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Tolerance of *Staphylococcus epidermidis* grown from indwelling vascular catheters to antimicrobial agents

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During a prospective study of indwelling vascular catheter-related infections, 134 isolates of *Staphylococcus epidermidis* were grown from 700 catheter tips. *In vitro* antimicrobial susceptibility testing of these isolates to oxacillin, vancomycin and ofloxacin was performed using the standard broth microdilution technique. These results were compared to those for the same organisms grown in biofilm before the addition of antimicrobial agents. In 96-well flat bottom microtiter plates, 10^4–10^5 colony forming units of *S. epidermidis* in 0.1 ml broth were grown for 18 h at 37° C, at which time a biofilm was observed for all isolates. Different concentrations of antimicrobial agents (0.1 ml) were then added to the plates. The plates were incubated for 18 h at 37° C. Since MICs could not be estimated in these plates, all the wells were subcultured after mixing the biofilm with the broth. Minimum bactericidal concentrations (MBCs) were defined as 99.9% reduction in colony forming units. For organisms grown in suspension, 100% of the isolates were susceptible to vancomycin, 81% to ofloxacin and 40% to oxacillin. MBCs of susceptible isolates were within four-fold differences for vancomycin (53%), oxacillin (50%), and ofloxacin (51%). When grown as a biofilm, 78%, 93% and 71% of isolates had MBCs of $\geq$2048 μg ml^{-1} of oxacillin, vancomycin and ofloxacin respectively. These data demonstrate the reduced bactericidal activity of antimicrobial agents against *S. epidermidis* in a biofilm and a simple method for its detection in the microbiology laboratory.

Keywords: biofilms; *Staphylococcus epidermidis*; vascular catheters; antimicrobial susceptibility; bactericidal activity

Introduction

Staphylococcus epidermidis is now the most frequent bacterial species causing nosocomial blood stream infections [3,9,32,34,38]. The increase in infections caused by coagulase-negative *Staphylococci* including *S. epidermidis* is the consequence of extensive use of prosthetic devices, especially indwelling intravascular catheters. The factors contributing to these infections include the ability of *S. epidermidis* to adhere to catheter surfaces and the formation of biofilms that interfere with the activity of host defense mechanisms and antimicrobial agents [5,13,19,23]. Biofilm-associated bacteria are difficult to eradicate and frequently removal of the prosthetic device is required for effective treatment of device-related infections [11,12,15,16,27–29]. Some recent studies showed that patients with catheter-related bacteremia due to coagulase-negative staphylococci can be treated successfully without catheter removal [4,22,30,36]. However, if the central venous catheter (CVC) was not removed, there was a 20% chance of recurrence of bacteremia compared with only a 3% risk of recurrence if the CVC was removed ($P = <0.05$).

The widely used automated methods and the standard broth dilution methods as described by the National Committee for Clinical Laboratory Standards for determination of antimicrobial susceptibility of clinical isolates are not optimal to evaluate the efficacy of antimicrobial agents against bacteria in well formed biofilms. These methods use bacteria in suspension and add the bacteria and the antimicrobial agents to the test system at the same time.

In this study, we compared the *in vitro* susceptibility of 134 isolates of *S. epidermidis* (isolated from indwelling vascular catheters) using the standard broth microdilution method and a biofilm method in which bacteria were grown for 18 h prior to the addition of antimicrobial agents.

Materials and methods

Bacterial isolates: *S. epidermidis*, 134 isolates used in this study were cultured from 700 vascular catheter tips obtained during a prospective study of vascular catheter-related infections. Isolates were identified using the API Staphy-Ident System (Analytab Products permit to bioMérieux Vitek Inc, Hazelwood, MO, USA). The isolates were stored at −70° C in Mueller-Hinton broth (Difco Labs, Detroit, MI, USA) supplemented with 20% glycerol. Before testing the isolates were thawed and subcultured on sheep blood agar (Microbiological Media, Springfield, IL, USA). *Staphylococcus aureus* ATCC strain 29213 and *Enterococcus faecalis* ATCC strain 29212 were used as controls.

Antimicrobial agents: Oxacillin and vancomycin were obtained from Sigma Chemicals Company, St Louis, MO, USA. Ofloxacin was supplied by RW Johnson Pharmaceutical Research Institute (Rahway, NJ, USA). Sterile solutions were prepared according to manufacturers' instructions and frozen at −70° C. Serial two-fold dilutions of oxacillin and ofloxacin were made in Mueller-Hinton broth.

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Mueller-Hinton broth supplemented with cations (25 μg ml^{-1} of magnesium and 50 μg ml^{-1} of calcium) was used to make serial two-fold dilutions of vancomycin.

