Comparative Efficacies of Quinupristin-Dalfopristin, Linezolid, Vancomycin, and Ciprofloxacin in Treatment, Using the Antibiotic-Lock Technique, of Experimental Catheter-Related Infection Due to *Staphylococcus aureus*

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We performed *in vitro* studies to elucidate the bactericidal activity of the antibiotics in an adherent-cell biofilm model. Efficacy studies were performed in a staphylococcal central venous catheter (CVC) infection rat model. Silastic catheters were implanted into the superior cava. Via the CVC the rats were challenged with 1.0×10^6 CFU of a live *Staphylococcus aureus* strain. Twenty-four hours later, the antibiotic-lock technique was started. All animals were randomized to receive daily isotonic sodium chloride solution, quinupristin-dalfopristin (Q/D), linezolid, vancomycin, or ciprofloxacin at the minimal bactericidal concentration (MBC) and at 1,024 $\mu\text{g/ml}$ in a volume of 0.1 ml that filled the CVC. The main outcome measures were MICs and MBCs for both planktonic and adherent cells, quantitative culture of the catheters and surrounding venous tissues, and quantitative peripheral blood cultures. The killing activities of all antibiotics against the adherent bacteria were at least fourfold lower than those against freely growing cells, with the exception of Q/D, which showed comparable activities against both adherent and planktonic organisms. Overall, Q/D at 1,024 $\mu\text{g/ml}$ produced the greatest reduction in the number of cells recovered from the catheters, while at the same concentration, Q/D and vancomycin demonstrated higher activities than ciprofloxacin or linezolid in reducing the number of organisms recovered from the blood cultures. This study points out that treatment outcome of device-related infections cannot be predicted by the results of a standard susceptibility test such as the MIC. Our findings suggest that the clinically used antibiotics cannot eradicate the CVC infection through the antibiotic-lock technique, even at a concentration of 1,024 $\mu\text{g/ml}$.

Central venous catheters (CVCs) pose a greater risk of device-related infection than does any other indwelling medical device (8). Organisms colonizing CVCs include coagulase-negative staphylococci (mainly *Staphylococcus epidermidis*), *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Candida albicans* (2). Initial colonization is followed by development of a biofilm structure, where the organisms are encased in a polysaccharide matrix. Actually, bacteria, especially staphylococci, on central venous catheters are most often found in biofilms: this matrix protects them from the attack of antimicrobial therapy and from the immune system. For these reasons, infections associated with biofilms are difficult to treat, and it is estimated that sessile bacteria in biofilms are at least 1,000 times more resistant to antibiotics than their planktonic counterparts (7, 12, 13, 22). This antibiotic resistance of biofilms often leads to the failure

of conventional antibiotic therapy and necessitates the removal of infected devices.

The management of those CVCs that do become associated with biofilm-based infection remains problematic (7, 9, 20, 22). Systemic antibiotic therapy is usually administered, but, although effective in eliminating circulating bacteria, it usually fails to sterilize the surfaces of the catheters, leaving the patient at continuing risk of complications or recurrence (4, 10, 14, 18). For these reasons, attention has turned to the *in situ* treatment of colonized catheters, named the lock technique, also known as intraluminal therapy. This technique involves the instillation of a concentrated antibiotic solution into a colonized catheter, in a volume chosen to fill the lumen but not to spill out into the circulation (4).

Two advantages of the lock technique are the possibility to obtain high concentrations of antimicrobial agents in the site of infection and the likelihood of low incidence of antibiotic toxicity. Vancomycin, teicoplanin, gentamicin, amikacin, minocycline, clindamycin, and several other drugs have been used as antibiotic locks (3–5, 18). On this basis, new bactericidal agents might be used therapeutically in this way. Furthermore, the

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emergence in recent years of organisms that are resistant to many conventional antibiotics has stimulated the search for naturally occurring antimicrobial agents that may have clinical usefulness.

