 1. This OD ratio (ODr) was used to measure changes in the biomass ("thickness") of the biofilms with increasing concentration of the antibiotics tested.

Bactericidal Efficacy of the Antimicrobial Agents within Biofilms in All Experiments

To test for viable *Staphylococcus epidermidis* in the biofilms in all experiments, the biofilms were not fixed and dyed, but scraped off and resuspended in MHB, seeded to Columbia agar, and examined for growth. The numbers of *Staphylococcus epidermidis* in suspension were enumerated by serial dilutions, and 0.1 ml of each dilution was inoculated onto blood agar plates. The plates were then incubated at 35°C in ambient air and read after 48 h.

Statistical Methods

The significance of difference was assessed by means the *t*-test for continuous variables. To compare groups with a small sample size and non-normally distributed variables, the Wilcoxon ranks sum (Mann–Whitney *U*-test) test was used. To assess the changes of the biofilm ODr at the different concentrations of the antibiotics, the general linear model for repeated measurements (repeated measurements ANOVA) was used. All tests were performed using SPSS for Windows, release 15. A $p < 0.05$ for a two-sided analysis was considered significant.

RESULTS

Susceptibility testing using the standard method to determine the minimal inhibitory concentrations of antibiotics in planktonic bacteria revealed low MICs for vancomycin (90% of all isolates 2 mg/l and below), daptomycin (100% below or equal to 1 mg/l), and tigecycline (100% below or equal to 0.5 mg/l), suggesting high probability of therapeutic success. However, only 60% of the isolates exhibited MICs below 64 mg/l for fosfomycin. For ceftriaxone, 21 of 55 tested isolates exhibited MICs of 4 mg/l and below, indicating resistance to the majority of the isolates. Staphylococcal hospital isolates from patients with implant infections or catheter-related bacteremia exhibited higher MICs, indicating clinical resistance. Isolates from the healthy volunteers had significantly lower MICs for ceftriaxone and fosfomycin than the isolates of patients with infections (Table 1).

Incubation of the staphylococcal biofilms with the antibiotics starting at the concentration of the determined MIC at planktonic conditions and escalating up to 128 × MIC, decreased the biofilm density ratio (ODr at baseline = 1) significantly for ceftriaxone (0.83 ± 0.48; mean ± SD) but minimally only for fosfomycin (0.96 ± 0.64), daptomycin (1.05 ± 0.59), tigecycline (1.18 ± 0.66), and vancomycin (0.98 ± 0.44) at the concentration of 128 × MIC. However, some reduction of the ODr was observed at 16 × MIC for fosfomycin (0.91 ± 0.41), daptomycin (0.93 ± 0.33), and tigecycline

Table 1. Minimal Inhibitory Concentration (MIC) for the *Staphylococcus epidermidis* Isolates (MIC_{50}–median; range) Split Up into Isolates from Implant Infections, Catheter-Associated Bacteremia, or Skin of Healthy Volunteers, and Antibiotic Concentrations Used in the Biofilm Experiments

Bacterial isolates	Vancomycin	Fosfomycin	Tigecycline	Daptomycin	Ceftriaxone
MIC mg/l (MIC_{50}: range)					
ATCC 29232	1	2	0.25	0.125	4
Implant infection ($n = 19$)	2; 1–2	32; 1–≥256	0.125; 0.03–0.5	0.25; 0.06–0.5	32; 1–>256
Catheter-associated bacteremia ($n = 21$)	2; 0.25–4	32; 1–≥256	0.125; 0.01–0.5	0.125; 0.06–1	16; 1–>256
Skin isolates of healthy volunteers ($n = 15$)	1; 0.06–2	16; 1–128	0.06; 0.06–0.25	0.06; 0.03–0.25	2; 0.25–32
All	1; 0.06–4	24; 1–≥256	0.125; 0.01–0.5	0.125; 0.03–1	16; 1–≥256
Antibiotic concentrations used on biofilms in mg/l (median; range)					
1 × MIC	1; 0.06–4	24; 1–256	0.125; 0.01–0.5	0.125; 0.03–1	16; 1–256
2 × MIC	2; 0.12–4	48; 2–512	0.25; 0.02–1	0.25; 0.06–2	32; 2–512
4 × MIC	4; 0.24–8	96; 4–1,024	0.5; 0.04–2	0.5; 0.12–4	64; 4–1,024
8 × MIC	8; 0.48–16	192; 8–2,056	1; 0.08–4	1; 0.24–8	128; 8–2,056
16 × MIC	16; 0.96–32	384; 16–4,112	2; 0.16–8	2; 0.48–16	256; 16–4,112
32 × MIC	32; 1.92–64	768; 32–8,224	4; 0.32–16	4; 0.96–32	512; 32–8,224
64 × MIC	64; 3.84–64	1,536; 64–16,448	8; 0.64–32	8; 1.92–64	1,024; 64–16,448
128 × MIC	128; 7.68–128	3,072; 128–32,896	16;1.32–64	16; 3.84–128	2,056; 128–32,896

