*Journal of Antimicrobial Chemotherapy* (2001) **48**, 793–801

JAC

# Synergy of different antibiotic combinations in biofilms of *Staphylococcus epidermidis*

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**The *in vitro* effect of nine antibiotic combinations was investigated in *Staphylococcus epidermidis* biofilms using ATP-bioluminescence for viable bacterial cell quantification. Four slime-producing (SP) strains were used to form biofilms 6, 24 and 48 h old. These biofilms were exposed for 24 h to antibiotics at 4 ×, 2 ×, 1 × and 0.5 × MIC. Combinations involving tetracycline together with another antibiotic were the most efficient at the biofilm age and concentration range under study. The combination vancomycin–rifampicin produced the highest bactericidal effect on 6 h biofilms at 4 × MIC, but this effect decreased dramatically in older biofilms. To detect possible antibiotic synergy in combinations that had a significant killing effect, antibiotics were studied not only in combination but also individually. Synergic effects were observed in all the combinations tested. Differences between the effect in combination and the sum of individual antibiotic effects (degree of synergy) were significant (mostly $P < 0.001$) and exceeded 1 $\log_{10}$ cfu/mL in the majority of cases. In 48 h biofilms, antibiotics caused a significant bactericidal effect when applied in combination, but never when used individually. These results indicate that the biofilm test applied allows the detection of synergy between antibiotics and suggests that this assay could be useful in clinical and extensive synergy studies on *S. epidermidis* biofilms.**

## Introduction

Antibiotic combinations represent a therapeutic option in the treatment of *Staphylococcus epidermidis* infections, as a result of the increasing appearance of multi-resistant microorganisms. In treatments involving antibiotics like rifampicin, combination therapy is used to avoid the appearance of antimicrobial resistance in the infectious agent.[1–5] In other treatments, combinations are used in order to enhance the effect of individual antimicrobials by means of synergic interactions. This enhancement has been very useful in clinical practice involving treatment of chronic staphylococcal infections, frequently associated with the formation of biofilms on the biomaterials used in implants or prostheses and the consequent decreased susceptibility.[6–9]

The optimal antibiotic combinations are commonly obtained from classical susceptibility tests based on diffusion and dilution (broth microdilution test), as described by the NCCLS,[10] but they do not involve biofilm studies. Therefore, these tests yield useful information on antibiotic resistance in individual bacterial cells, but additional testing is required to determine the relative performance of different antibiotics when bacteria are forming biofilms. Various microorganisms, including staphylococci,[11–15] have been studied in biofilm susceptibility assays to determine the viable cell quantification by a variety of methods.[16–18] Some of them allow automation[15,16,19] or use continuous flow devices for biofilm formation.[20–23] However, most of them present problems in the feasibility of multiple comparison studies and in reproduction of medium composition, flow speed, oxygen availability, free radical formation and immune mediator activities of the heterogeneous conditions found in *in vivo* infections at different body sites.

Furthermore, the diversity of methodologies applied in

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combination or synergy work leads to difficulties in comparing the results obtained in different studies. Difficulties include the use of different exposure periods and antibiotic concentrations,[9,24] bacteria sometimes in suspension[7,25] or adherent,[26] isolates with different degrees of adherence, different biofilm support surfaces, biofilms of different age and developed with different growth media. There is a need for a test that, like the classical tests[10] (diffusion and dilution), would facilitate comparisons.

The purpose of this work was to study nine commonly applied antibiotic combinations in *S. epidermidis* biofilms, using *in vitro* ATP-bioluminescence.[15] Differences in efficacy were determined as a function of the antibiotic combinations, antibiotic concentrations, isolates, age of the biofilm and exposure periods applied. The synergy that may exist between the antibiotics involved in each combination was analysed.