The aim of the present study was to assess the efficacy of four clinically available antibiotics in a rat model of CVC infection using the antibiotic-lock technique (19). In addition, *in vitro* studies were performed to elucidate the bactericidal activity of the antibiotics in an adherent-cell biofilm model (21).

MATERIALS AND METHODS

Organisms. *S. aureus*, strain Smith diffuse, kindly provided by N. Balaban (Department of Biomedical Sciences, Division of Infectious Diseases, School of Veterinary Medicine, Tufts University, North Grafton, MA), was used. This is a highly encapsulated, slime-producing strain with exopolysaccharides which are antigenically identical to those of many clinical *S. aureus* strains tested (16).

Antibiotics used in the study. Vancomycin (Sigma-Aldrich S.r.l., Milan, Italy), quinupristin-dalfopristin (Q/D) (Aventis Pharma, S.p.A., Lainate, Milan, Italy), ciprofloxacin (Bayer S.p.A., Milan, Italy), and linezolid (Pharmacia S.p.A., Milan, Italy) solutions were made fresh on the day of assay.

Susceptibility testing with planktonic cells. MIC and minimal bactericidal concentration (MBC) were determined according to the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI [formerly NCCLS]) (17). The MIC was taken as the lowest drug concentration at which observable growth was inhibited. The MBC was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculum. Experiments were performed in triplicate.

Susceptibility testing with adherent cells. For use in the biofilm test, the biofilm MIC and MBC (MIC^b and MBC^b, respectively) were determined with modifications. Biofilms (prepared as described below) were washed with phosphate-buffered saline (PBS) in order to discard unbound bacteria. Subsequently, serial twofold dilutions of antibiotics in Mueller-Hinton (MH) broth were added to wells containing adherent organisms. The polystyrene plates were incubated for 18 h at 37°C in air. The MIC^b was taken as the lowest drug concentration at which observable growth was inhibited. To determine the MBC^b, the MH broth containing antibiotics was removed from each well and replaced with antibiotic-free MH broth; the plates were incubated again for 18 h at 37°C in air. The MBC^b was taken as the lowest concentration of each drug that resulted in no bacterial growth following removal of the drug (13, 21).

Adherent biofilm formation for susceptibility testing. To develop biofilms, 50 µl of tryptic soy broth (TSB) (Oxoid S.p.A., Milan, Italy) containing 10⁶ CFU/ml of bacteria was added under aseptic conditions to each well of a tissue culture-treated polystyrene 96-well plate (Becton Dickinson) containing 150 µl of TSB–2% glucose. After 24 h of incubation at 37°C, the growth medium was discarded and each well was washed three times with PBS under aseptic conditions to eliminate unbound bacteria. To evaluate the formation of adherent biofilm, the remaining attached bacteria were fixed with 0.2 ml of 99% methanol per well, and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 0.2 ml of 2% crystal violet per well. Excess stain was rinsed off by placing the plate under running tap water (6, 21). The plates were air dried, and the dye bound to the adherent cells was resuspended with 0.2 ml of 33% (vol/vol) glacial acetic acid per well. The optical density (OD) of each well was determined photometrically at 570 nm by using the MR 700 Microplate Reader (Dynatech Laboratories, Guernsey, United Kingdom). The 0.00 value (negative control) was determined for every plate measuring the optical density of a well filled with PBS solution. The cutoff OD for the microtiter plate test was defined as 3 standard deviations above the mean OD of the negative control. Tests were performed in duplicate.

Animals. Adult male Wistar rats weighting 200 to 300 g were used for all the experiments. The study was approved by the animal research ethics committee of the INRCA-IRRCs, Università Politecnica delle Marche, Ancona, Italy.

Preparation of inoculum. *S. aureus* strain Smith diffuse was grown in brain heart infusion broth. When bacteria were in the log phase of growth, the suspension was centrifuged at 1,000 × g for 15 min, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline to achieve a concentration of approximately 1 × 10¹⁰ CFU/ml.