(0.98 ± 0.49), but not for vancomycin (1.01 ± 0.5) (Fig. 1a).

With regard to the bactericidal effects of the antimicrobial agents tested on the biofilms, no reduction of the number of the viable bacteria was found for vancomycin, daptomycin, and tigecycline. A mean reduction of the bacterial growth by 0.5 log counts was observed only for fosfomycin and ceftriaxone. In two and four isolates, respectively, a reduction of the log count of the viable bacteria by one was observed for fosfomycin and ceftriaxone. This reduction was only observed in isolates with low MIC (≤4 mg/l for fosfomycin and ≤1 mg/l for ceftriaxone) (Fig. 1b). Figure 2 illustrates the missing decrease of the biofilm ODr and of the bacterial growth in relation to the absolute concentrations of ceftriaxone, tigecycline, vancomycin, and daptomycin, even when escalated to concentrations highly above the clinically achievable concentrations in serum.

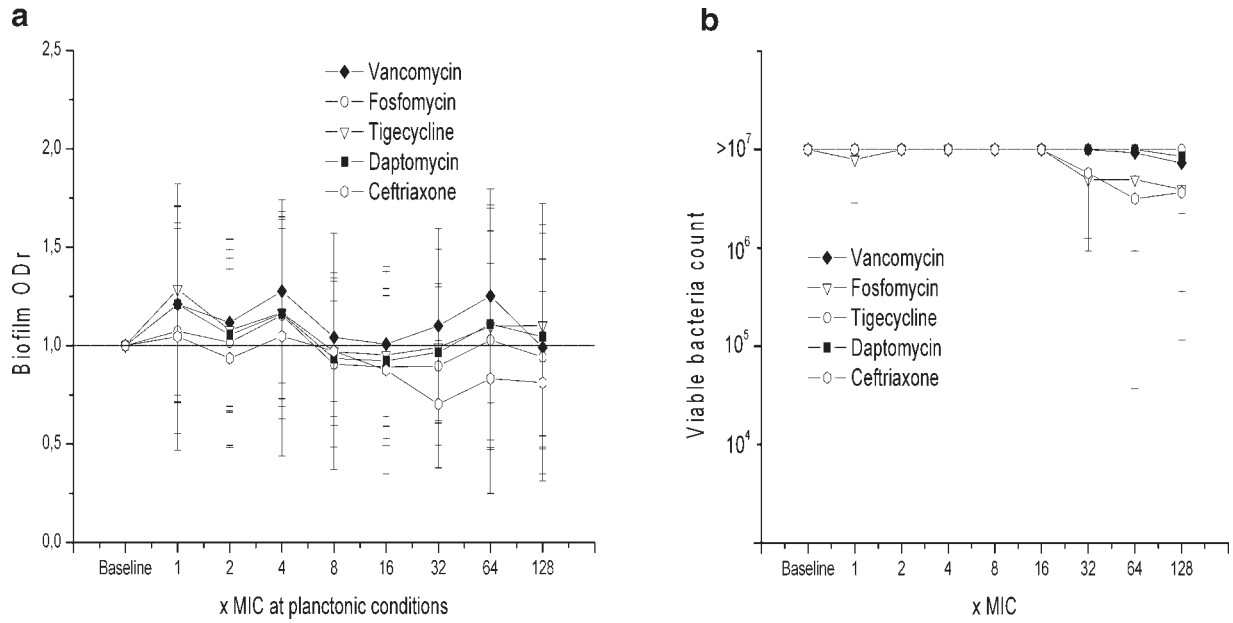