## Materials and methods

### *Organisms*

*S. epidermidis* isolate ATCC 12228 and three clinical isolates (strains 12, 19 and 60) obtained from patients with staphylococcal infections at the University Clinics of Navarra, were used. All of them were poor slime producers, as they formed smooth colonies when grown in Congo Red agar.[27] For this reason, before carrying out biofilm studies, bacteria were subcultured for enhancement of adherence and biofilm formation, as described previously for *Staphylococcus aureus*.[28] Briefly, bacteria were cultured in tryptone–soy broth (TSB)–2% glucose (Difco, Detroit, MI, USA). Culture tubes contained 4 mg/mL of microcarrier beads (150–210 μm diameter; Sigma, St Louis, MO, USA) to which bacteria adhered. After various empty–refill cycles, selecting for bacteria adhered to beads and discarding the supernatant, all the bacteria on the beads had a rough colony morphology in Congo Red agar, commonly associated with high slime production. At this stage, bacteria had an increased adherence and ability to produce biofilms[19] and were then used in antibiotic susceptibility tests involving biofilm formation. Ribotyping and pulsed-field gel electrophoresis (PFGE) were applied in order to discard the possibility of bacterial contamination throughout the subculturing process. Ribotyping was performed according to a procedure described previously.[29] PFGE was performed on a Chef-DR II (Bio-Rad, Hercules, CA, USA) and DNA extraction from agarose gels was carried out according to the manufacturer's instructions (Bio-Rad-GenePath Group1 Reagent Kit).

### *Biofilm formation*

The procedure was carried out as described previously in *S. aureus*.[15] Briefly, bacteria were grown overnight in TSB at 37°C. Aliquots (25 μL) from each culture (18–24 h growth in TSB) were added to wells of a 96-well microtitre plate (tissue culture treated, flat bottom plates; Corning, New York, NY, USA). Wells were filled with 175 μL of TSB–2% glucose. Bacteria were allowed to form biofilms (at 37°C) for 6, 24 or 48 h. In 24 or 48 h biofilms, the growth medium was discarded and fresh medium added every 12 h. At the end of the incubation period, a biofilm with clear boundaries had been formed.

### *Calibration curve*

In order to quantify biofilm bacteria by ATP-bioluminescence, a calibration curve[30] (bacterial ATP versus cfu/mL of sample) was produced for *S. epidermidis* before carrying out the antibiotic studies in biofilms. This curve allowed the conversion of moles of ATP obtained by bioluminescence to conventional cfu in this species. In this calibration study, a linear relationship was found between the amount of bacterial ATP detected and the number of bacteria (cfu/mL) in the interval between $2.2 \times 10^5$ cfu/mL and $3.6 \times 10^{10}$ cfu/mL, a high correlation being observed between both variables ($r = 0.99$). For this reason, killing results within this interval are provided in cfu/mL.

### *Antibiotic susceptibility by classical methods*

Antibiotic susceptibility (resistance profile) was first determined for cells in suspension, by two classical methods: diffusion and broth microdilution.[10] Six antibiotics were used in this experiment to determine MICs and MBCs by broth microdilution (Table 1): cefalothin, tetracycline, rifampicin, vancomycin, clindamycin (Sigma) and trimethroprim–sulfamethoxazole (Almirall, Barcelona, Spain). When the MIC differed between isolates, the highest value obtained was always chosen for biofilm studies.

### *Susceptibility assay for biofilm bacteria*

Biofilms were washed with distilled water in order to discard unbound bacteria. Subsequently, 50 μL of each antibiotic involved in the combination, together with 100 μL of Mueller–Hinton broth, were added per well to the desired antibiotic concentration. After incubation at 37°C for 24 h, the culture medium was removed and ATP-bioluminescence was used to quantify viable bacteria within the biofilm, as described previously.[15] Tests were carried out in triplicate and on four dates. A control (untreated) group using Mueller–Hinton broth without antibiotic was included in all cases.