Rat CVC-associated infection model. Animals underwent catheterization. Briefly, a Silastic catheter was inserted into the jugular vein and was advanced into the superior vena cava. The proximal portion of the catheter was tunneled

TABLE 1. Antimicrobial activities of Q/D, linezolid, vancomycin, and ciprofloxacin against *Staphylococcus aureus* strain Smith planktonic and adherent cells

Agent	Activity against:			
	Planktonic organisms		Adherent organisms	
	MIC (µg/ml)	MBC (µg/ml)	MIC ^b (µg/ml)	MBC ^b (µg/ml)
Q/D	1.00	2.00	2.00	4.00
Vancomycin	1.00	2.00	2.00	16.00
Ciprofloxacin	0.25	0.50	1.00	4.00
Linezolid	2.00	4.00	4.00	32.00

subcutaneously to exit in the midscapular space. A rodent restraint jacket was used to protect the catheter and to allow access to it. Twenty-four hours after CVC placement, blood cultures were obtained from the catheters to ensure sterility (19). Via the CVC, the rats were challenged with 1.0 × 10⁶ CFU of live *S. aureus* strain Smith diffuse. Six animals (control group 24 h) were sacrificed 24 h after the bacterial challenge to verify the presence of infection by quantitative culture of the catheters and surrounding venous tissues. Contemporaneously, the antibiotic-lock technique was started for the other groups: the drugs were allowed to dwell for 1 h, and the catheters were then flushed with a heparin solution. Twelve animals for each group were randomized to receive daily isotonic sodium chloride solution, Q/D, linezolid, vancomycin, or ciprofloxacin at the MIC^b and 1,024 mg/ml in a volume of 0.1 ml that filled the CVC. As described above, each compound was allowed to dwell for 1 h and the catheter was then flushed with a sterile heparin solution.

Evaluation of treatment. The measurements of the *in vivo* results were quantitative peripheral blood cultures and quantitative culture of the catheters and surrounding venous tissues.

On day 7 the animals were killed. Peripheral blood was obtained by aseptic percutaneous transthoracic cardiac puncture and quantitatively cultured on sheep blood agar. All plates were incubated at 37°C for 48 h and evaluated for the presence of the staphylococcal strain. The organisms were quantitated by counting the number of CFU per plate.

The location of the distal tip of the CVC in the superior vena cava was confirmed visually, and the catheters and surrounding venous tissue were removed aseptically. The explanted catheters or venous tissues were placed in tubes containing 10 ml of PBS solution and sonicated for 5 min to remove the adherent bacteria. The wash fluid was quantitatively cultured by performing serial dilutions (0.1 ml) of the bacterial suspension in 10 mM of sodium HEPES buffer (pH 7.2) (Sigma-Aldrich) to minimize the carryover effect and by culturing each dilution on blood agar plates. As mentioned above, all plates were incubated at 37°C for 48 h and evaluated for the presence of the staphylococcal strains.

The limit of detection for both methods was approximately 1 log₁₀ CFU/ml.

Statistical analysis. MICs are presented as the geometric mean of three separate experiments. Quantitative culture results from all groups are presented as the mean ± standard deviation. The bacterial counts obtained from peripheral blood, explanted CVCs, and peripheral tissues were compared by using the Kruskal-Wallis test. Significance was accepted when the *P* value was ≤0.05.

RESULTS

Susceptibility testing with planktonic cells. According to the broth microdilution method recommended by the CLSI, Q/D, vancomycin, ciprofloxacin, and linezolid exhibited MICs of 1.00, 1.00, 0.25, and 2.00 µg/ml, respectively. The pattern of susceptibility was confirmed by the MBC determination, which exhibited for all antibiotics values twofold higher (Table 1).

