

Microbiological tests to predict treatment outcome in experimental device-related infections due to *Staphylococcus aureus*

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Treatment outcome of experimental device-related infections cannot be predicted by the results of standard susceptibility tests such as MIC. Microorganisms involved in such infections have a slow growth rate and adhere to surfaces. Therefore, laboratory tests were developed taking into account these properties and compared with the treatment outcome in an animal model. Vancomycin, teicoplanin, ciprofloxacin and fleroxacin were tested alone, or in combination with rifampicin for their ability to cure experimental device-related infections in guinea pigs due to *Staphylococcus aureus* ATCC 29213. Rifampicin alone or in combination was significantly more effective than the other four drugs ($P < 0.001$). Combined treatment with rifampicin had a higher cure rate than rifampicin alone. Treatment success was not predicted by an antibiotic trough level exceeding the MIC at site of infection. In contrast, drug efficacy was predicted if the stationary-phase MBC was in the sensitive range, and if glass-adherent *S. aureus* was killed by low drug concentrations

Introduction

Infection associated with implants is a rare event (Jara *et al.*, 1979; Rand & Bryan, 1983; Grogan *et al.*, 1986). However, when it occurs treatment outcome with antimicrobial agents alone is poor (Fitzgerald, Nolan & Ilstrup, 1977; Rand & Bryan, 1983). According to the traditional concept, implants have to be replaced in order to cure infection (Dougherty, 1988; Ivey *et al.*, 1990). Since joint replacement is mainly performed in the elderly, the replacement of an infected joint may carry an unacceptable risk for the patient. Therefore, it would be a great medical progress if device-related infections could be cured by antibiotics, without removal of the device. Up to now, the correlation between standard susceptibility testing and clinical outcome of such infections is poor. We observed in experimental device-related infections that the MIC of an antibiotic completely fails to predict treatment outcome (Widmer *et al.*, 1990*a, b*; 1991). The lack of correlation between drug efficacy *in vitro* and *in vivo* may be due to several factors: intracellular persistence of the pathogen, high bacterial density (inoculum effect), low growth rate, protection by a biofilm, surface adherence, and unfavourable conditions in abscesses (Brown, Allison & Gilbert, 1988; Gilbert, Collier & Brown, 1990; Widmer *et al.*, 1991).

The aim of the present study was to define antimicrobial susceptibility tests which more accurately predict the treatment outcome of experimental tissue-cage infections

due to *Staphylococcus aureus*. We evaluated the MIC in relation to local drug levels, the MBC of growing and stationary-phase bacteria, and the checkerboard assay for drug combinations. In addition, we developed an assay to determine the killing of surface adherent bacteria. Finally, we correlated these in-vitro data with the in-vivo results.

Material and methods

Bacterial strain and antibiotics

All experiments were performed with *S. aureus* ATCC 29213. Working cultures were maintained on blood agar and were subcultured weekly. A fresh aliquot of the strain, stored in skim milk at -70°C , was cultured every 3 months. The following antimicrobial agents were kindly provided by the indicated manufacturers: vancomycin (Eli Lilly, Indianapolis, USA), teicoplanin (Merrel-Dow, Thalwil, Switzerland), ciprofloxacin (Bayer AG, Wuppertal, Germany), fleroxacin (Hoffmann-La Roche, Basle, Switzerland), and rifampicin (Ciba-Geigy, Basle, Switzerland).

Animal studies

We used a previously described animal model of foreign body infection (Zimmerli *et al.*, 1982; Zimmerli, 1993). In brief, four sterile polytetrafluoroethylene (Teflon) tubes (32×10 mm) perforated by 130 regularly spaced holes 1 mm diameter (Ciba-Geigy) were aseptically implanted into the flanks of albino guinea pigs (weight 800–1000 g). Experiments were started after complete healing of the wound, (3 to 6 weeks after surgery). Before each experiment, the interstitial fluid which had accumulated in the tissue cages was checked for sterility. On day zero, tissue cages of the treatment group were infected by inoculating with 2×10^4 cfu of *S. aureus*. Established infection was confirmed by quantitative cultures of the tissue-cage fluid at 24 h, just before the first injection of antibiotic. Each drug was given intraperitoneally every 12 h for 4 days. Tissue-cage fluid was aspirated 2 and 10 days after completion of antibiotic therapy.