Determination of minimum inhibitory concentrations: MICs were determined using the standard broth microdilution technique described by the National Committee for Clinical Laboratory Standards [24]. Briefly, 18-h broth cultures were adjusted to 10^5–10^6 colony forming units ml^{-1} using McFarland standards. One hundred microliters of the inoculum were added to each of the wells of microtiter plates containing serial dilutions of the antimicrobial agents. The plates were incubated aerobically at 37° C for 18 h. The range of concentrations tested was 0.0625 μg ml^{-1} to 512 μg ml^{-1} for oxacillin and ofloxacin and 0.0625 μg ml^{-1} to 1024 μg ml^{-1} for vancomycin. The MIC was defined as the lowest concentration of the antibiotic that prevented visible growth.

Bactericidal activity

In suspension: After determination of MICs in suspension as described above, the contents of all wells showing no visible growth were thoroughly mixed and 10 μl were subcultured on sheep blood agar plates. The MBC was defined as the lowest concentration of the antibiotic that killed at least 99.9% of the original inoculum. All experiments were repeated three times.

In biofilm: Bacterial suspensions after 18 h of growth in broth were standardized to contain 10^5–10^6 colony forming units ml^{-1}. The suspensions, 100 μl per well in Mueller-Hinton broth, were added to flat-bottom microtiter plates. We used Micro Test III (Fallon No 3072, Becton Dickinson, Oxnard, CA, USA) microtiter plates. These plates are sterilized with gamma irradiation and the surfaces electrically charged to diminish the hydrophobicity of the polystyrene. The plates were incubated aerobically for 18 h at 37° C. After incubation, a layer of bacterial growth was visible in all wells. A cell count was done to determine the inoculum size and set again at 10^5–10^6 at CFU ml^{-1}. Serial two-fold dilutions, 100 μl well^{-1} of antimicrobial agents were added to the wells containing the biofilms. The plates were then incubated aerobically at 37° C for 18 h. The range of concentrations tested was 8 μg ml^{-1} to 2048 μg ml^{-1} for all three agents. Since all wells showed visible growth, MICs could not be determined. The contents of all wells were mixed thoroughly and subcultured on sheep blood agar. The MBC was defined at the lowest concentration of the antimicrobial agent that killed at least 99.9% of the original inoculum.

Results

Minimum inhibitory and minimum bactericidal concentrations of oxacillin, ofloxacin and vancomycin for 134 isolates of *S. epidermidis* grown in suspension are shown in Tables 1 and 2. Vancomycin was the most effective agent, 100% of the isolates being susceptible at ≤4 μg ml^{-1}. The susceptibility to ofloxacin was 81% (MIC ≤ 2 μg ml^{-1}) and to oxacillin 40% (MIC ≤ 2 μg ml^{-1}). Of the isolates resistant to oxacillin, 25% were also resistant to ofloxacin, 5% were moderately susceptible and 70% were susceptible to ofloxacin. Tolerance, MIC to MBC ratio of 1 : 32 or greater was seen in 14% of isolates to oxacillin, 3% for ofloxacin and 5% for vancomycin. The mean CFU ml^{-1} were $1.4 \pm 0.6 \times 10^7$ in the biofilm wells compared to $9.8 \pm 5 \times 10^4$ in the suspension. The CFU ranged from 0.56×10^7 to 2.7×10^7 ml^{-1} in the biofilm wells compared to 4×10^4 to 20×10^4 ml^{-1} in suspension. As shown in Table 3, the MBC of oxacillin for all isolates grown as biofilm was greater than 128 μg ml^{-1}, and 104 isolates showed growth at ≥2048 μg ml^{-1} compared to 125 isolates for vancomycin and 95 isolates for ofloxacin. Tolerance, MIC to MBC ratio of 1 : 32 or greater was seen in 88%, 100% and 100% of the isolates to oxacillin, ofloxacin and vancomycin respectively when the organisms were grown in a biofilm prior to the addition of the antimicrobial agents (Table 4).

Table 1 *In vitro* susceptibility of 134 isolates of *Staphylococcus epidermidis* grown in suspension

Antimicrobial agent	MIC_{50} (μg ml^{-1})	MIC_{90} (μg ml^{-1})	MBC_{50} (μg ml^{-1})	MBC_{90} (μg ml^{-1})	% Susceptible
Oxacillin	4.0	64.0	16.0	512.0	40
Vancomycin	2.0	2.0	8.0	32.0	100
Ofloxacin	0.5	8.0	2.0	32.0	81

Table 2 Ratio of MIC to MBCs for 134 isolates of *Staphylococcus epidermidis* grown in suspension

	Percentage of isolates with MIC : MBC ratio					
Antimicrobial agent	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	>1 : 32
Oxacillin	25	25	13	10	10	4
Vancomycin	28	25	15	8	2	3
Ofloxacin	29	22	16	4	2	1