Susceptibility testing with adherent cells. The production of the biofilm was photometrically confirmed: the strain showed a mean OD₅₇₀ of 0.415 ± 0.031. The activities of all antibiotics against the adherent bacteria were at least two- to fourfold lower than those for freely growing cells. In detail, Q/D, vancomycin, ciprofloxacin, and linezolid exhibited against the adherent organisms MIC^b values of 2.00, 2.00, 1.00, and 4.00

TABLE 2. Efficacy of Q/D, vancomycin, ciprofloxacin, and linezolid in a rat model of CVC infection induced by *S. aureus* strain Smith diffuse

Treatment	Result for culture ^a :	
	Quantitative blood (CFU/ml)	Quantitative catheters/venous tissues (CFU/ml)
Control group (24 h)		$6.5 \times 10^6 \pm 1.8 \times 10^6$
Isotonic sodium chloride solution	$8.6 \times 10^3 \pm 1.7 \times 10^3$	$9.5 \times 10^7 \pm 1.4 \times 10^7$
Q/D		
1.00 µg/ml	$4.8 \times 10^3 \pm 1.1 \times 10^3$	$8.5 \times 10^6 \pm 2.2 \times 10^6$
1,024 µg/ml	$3.6 \times 10^1 \pm 0.9 \times 10^{1b}$	$1.9 \times 10^3 \pm 0.6 \times 10^{3b,c}$
Vancomycin		
16.00 µg/ml	$3.7 \times 10^3 \pm 1.4 \times 10^3$	$6.8 \times 10^6 \pm 2.1 \times 10^6$
1,024 µg/ml	$5.5 \times 10^1 \pm 1.3 \times 10^{1b}$	$5.8 \times 10^5 \pm 1.5 \times 10^{5b}$
Ciprofloxacin		
4.00 µg/ml	$5.6 \times 10^3 \pm 2.0 \times 10^3$	$9.9 \times 10^6 \pm 2.7 \times 10^6$
1,024 µg/ml	$8.7 \times 10^1 \pm 2.3 \times 10^{1b}$	$7.7 \times 10^5 \pm 1.9 \times 10^{5b}$
Linezolid		
32.00 µg/ml	$2.9 \times 10^3 \pm 0.8 \times 10^3$	$5.7 \times 10^6 \pm 1.1 \times 10^6$
1,024 µg/ml	$2.3 \times 10^1 \pm 0.5 \times 10^{1b}$	$8.7 \times 10^5 \pm 1.9 \times 10^{5b}$

^a Mean $\pm$ standard deviation.^b $P < 0.05$ versus the isotonic sodium chloride solution-treated group.^c $P < 0.05$ versus all other treated groups.

µg/ml, respectively. In contrast, the MBC^b, defined as the lowest concentration of each drug that resulted in no bacterial growth following removal of the drug, showed an important increase in the values able to kill the cells, with the exception of Q/D, which showed comparable activities against both adherent and planktonic organisms (Table 1).

Evaluation of treatment. All animals included in the control group at 24 h demonstrated evidence of CVC infection, with quantitative culture results from catheters and surrounding tissues of $6.5 \times 10^6 \pm 1.8 \times 10^6$ CFU/ml.

Quantitative peripheral blood cultures obtained on day 7 by transthoracic cardiac puncture provided evidence that at the MBC^b all drugs exerted weak antimicrobial activity, with results not significantly different from those for untreated controls.

On the contrary, at 1,024 µg/ml, the antimicrobial activity of all compounds was evident. In particular, Q/D demonstrated higher activities than ciprofloxacin, linezolid, and vancomycin in reducing the number of organisms recovered from the blood cultures (Table 2).

Similar results were obtained when the quantitative cultures were performed with the explanted catheters or venous tissues. Nevertheless, under these conditions, Q/D at 1,024 µg/ml produced the greatest reduction in the bacterial numbers: its activity was statistically higher than those of other drugs (Table 2).