Figure 1. (a) Decrease of the biofilm density given as the change of the optical density ratio (ODr) measured after 24 h incubation. (b) Decrease of the colony count with increasing antibiotic concentration (vancomycin, filled diamonds; fosfomycin, open triangles top down; tigecycline, open circles; daptomycin, filled squares; ceftriaxone, open hexagons). The symbols represent the mean; the whiskers represent the standard deviation.

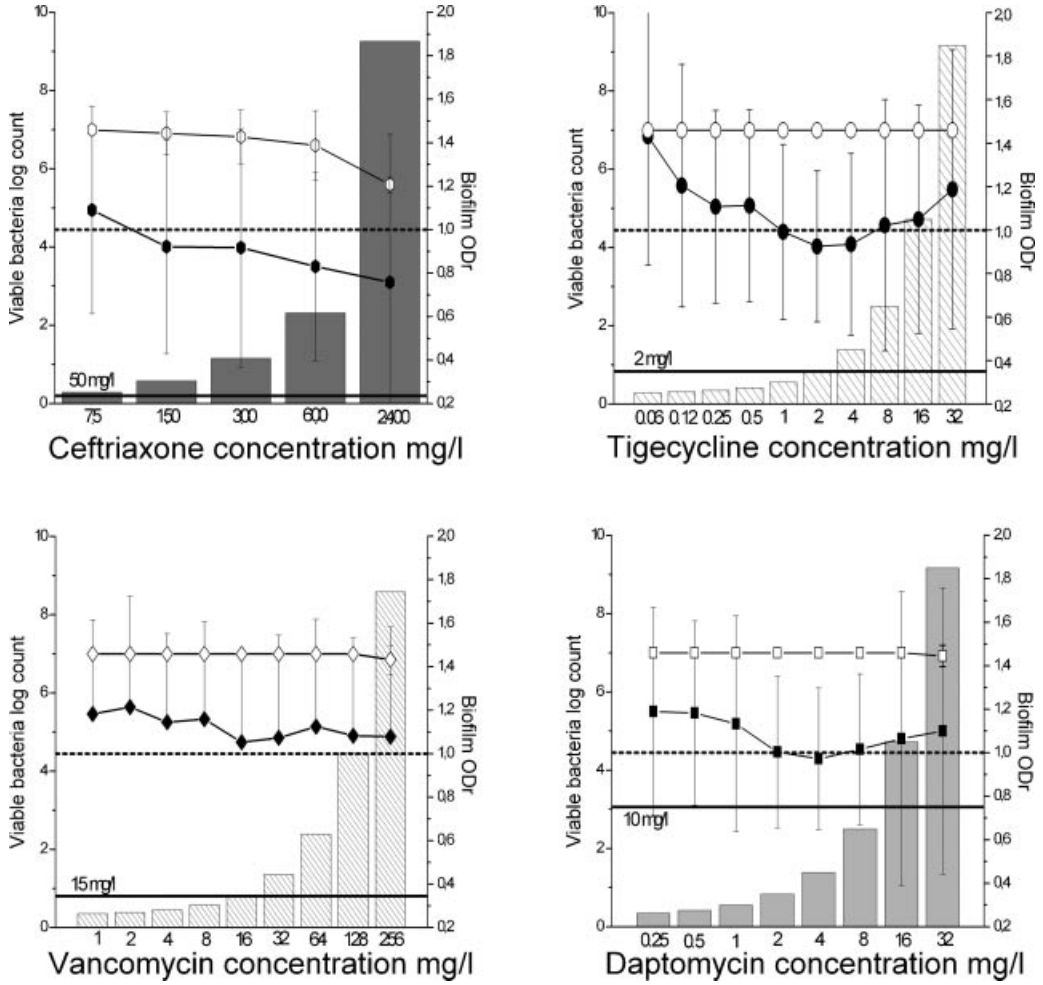

Figure 2. Changes of the biofilms ODr (filled symbols referring to the y2 axis) and the bacterial growth (given as log count; open symbols referring to y1 axis) with regard to the absolute concentrations of the antibiotics tested (bars, mg/l). The solid line represents the concentrations to be expected in human serum after standard dosing; the dotted line represents the untreated biofilm ODr. The symbols represent the mean; the whiskers represent the standard deviation.