Ten days after treatment, tissue cages were removed under strict aseptic conditions from animals anaesthetized with fentanyl-droperidol. Tissue cages were placed in trypticase soy broth (TSB) and were incubated for 48 h. A positive culture identified as *S. aureus* was defined as a treatment failure, if the isolated strain had the same antimicrobial susceptibility pattern (except for the treatment agents) as the inoculated strain. Each antibiotic regimen was tested with 12 tissue cages, except fleroxacin and combinations ($n = 8$) and rifampicin ($n = 20$).

Measurements of drug concentrations

Pharmacokinetic studies were performed in sterile tissue cages. Each drug was injected intraperitoneally. Peak levels in tissue-cage fluid were determined 3 to 5 h after antibiotic administration, and trough levels 12 h after antibiotic administration. The results of these studies were previously published (Widmer *et al.*, 1990a, 1991).

MIC/MBC determinations

MICs were determined in Mueller-Hinton broth (MHB; Difco 0757-01-4) by a tube macrodilution method with a standard inoculum of 5×10^5 cfu/mL (National

Committee for Clinical Laboratory Standards, 1988). MBCs were determined as described in the Manual of Clinical Microbiology (Schoenknecht, Sabath & Thornsberry, 1985). The antibiotic concentration which reduced the original inoculum by $\geq 99.9\%$ was defined as the MBC.

MBCs were also determined with bacteria in a stationary phase of growth. Overnight cultures of *S. aureus* were centrifuged at 2000 g for 10 min, and were then resuspended in a medium containing 1% glucose supplemented phosphate buffered saline (PBS; pH 7.4) with 4% MHB. In this medium, bacterial counts remained stable for up to 36 h. After incubation with different antibiotic concentrations for 24 h, the concentration which reduced the inoculum by $\geq 99.9\%$ was defined as the MBC of antibiotic for non-growing bacteria.

Killing of adherent bacteria

This test was modified from an assay recently described by Vergères & Blaser (1992). In order to get an adherent inoculum, sinter glass beads containing pores of 60–300 μm (Sikuf 0.23/300/A; Schott Schleifer, Muttentz, Switzerland) were placed into a 100 mL flask containing 1 mL medium per incubated bead. In order to get an adherent inoculum of approximately 5×10^5 cfu per bead, the composition of the medium was chosen as follows: 1% glucose and 4.2% TSB supplemented with 50 mg/L Ca^{++} and 25 mg/L Mg^{++} in PBS. This medium was inoculated with two colonies taken from a blood agar plate and incubated overnight. After 24 h, the medium was aspirated and replaced with saline twice. Each bead was placed with a sterile forceps on a filter (0.45 μm), and washed twice with saline in order to avoid a carry over of bacteria suspended in the incubation medium. The number of *S. aureus* adhering on the sinter glass beads (the adhering inoculum) was determined by placing washed glass beads in 2 mL of physiological saline containing EDTA (0.15%) and Triton-X (0.1%). Bacteria adhering to the beads were removed by vigorously mixing the tubes three times for 15 sec. Thereafter, the tubes were placed in a ultrasonic bath, and sonicated for 3 min at 110 W (Labsonic 2000, Bender & Hobein, Zürich, Switzerland). After an additional mixing, 100 μL aliquot was diluted for quantitative bacterial culture on Mueller-Hinton agar. Using this procedure, $97 \pm 2\%$ of the adherent bacteria were removed, as verified with [^3H]thymidine labelled *S. aureus* ATCC 29213 (R. Spörri, personal communication). In each experiment the geometric mean cfu removed from four different sinter glass beads was defined as the adherent inoculum for calculating antibiotic bactericidal activity. The mean coefficient of variation in individual experiments (intraassay variation) was 32%. The coefficient of variation in 9 different experiments (interassay variation) was 45%. The geometric mean of the inoculum was 4.5×10^5 cfu/bead ($n = 9$), range $2.5\text{--}8 \times 10^5$ cfu/bead.