*Antibiotic combination study in biofilm assays.* Antibiotics were diluted in Mueller–Hinton broth at supra-inhibitory ($4 \times$ MIC and $2 \times$ MIC), inhibitory (MIC) and sub-inhibitory ($0.5 \times$ MIC) concentrations. After dilution, antibiotics were filter-sterilized (pore diameter 0.22 μm; Millipore, Hertfordshire, UK) and stored at 4°C before use

**Table 1.** MIC and MBC (mg/L) of six antibiotics determined by the broth microdilution method,[10] using cell suspensions of four *S. epidermidis* isolates

| | Isolate 12 | | Isolate 19 | | Isolate 60 | | ATCC 12228 | |
|---|---|---|---|---|---|---|---|---|
| Antibiotic | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| Cefalothin | 0.5 | 4 | 2 | 4 | 1 | 4 | 1 | 1 |
| Clindamycin | R | | 0.5 | 2 | 0.5 | 2 | 0.5 | 16 |
| Rifampicin | 0.5 | 0.5 | r | | R | | 0.5 | 0.5 |
| Tetracycline | R | | 2 | 8 | 1 | 8 | 0.5 | 32 |
| SXT | 0.4/2 | 1.6/8 | r | | R | | 0.2/1 | 0.4/2 |
| Vancomycin | 4 | 4 | 4 | 8 | 4 | 4 | 2 | 4 |

SXT, trimethroprim–sulfamethoxazole; R, resistant in broth microdilution and diffusion tests;[10] r, resistant in broth microdilution test, but susceptible in diffusion test.[10] R or r isolates were not included in the biofilm study of that antibiotic.

in the biofilm test. The study was carried out in decreasing order of concentration; those concentrations below the one that did not have a significant effect on biofilm bacteria were omitted from the study.

*Synergy study*. Synergy was studied in biofilms of 6 and 48 h. Only combinations and concentrations producing in the biofilm assay a significant decrease in viable biofilm bacteria with respect to the cfu present in untreated controls ($\Delta$ value $> 0.5$ $\log_{10}$ cfu/mL or 0.4 $\log_{10}$ cfu/mL for 6 and 48 h biofilms, respectively) were included in the synergy study. The reason for lowering the $\Delta$ threshold value to 0.4 $\log_{10}$ cfu/mL in 48 h biofilms was the scarcity of values $>0.5$ $\log_{10}$ cfu/mL in biofilms of this age. Antibiotics were also studied individually at the concentrations included in this synergy analysis in order to determine whether the combination was synergic (i.e. whether the bactericidal effect of the combination treatment was significantly higher, $P < 0.05$, than the sum of effects of individual antibiotic treatments).[9,31]

*Exposure period study*. Only combinations with significant $\Delta \geqslant 0.4$ $\log_{10}$ cfu/mL in 24 h biofilms were included in this study. The exposure period was also reduced from 24 h to 6 and 3 h, while keeping the age of the biofilms (24 h) and the antibiotic concentrations (4 × MIC and MIC) constant.

### *Statistical analysis*

In the antibiotic combination studies, all the comparisons were individually carried out per isolate on the basis of the $\Delta$ values obtained. Analysis of variance was used to study the effect of different antibiotics, antibiotic concentrations and biofilm ages on biofilm cells, using the Statview program for Macintosh (Scheffe's *F*). In the synergy study, a one-tailed Student's *t*-test was used to analyse differences ($P < 0.05$) in $\Delta$ values when comparing effects of antibiotic combinations with the sum of individualized antibiotic effects (in this study, the variance of the sum was calculated as the sum of the individual variances). Satterthwhaite's correction was applied to the Student's *t*-test values when there were significant differences (according to Snedecor's *F*-test) between the variances of the compared means.

## Results

Only *S. epidermidis* strains susceptible to antibiotics in classical tests (Table 1) were used in the biofilm studies. Biofilm age and antibiotic concentration affected the efficacy of the antibiotic treatment in biofilms (Table 2). Specifically, the effect ($\Delta$ value) of the antibiotic combination increased as the antibiotic concentration increased and the age of the biofilm decreased (especially from either 24 or 48 h to 6 h).