DISCUSSION

Experimental data regarding effects of antibiotics' staphylococcal biofilms showed promising results: daptomycin, tigecycline, and linezolid reduced the biofilm burden and the number of viable bacteria within the biofilms significantly. When incubated repeatedly, even eradication of the viable bacteria was achieved in this silicone disk model loaded with thin biofilms consisting of 5×10^3 bacteria/ml.[14] In the present study, the static microtiter plate biofilm was used, mimicking the infection of the implant coated with an established biofilm with densely packed cells. In our model, the antibiotic treatment did not have a clear effect on the biofilm density overall. The reduction of the log count of the viable bacteria meaning eradication was minimal, even when the antibiotics were used at concentrations up to $128 \times$ MIC. Generally, these concentrations are far beyond any concentration that can be achieved after administration of standard therapeutic doses (Fig. 2). For pathogens with very low MICs, the option of eradication may be possible because concentrations needed are likely to be achieved using higher doses (Table 1).

The effects of the antibiotics tested on the staphylococcal biofilms were differential. Vancomycin is the standard antimicrobial agent used in the treatment of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*.[15,16] Although reported as minimally effective against bacterial biofilms,[9] vancomycin reduced the biofilm thickness at the higher concentrations in our experiment. However, vancomycin has the potency of nephro- and ototoxicity, particularly at higher concentrations, that may limit the use of higher doses in the clinical practice.[15] Alternative agents are daptomycin and tigecycline, both with excellent activity against methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. However, as these agents are in clinical use for a short time only, the extent of toxicity is yet to be experienced. In our experiments, daptomycin was used at comparatively low concentrations and did neither reduce the biofilm ODr nor the bacterial log count. Daptomycin at a very high dosage of 30 mg/kg was shown to be effective in experimental implant infection. In the clinical practice, doses up to 8 mg/kg have been used, yet a daily dose of 6 mg/kg is

recommended.[17,18] Tigecycline is used for treatment of nosocomial abdominal infections caused by multi-resistant organisms at a daily dose of 100 mg with mean serum levels up to 2 mg/l. Very low MICs of most clinical isolates, including the *Staphylococcus epidermidis* isolates examined in this study, do not demand higher doses. At medium concentrations ($8-32 \times$ MIC), tigecycline reduced the biofilm film density but had no effect on the bacterial growth. Fosfomycin, similarly to vancomycin, reduced the biofilm density and, in selected isolates, the count of the viable bacterial cells. Noteworthy, the beta-lactam antibiotic ceftriaxone reduced the growth of isolates with low planktonic MICs. This may indicate that a dose escalation might be more effective in the treatment of biofilm-embedded bacteria, particularly because beta-lactam antibiotics are generally well tolerated. For both antibiotics, ceftriaxone and fosfomycin, increasing the doses up to $32 \times$ the MIC may result in concentrations far beyond 10 g/l (Table 1). For these concentrations to be achieved in blood or elsewhere in the body after administration of standard doses, or even double-standard doses, is unrealistic (Fig. 2). Moreover, plasma-protein-binding (of ceftriaxone) or toxicity of higher concentrations has to be taken into account.