The killing of adherent bacteria was measured in parallel experiments. All antibiotic regimens were simultaneously tested on three different occasions. Eight regimens were tested in three additional series of experiments. One sinter glass bead was incubated in MHB, together with the adherent bacteria and drug concentrations corresponding to the tissue-cage fluid peak and trough level. After incubation for 24 h at 37°C, the sinter glass beads were washed as described above. After washing, the remaining surface adherent bacteria were removed with the identical protocol as described above. The log killing of adherent bacteria was calculated as follows: log cfu of untreated beads (inoculum) minus log cfu after antibiotic treatment of the beads.

Chequerboard assay

The activity of drug combinations *in vitro* was tested with a chequerboard method (Eliopoulos & Moellering, 1991). The fractional inhibitory concentration index (FIC-index) was calculated by summation of the separate FICs of each of the antibiotics present in a well.

Statistics

Fisher's exact test was used to determine the significance of changes in proportions in the animal studies. If combination regimens were different from single antibiotic therapy at a p -value < 0.05 , the combination was defined as synergistic or antagonistic. The correlation between killing of adherent *S. aureus* and cure rate was determined by linear regression.

Results

Experimental infections

At the beginning of antibiotic treatment, mean bacterial counts in tissue-cage fluid were 9.4×10^5 cfu/mL (geometric mean, $n = 108$). Treatment results are summarized in the Figure. Combination regimens were significantly better than single drug regimens. Rifampicin was the only drug with reasonable efficacy when given alone. As anticipated, in two out of 20 infected cages, rifampicin-resistant *S. aureus* emerged during treatment. One of the failures following treatment with vancomycin and rifampicin was also due to the emergence of a rifampicin-resistant strain. In all other treatment failures, the strain was still susceptible to the respective antimicrobial agents.

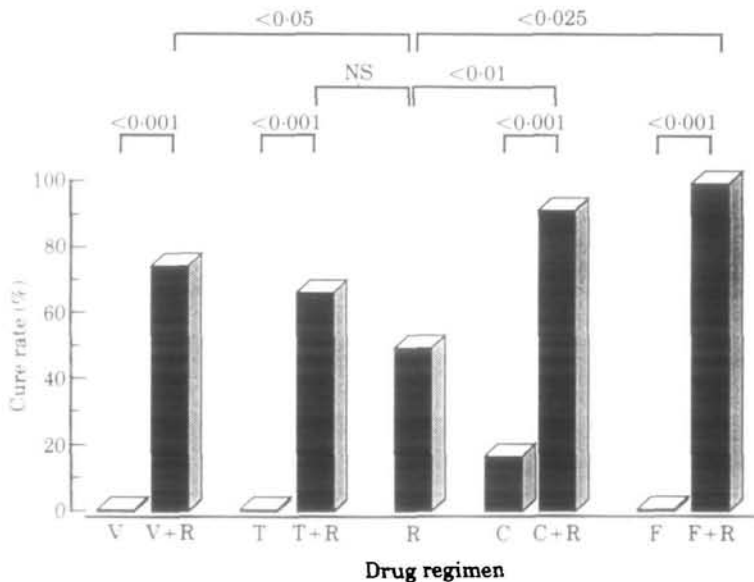


Figure. Cure rate of tissue-cage infections with *S. aureus* ATCC 29213. Guinea pigs with tissue-cage implants were treated with antibiotics as indicated on the x-axis. V, Vancomycin; R, rifampicin; T, teicoplanin; C, ciprofloxacin; F, fleroxacin. Number of infections: 12 each, except rifampicin 20, and fleroxacin alone and in combination eight.

Table I. Drug levels in tissue cage fluid and MICs of *S. aureus* ATCC 29213

Drug (mg/kg/day) ^a	Tissue cage level ^b		MIC (mg/L)	Ratio of trough level to MIC
	peak (mg/L)	trough (mg/L)		
Vancomycin (15)	8.6	2.8	1.09	2.6
Teicoplanin (6.6)	9.6	7.7	0.63	12.2
Ciprofloxacin (10)	0.95	0.11	0.44	0.25
Fleroxacin (10)	3.1	0.23	0.69	0.33
Rifampicin (25)	8.3	1.88	0.07	26.9

^aDoses were given intraperitoneally twice daily.