Discussion

Biomaterial-related infections are often refractory to antimicrobial therapy, which is understandable based on standard suspension culture MIC and MBC results. The mech-

Table 3 Minimum inhibitory concentations of 134 isolates of *S. epidermidis* grown in biofilm

	Number with MBC (μg ml^{-1})						
Antimicrobial agent	>2048	2048	1024	512	256	128	64
Oxacillin	88	16	20	6	4	0	0
Vancomycin	113	12	4	1	1	2	1
Ofloxacin	34	61	23	5	5	2	3

Table 4 Ratio of MIC[a] to MBC[b] for 134 isolates of *S. epidermidis* grown in biofilm

Antimicrobial agent	Percentage of isolates with MIC : MBC ratio		
	1 : 16	1 : 32	>1 : 32
Oxacillin	3	1	88
Vancomycin	0	0	100
Ofloxacin	0	0	100

[a] MIC – in suspension; [b] MBC – in biofilm

anism of this resistance has not been well characterized. A biofilm barrier effect has been proposed [6,7,14,20,39]. The bacterial exopolysaccharides that encase the microcolonies are considered important factors in the resistance of biofilm-associated bacteria to antimicrobial agents [17,18]. The sticky alginate slime produced by mucoid *Pseudomonas aeruginosa* can bind antibiotic molecules, thereby significantly reducing penetration of antibiotics through the glycocalyx matrix [8,26,31]. Other studies showed that glycocalyx *per se* does not reduce the penetration of antibiotics but it is the altered physiology of bacterial cells in the biofilm mode of growth that results in the changes in permeability of antimicrobial agents [37]. Using disks of stainless steel, polymethylmethacrylate and polyethylene as the substratum, Gristina *et al* [21] demonstrated greater resistance of adherent bacteria compared to the susceptibility of the same strains grown in suspension. The resistance was independent of slime production and was related to the biomaterial used. However, biomaterial-adherent strains yielded 10 times more colony forming units per disk than the non-slime producing strain. Independent of the mechanism involved, failure of antimicrobial therapy in biomaterial-associated infections is well documented. Although bacteremias/septicemias originating from a catheter may be treated successfully by antimicrobial therapy based on *in vitro* results on bacterial suspensions, the persistent bacteria on the catheter remain a source of recurrent bacteremia. Exposure of the large number of bacteria in the biofilms to concentrations of antimicrobial agents that are not able to kill them can lead to induction of resistance [2]). It is therefore of great importance to develop methodology that gives antimicrobial susceptibility results relevant to the biofilm-associated bacteria.

A number of techniques, including the use of biomaterial disks and an *in vitro* chemostat system with defined growth environment have been able to determine the *in vitro* susceptibility of biofilm-associated bacteria [1,25]. Although these experimental methods are very useful in demonstrating the tolerance of biofilm-associated bacteria, their use in clinical laboratories is not feasible. We have demonstrated a significant difference in the susceptibility of bacteria in suspension and in biofilms using the wells of microtiter plates that are routinely used in antimicrobial susceptibility testing. The method involves growing the bacteria in the wells for 18–24 h prior to the addition of antimicrobial agents, but otherwise is similar to the broth microdilution technique. This simple method can be used by diagnostic laboratories on blood isolates from patients suspected of having device-related infections and can help in making a decision for removal of the device.

We have used a large number (134) of clinical isolates actually recovered from vascular catheter tips and our results are similar to those observed with small numbers of well characterized bacterial strains. Although not all of these isolates were actually associated with an infectious episode, a large number of those that were not had survived antimicrobial therapy given for other reasons. The option of using higher dosages of currently available antimicrobial agents, although attractive, is not feasible. The concentration that would be required to cure device-related adherent infections without their removal are much higher than the safe therapeutic levels. The length of time of exposure of the biofilms to antibiotics is also an important clinical consideration. In our experiments, the biofilms were exposed to high concentrations of antimicrobial agents for 18 h without any significant killing. In the clinical situation, the bacteria on the catheter surface are exposed to varying concentrations of antimicrobial agents depending on their pharmacokinetics. More recent studies have focused on the use of enzymatic degradation of the exopolysaccharide matrix to improve contact between bacteria in biofilms and the antimicrobial agents [10,33,35]. Others have explored the use of non-antibiotic properties of some of the available antimicrobial agents in improving the outcome of antimicrobial therapy of device-related infections [38,39]. The colonized device could be irrigated with a solution of high concentration of antimicrobial agents as an adjunct to therapeutic doses of systemic therapy. The potential availability of antimicrobial agents with the inherent property of penetrating the biofilms remains an extremely attractive option for the future.

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