Interestingly, the three treatments involving the use of tetracycline in combination with another antibiotic resulted in significant killing even when low antibiotic concentrations ($<4 \times$ MIC) and old (24 or 48 h) biofilms were used.

The strongest effect was observed when studying the combination vancomycin–rifampicin at 4 × MIC on 6 h biofilms, but this effect decreased dramatically in older biofilms, and was negligible at 2 × MIC (Table 2). A similar age-related decrease was observed in the combinations cefalothin–rifampicin and cefalothin–vancomycin.

The differences in susceptibility observed between isolates, ATCC 12228 having a generalized resistance in biofilms, disappeared as the age of the biofilm increased and the antibiotic concentration decreased (Table 2). The existence of isolate differences encouraged the independent analysis of data on individual isolates when studying synergy between antibiotics.

**Table 2.** Study of *S. epidermidis* isolates showing the effect of 24 h antibiotic treatments on viability decrease in biofilms (Δ for differences between control and treated samples in $\log_{10}$ cfu/mL)[a]

| | | 4 × MIC[b] | | | | 2 × MIC | | | | MIC | | | | 0.5 × MIC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Δ in biofilms of | | | | Δ in biofilms of | | | | Δ in biofilms of | | | | Δ in biofilms of | | |
| Combination | Strain | mg/L[c] | 6 h | 24 h | 48 h | mg/L[c] | 6 h | 24 h | 48 h | mg/L[c] | 6 h | 24 h | 48 h | mg/L[c] | 6 h | 24 h | 48 h |
| Cefalothin–rifampicin | 12 | 8/2 | −1.7* | −0.33* | −0.2* | 4/1 | −0.8* | 0.01 | −0.18 | | | | | | | | |
| | ATCC | | −1.2* | NT | −0.22* | | −0.66* | −0.34* | 0.01 | | | | | | | | |
| Cefalothin–vancomycin | 12 | 8/16 | −1.8* | −0.08 | 0.01 | | | | | | | | | | | | |
| | ATCC | | −0.1 | −0.01 | 0.04 | | | | | | | | | | | | |
| Clindamycin–rifampicin | ATCC | 2/2 | 0.15 | 0.14 | 0.13 | | | | | | | | | | | | |
| Clindamycin–tetracycline | 19 | 2/8 | −1.41* | −0.5* | −0.2* | 1/4 | −1.06* | −0.6* | −0.46* | 0.5/2 | −0.75* | −0.19 | −0.13 | 0.25/1 | −0.23* | 0.10 | 0.28 |
| | 60 | | −1.11* | −0.54* | −0.18* | | −0.66* | −0.61* | −0.24* | | −0.35* | 0.08 | −0.02 | | −0.14 | −0.19 | 0.21 |
| | ATCC | | −0.88* | −0.38* | 0.03 | | −0.57* | −0.41* | NT | | −0.43* | −0.25 | 0.06 | | −0.08 | 0.26 | 0.17 |
| Clindamycin–vancomycin | 19 | 2/16 | −0.27* | 0.16 | 0.01 | | | | | | | | | | | | |
| | 60 | | −0.38* | −0.03 | −0.15 | | | | | | | | | | | | |
| | ATCC | | −0.07 | 0.01 | −0.06 | | | | | | | | | | | | |
| Tetracycline–rifampicin | ATCC | 8/2 | −0.9* | −0.58* | −0.48* | 4/1 | −0.83* | −0.63* | −0.05 | 2/0.5 | −0.86* | −0.45* | 0.08 | 1/0.25 | −0.3* | 0.13 | 0.18 |
| Tetracycline–vancomycin | 19 | 8/16 | −1.19* | −0.62* | −0.77* | 4/8 | −0.76* | −0.39* | −0.34* | 2/4 | −0.61* | −0.64* | −0.48* | 1/2 | −0.35* | 0.09 | 0.19 |
| | 60 | | −1.23* | −0.76* | −0.75* | | −0.69* | −0.52* | −0.36* | | −0.77* | −0.56* | −0.2* | | −0.17* | −0.2* | 0.18 |
| | ATCC | | −0.76* | −0.47* | −0.52* | | −0.38* | −0.39* | −0.3* | | NT | −0.41* | −0.22* | | −0.06 | 0.21 | 0.24 |
| SXT–rifampicin | 12 | 1.6–8/2 | −1.34* | −0.61* | −0.41* | 0.8–4/1 | −0.08 | −0.05 | −0.19 | | | | | | | | |
| | ATCC | | −0.67* | −0.38* | −0.46* | | −0.54* | −0.04 | −0.01 | | | | | | | | |
| Vancomycin–rifampicin | 12 | 16/2 | −2.19* | −0.54* | −0.39* | 8/1 | −0.39* | −0.02 | −0.13 | | | | | | | | |
| | ATCC | | −1.25* | −0.22* | −0.22* | | −0.24* | −0.15* | −0.02 | | | | | | | | |