^bPublished results from Widmer *et al.* (1990a, 1991)

Drug levels, MICs and MBCs

For each antimicrobial agent, a dosage was chosen which resulted in peak serum levels near those suggested by the manufacturer (in humans). Table I summarizes antibiotic concentrations in tissue-cage-fluid and MICs of antibiotics for the test strain. With three (rifampicin, teicoplanin and vancomycin) out of five antibiotics, antibiotic concentrations at the site of infection exceeded the MIC throughout the whole 4-day treatment period. However, comparison of these data with cure rates (Figure) was not useful in predicting treatment success. We therefore looked for microbiological tests which would better predict drug efficacy in the device-related infection model. MBCs of antibiotics for *S. aureus* in stationary phase were 7.7–167-fold higher than in logarithmic phase (Table II). Rifampicin was the only antibiotic which achieved higher levels in tissue cage fluid than the stationary phase MBC.

Killing of adherent *S. aureus*

At tissue-cage-fluid peak concentrations, each antibiotic killed adherent bacteria well *in vitro* (Table III). However, at trough concentrations, four out of five antibiotics did not kill glass-adherent bacteria. Only rifampicin and its combinations killed adherent bacteria. The coefficient of correlation between antibiotic killing *in vitro* and *in vivo* was $r^2 = 0.851$.

Table II. Minimum bactericidal concentration (MBC) of *S. aureus* ATCC 29213 in different growth phases

Antibiotic	Phases of bacterial growth		Fold increase
	logarithmic ^a (mg/L)	stationary ^b (mg/L)	
Vancomycin	3.4	263	77.4
Teicoplanin	1.9	94	49.5
Ciprofloxacin	0.75	125	167
Fleroxacin	5.0	333	66.6
Rifampicin	0.44	3.4	7.7

^aIncubation in Mueller-Hinton broth.

^bIncubation in phosphate buffered saline supplemented with 1% glucose and 4% Mueller-Hinton broth.

Table III. Log killing of glass-bead adherent *Staphylococcus aureus* ATCC 22913 at tissue-cage-fluid peak and trough level of the indicated antibiotics

Antibiotic	Log killing ^a at tissue-cage-fluid	
	peak level ^b mean \pm S.D.	trough level ^b mean \pm S.D.
Vancomycin (V)	3.13 \pm 0.56	-0.23 \pm 1.77
Teicoplanin (T)	4.10 \pm 0.98	-1.20 \pm 1.59
Ciprofloxacin (C)	3.66 \pm 1.55	-0.36 \pm 1.62
Fleroxacin (F)	3.28 \pm 0.75	-2.59 \pm 0.01
Rifampicin (R)	2.62 \pm 0.75	2.02 \pm 0.48
V + R	1.93 \pm 0.33	1.86 \pm 0.34
T + R	3.02 \pm 0.31	2.71 \pm 0.23
C + R	3.26 \pm 0.54	2.58 \pm 0.31
F + R	3.09 \pm 0.90	2.02 \pm 0.48

^aLog killing was calculated as follows: Log cfu (geometric mean) of inoculum - log cfu (geometric mean) of antibiotic-incubated beads. Results are the geometric means of at least three different experiments. A positive value means 'killing'; a negative value indicates growth.

^bSee Table I

Chequerboard test

All four combination regimens were indifferent in this assay with FIC-indices of 1.3 of vancomycin/rifampicin, 0.69 for teicoplanin/rifampicin, 0.75 for ciprofloxacin/rifampicin, and 0.63 for fleroxacin/rifampicin. Therefore, the synergistic effect *in vivo* was not predictable by the results of the chequerboard assay.

Discussion

According to clinical observations, routine antimicrobial susceptibility tests poorly predict treatment outcome of bacterial infections (Greenwood, 1981; Zak, Tosch & Sande, 1985; Drusano, 1988). In device-related infections, the correlation is especially poor (Widmer *et al.*, 1990b, 1991), leading to the dogma that infected implants have to be removed in order to achieve cure. The main characteristics of such infections are the microbial adherence effected by the biofilm and the low growth-rate of surface adherent microorganisms (Brown *et al.*, 1988; Gilbert *et al.*, 1990). The phenomenon of bacteria-laden biofilms has been repeatedly demonstrated with electron-microscopy (Gristina & Costerton, 1985; Gristina, 1987; Widmer *et al.*, 1990b). Bactericidal activity of various antibiotics depends on bacterial growth (Hobby, Meyer & Chaffee, 1942; Tuomanen *et al.*, 1986). The discrepancy between the results of routine antibiotic susceptibility testing and treatment success in device-related infections may therefore be due to the fact that bacterial biofilms have different resistance pattern compared with planktonic bacteria (Brown *et al.*, 1988; Anwar, Dasgupta & Costerton, 1990; Gilbert *et al.*, 1990).