In conclusion, bacterial cells within biofilms are highly resistant to antibiotics, even when very low MICs determined in the growing phase predict high susceptibility to the antimicrobial agent. Increasing the antibiotic to the very high concentrations causes some reduction of the biofilm density, particularly for ceftriaxone and tigecycline. However, the overall bactericidal effects of all antibiotics tested on bacterial cells within these thick, established biofilms are little. Standard dosing regimens result in substantially lower plasma and tissue concentrations of antibiotics, as tested in this study. Moreover, for pathogens with high MICs, concentrations up to $128 \times$ the MIC will not be achieved at the infection site when administered at standard dosages. Thus, the chance to treat implant infections is very low once infection involving bacterial biofilms has begun. As a consequence, additional measures are needed to achieve bacterial eradication and cure. Up to now, debridement and removal of a heavily infected implant plus antimicrobial treatment are the only options.[19] However, future experimental work has to be performed to investigate the level of debridement and the optimal type and dosage of antimicrobial substance needed for the eradication of the bacterial biofilm.

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REFERENCES

1. Siegenthaler MP, Martin J, Beyersdorf F. 2003. Mechanical circulatory assistance for acute and chronic heart failure: a review of current technology and clinical practice. J Interv Cardiol 16:563–572.
2. Hebert CK, Williams RE, Levy RS, et al. 1996. Cost of treating an infected total knee replacement. Clin Orthop 331:140–145.
3. Darouiche RO. 2004. Treatment of infections associated with surgical implants. N Engl J Med 350:1422–1429.
4. Sohail MR, Uslan DZ, Khan AH, et al. 2007. Risk factor analysis of permanent pacemaker infection. Clin Infect Dis 45:166–173.
5. Fisman DN, Reilly DT, Karchmer AW, et al. 2001. Clinical effectiveness and cost-effectiveness of 2 management strategies for infected total hip arthroplasty in the elderly. Clin Infect Dis 32:419–430.
6. Schulin T, Voss A. 2001. Coagulase-negative staphylococci as a cause of infections related to intravascular prosthetic devices: limitations of present therapy. Clin Microbiol Infect 7(Suppl 4):1–7.
7. Pfaller MA, Herwaldt LA. 1988. Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. Clin Microbiol Rev 1:281–299.
8. Olson ME, Ceri H, Morck DW, et al. 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res 66:86–92.
9. Monzon M, Oteiza C, Leiva J, et al. 2002. Biofilm testing of Staphylococcus epidermidis clinical isolates: low performance of vancomycin in relation to other antibiotics. Diagn Microbiol Infect Dis 44:319–324.
10. Presterl E, Lassnigg A, Parschalk B, et al. 2005. Clinical behavior of implant infections due to Staphylococcus epidermidis. Int J Artif Organs 28:1110–1118.
11. Huang YT, Liao CH, Teng LJ, et al. 2007. Daptomycin susceptibility of unusual gram-positive bacteria: comparison of results obtained by the Etest and the broth microdilution method. Antimicrob Agents Chemother 51:1570–1572.
12. Frossard M, Joukhadar C, Erovic BM, et al. 2000. Distribution and antimicrobial activity of fosfomycin in the interstitial fluid of human soft tissues. Antimicrob Agents Chemother 44:2728–2732.
13. Christensen GD, Simpson WA, Younger JJ, et al. 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol 22:996–1006.
14. Raad I, Hanna H, Jiang Y, et al. 2007. Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant Staphylococcus bacteremic isolates embedded in biofilm. Antimicrob Agents Chemother 51:1656–1660.
15. Paterson DL. 1999. Reduced susceptibility of Staphylococcus aureus to vancomycin—a review of current knowledge. Commun Dis Intell 23:69–73.
16. Chi CY, Wong WW, Fung CP, et al. 2004. Epidemiology of community-acquired Staphylococcus aureus bacteremia. J Microbiol Immunol Infect 37:16–23.
17. Schaad HJ, Bento M, Lew DP, et al. 2006. Evaluation of high-dose daptomycin for therapy of experimental Staphylococcus aureus foreign body infection. BMC Infect Dis 6:74.
18. Sauermann R, Rothenburger M, Graninger W, et al. 2008. Daptomycin: a review 4 years after first approval. Pharmacology 81:79–91.
19. Trampuz A, Zimmerli W. 2006. Antimicrobial agents in orthopaedic surgery: prophylaxis and treatment. Drugs 66: 1089–1105.