SXT, trimethroprim–sulfamethoxazole. NT, not tested.

[a]Analyses involve three biofilm ages and four antibiotic concentrations. When significant ($P < 0.05$) Δ values (*) were observed in 24 h biofilms (or in 6 h biofilms in the case of the combination clindamycin–tetracycline), the study was extended to a lower concentration until reaching 0.5 × MIC.

[b]MIC obtained by microdilution.[10] Values correspond to the strain with the highest MIC.

[c]Values obtained for each antibiotic are in the order given in the first column (the value for the first antibiotic is on the left and that for the second on the right).

Synergy (Tables 3 and 4) was observed for all the isolates and all the combinations analysed. In 6 h biofilms, the difference attributed to synergy (in Δ values; Table 3), reached a significance level of $P < 0.001$, except in the case of the combination tetracycline–vancomycin, where the significance levels decreased ($P < 0.05$ and $P < 0.01$ at 4 × MIC and at 2 × MIC in biofilms of isolates 19 and 60, respectively). When 6 h biofilms were exposed to 4 × MIC, the difference attributed to synergy reached 1.65 $\log_{10}$ cfu/mL (in isolate 12 for the combination vancomycin–rifampicin) and in the majority of cases was ⩾1 $\log_{10}$ cfu/mL. This difference decreased to ⩽0.31 $\log_{10}$ cfu/mL in the combination tetracycline–vancomycin (isolates 19 and 60), probably because the sum of the individual antibiotic effects was already high. When 6 h biofilms were exposed to lower antibiotic concentrations (<4 × MIC), synergy was still observed in some isolates, even at the MIC.

When increasing the biofilm age to 48 h, few antibiotic combinations were able to produce a significant bactericidal effect >0.4 $\log_{10}$ cfu/mL (Table 2) among the isolates tested. However, all of them produced synergic effects in all cases (Table 4). In contrast, none of the individual antibiotics studied produced a significant effect.

Results on the effects of antibiotic combinations as a function of the exposure period applied are illustrated in the Figure. When the exposure period was <24 h, the bactericidal effect was <0.4 $\log_{10}$ cfu/mL even at 4 × MIC. However, in a few cases (combinations tetracycline–clindamycin and tetracycline–vancomycin), a low but significant effect was reached by 3 h. A low antibiotic concentration (MIC) had no significant effect when applied for short (<24 h) exposure periods.