We have recently shown that killing of non-growing and adherent *Staphylococcus epidermidis* and *Escherichia coli* determines drug efficacy in device-related infections (Widmer *et al.*, 1990a, 1991). The antibiotic susceptibility testing of adherent bacteria described in those studies was poorly reproducible when performed with *S. aureus*. We therefore looked for an alternative assay. Vergères & Blaser (1992) described a complex method to determine bactericidal activity of antibiotics on biofilms of bacteria adhering

to glass beads. For our purposes, this assay was simplified. We used only selected antibiotic concentrations instead of the pharmacokinetic model.

In the present study, rifampicin was the only antibiotic which achieved a reasonable cure rate when given alone in the animal model. All other single antibiotic regimens failed to achieve cure, despite the fact that all isolates remained susceptible. Combination treatment with rifampicin was significantly better compared with either antibiotic alone. The combination of rifampicin with quinolones or teicoplanin prevented the emergence of rifampicin-resistant bacteria. In contrast, resistance to rifampicin was observed after single antibiotic treatment, or combination treatment with vancomycin in both this study and in a similar rat model (Lucet *et al.*, 1990). This may be due to the different volume of distribution of these two drugs (Eng *et al.*, 1985; Brumfitt & Hamilton-Miller, 1989). Rifampicin, with the best efficacy in the short-term treatment, had by far the best ratio of in-vivo trough level to MIC. In contrast, vancomycin and teicoplanin which also had an excellent ratio of 2.6 and 12.2, respectively, failed to cure experimental device-related infections. Rifampicin was the only drug with a stationary-phase MBC which could be attained at the site of infection. In addition, only rifampicin alone or in combination had a good efficacy on sinter-glass biofilms, when tested at tissue-cage trough levels. Interestingly, at high concentrations (peak level), each antibiotic alone or in combination had an excellent killing activity. In control experiments we observed that the killing of adherent bacteria was not increased at antibiotic concentrations above a critical level (data not shown). Therefore, only the results of in-vitro experiments using low antibiotic concentrations were predictive of the in-vivo superiority of rifampicin.

Rifampicin has been successfully used in experimental *S. epidermidis* foreign-body infections (Widmer *et al.*, 1990a), experimental endocarditis (Vazquez & Archer, 1980; Kobasa *et al.*, 1983), and in experimental chronic *S. aureus* osteomyelitis (Norden, 1983). This indicates that not only in device-related infections, but also in endocarditis and chronic osteomyelitis, antibiotics with good bactericidal activity against non-growing and adherent bacteria may have a therapeutic advantage. However, the clinical use of rifampicin in non-tuberculous infections remains controversial (Sande, 1983; Morris, Brown & Sands, 1993). Few clinical studies have been published on the efficacy of rifampicin combinations in staphylococcal device-related infections. We reported a cure rate of 80% in ten patients with orthopaedic implant infections treated with rifampicin combinations, where the prosthesis was left *in situ* (Widmer *et al.*, 1992). This observation was confirmed in a large study on 47 patients treated with rifampicin plus ofloxacin (Drancourt *et al.*, 1993). However, both studies were uncontrolled case series. Therefore, it is still unclear whether the successful outcome was due to standardized long-term treatment, or indeed to the specific action of rifampicin. This question is being addressed by an ongoing double-blind controlled clinical trial comparing oral ciprofloxacin and ciprofloxacin plus rifampicin treatment, after an initial 2-week course with a standard iv-regimen.

In conclusion, cure rate in experimental device-related infections can be predicted by the in-vitro bactericidal effect of antibiotics on non-growing and adherent bacteria. It may be useful to perform these tests in patients with device-related infections.

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