DISCUSSION

In the industrialized world, biofilm-associated infections are becoming more important and staphylococci are the most frequently isolated organisms associated with biomaterials (7, 8). Biofilms have a variety of attributes that contribute synergisti-

cally to the process of antibiotic insensitivity. These attributes include, but are not limited to, a lower growth rate, an exopolysaccharide matrix, a change in gene expression, an optimal three-dimensional structure, and the production of potentially resistant genes (7). The resistance to antimicrobial agents of the sessile bacterial communities is at the basis of many persistent and chronic bacterial infections. Once a biofilm has been established, the embedded bacteria are less accessible and susceptible to a wide range of antibiotics. This ability to suppress antimicrobial efficacy is due to the thickness and chemical composition of the biofilm that prevents the perfusion of antibiotics up to inhibitory or bactericidal levels. In addition, the microenvironment of the biofilm or the metabolic state of the cells within the matrix may inhibit the efficacy of the antibiotics.

Several reports point out the problem of lack of reliability when conventional culture techniques are used to predict antibiotic susceptibilities of biofilm communities. This can explain part of the clinician's failure to eradicate biofilm-related infections. At present, antibiotic susceptibility is measured by standards set out by CLSI in which the MIC of an antibiotic is determined for bacteria in planktonic form only. Therefore, many attempts have been made to create experimental biofilm models that reproduce those formed in the human body so as to evaluate susceptibility tests in comparison to the classical method defined by CLSI (1, 11, 17).

The aim of the present study was to elucidate the bactericidal activity of four clinically available antibiotics in an adherent-cell biofilm model and to assess the efficacy of the compounds in a rat model of CVC infection using the antibiotic-lock technique. We examined the in vitro activities of Q/D, vancomycin, ciprofloxacin, and linezolid against *S. aureus* strain Smith diffuse both in the planktonic state and after adhesion to polystyrene 96-well plates. The staphylococcal strain displayed biofilm formation capabilities. Evidence was found in this work that significant discordance exists between data obtained by the CLSI methods using planktonic cells and those obtained testing matrix-embedded organisms. In fact, the cells in suspension were susceptible to the antibiotics as determined by CLSI guidelines. In contrast, the antimicrobial agents were less active against adherent bacteria.

These results have been confirmed by the in vivo experiments. In fact, even at 1,024 µg/ml no agent produced eradication of the staphylococcal infection of the device. Nevertheless, at this concentration, Q/D, the combination agent that demonstrated in vitro the highest activity, demonstrated the greatest reduction in the number of cells recovered from the catheters, with a 4-log₁₀ decrease of the bacterial count compared with that of the controls.

Today it is well known that biofilm-embedded bacteria are scarcely affected by antibiotic treatment even if they are defined as susceptible on the basis of in vitro tests. However, these organisms usually become rapidly susceptible to an antibiotic when dispersed from a biofilm, which suggests that resistance of bacteria in a biofilm is not acquired via mutation or mobile genetic elements. Organisms that colonize the CVC originate either from the skin insertion site, migrating along the external surface of the device, or from the hub, due to manipulation by health care workers, migrating along the inner lumen, and generally colonization and biofilm formation may

occur within 3 days of catheterization. It has been shown that catheters in place for less than 10 days tended to have more extensive biofilm formation on the external surface of the catheter; for longer-term catheters (up to 30 days), biofilms were more extensive on the internal lumen (11). Various antibiofilm strategies directed at disruption of adherent bacteria are the focus of intense research to improve the detection of biofilm organisms and their eradication. Our study indicates that treatment outcome of device-related infections cannot be predicted by the results of a standard susceptibility test such as the MIC. In addition, our findings highlight that the clinically used antibiotics cannot eradicate the CVC infection through the antibiotic-lock technique, even at a concentration of 1,024 µg/ml.

In conclusion, our data confirm that the clinicians should follow the dogma that infected devices have to be removed in order to achieve cure.

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