## Discussion

Differences in the efficacy of combinations and in the susceptibility between isolates when they are studied in

**Table 3.** Decrease (Δ) in $\log_{10}$ cfu/mL observed in 6 h biofilms upon individual and combined 24 h exposure antibiotic treatments, according to the strain[a] and concentration used

| Antibiotic | Antibiotic concentration | Strain | Sum of Δ values using individual antibiotics | Δ value using the combination[b] |
|---|---|---|---|---|
| Cefalothin–rifampicin | 4 × MIC | 12 | 0.31 | 1.7*** |
| | 4 × MIC | ATCC | 0.15 | 1.2*** |
| Clindamycin–tetracycline | 4 × MIC | 19 | 0.28 | 1.41*** |
| | 4 × MIC | 60 | 0.3 | 1.11*** |
| | 4 × MIC | ATCC | 0.04 | 0.88*** |
| | 2 × MIC | 19 | 0.08 | 1.06*** |
| | 2 × MIC | 60 | 0.06 | 0.66*** |
| | 2 × MIC | ATCC | 0.04 | 0.57*** |
| | MIC | 19 | 0.04 | 0.75*** |
| Tetracycline–vancomycin | 4 × MIC | 19 | 0.88 | 1.19* |
| | 4 × MIC | 60 | 0.95 | 1.23* |
| | 4 × MIC | ATCC | 0.04 | 0.76*** |
| | 2 × MIC | 19 | 0.53 | 0.76** |
| | 2 × MIC | 60 | 0.44 | 0.69** |
| | MIC | 19 | 0.04 | 0.61*** |
| | MIC | 60 | 0.22 | 0.77*** |
| SXT–rifampicin | 4 × MIC | 12 | 0.13 | 1.34*** |
| | 4 × MIC | ATCC | 0.07 | 0.67*** |
| | 2 × MIC | ATCC | 0.08 | 0.54*** |
| Vancomycin–rifampicin | 4 × MIC | 12 | 0.54 | 2.19*** |
| | 4 × MIC | ATCC | 0.06 | 1.25*** |
| Cefalothin–vancomycin | 4 × MIC | 12 | 0.63 | 1.8*** |
| Tetracycline–rifampicin | 4 × MIC | ATCC | 0.06 | 0.9*** |
| | 2 × MIC | ATCC | 0.04 | 0.83*** |
| | MIC | ATCC | 0.04 | 0.86*** |

SXT, trimethroprim–sulfamethoxazole.

[a]ATCC = ATCC 12228.

[b]*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, for the difference between the Δ value of the combination and the sum Δ values of the corresponding individual antibiotics.

**Table 4.** Decrease (Δ) in $\log_{10}$ cfu/mL observed in 48 h biofilms upon individual and combined 24 h exposure antibiotic treatments, according to the strain and concentration used

| Antibiotic | Antibiotic concentration | Strain | Sum of Δ values using individual antibiotics | Δ value using the combination |
|---|---|---|---|---|
| Tetracycline–rifampicin | 4 × MIC | ATCC | 0.02 | 0.48*** |
| Tetracycline–vancomycin | 4 × MIC | 19 | 0.02 | 0.77*** |
| | 4 × MIC | 60 | 0.02 | 0.75*** |
| | 4 × MIC | ATCC | 0.02 | 0.52*** |
| | 2 × MIC | 19 | 0.02 | 0.34*** |
| | MIC | 19 | 0.02 | 0.48*** |
| SXT–rifampicin | 4 × MIC | 12 | 0.02 | 0.41*** |
| | 4 × MIC | ATCC | 0.02 | 0.46*** |

SXT, trimethroprim–sulfamethoxazole.
See footnotes to Table 3.

biofilms were demonstrated. The results obtained strongly suggest that particular combinations are of potential therapeutic interest and show that the test applied is of potential use in studies on antibiotic synergy involving *S. epidermidis* biofilms. The methodological trend in biofilm susceptibility testing is automation, especially for large studies in research and clinical laboratories. The use of a discontinuous flow system rather than the continuous flow facility currently available[23] allowed multiple comparisons (hundreds of test samples per test date) under sterile and automated conditions.

The increased efficacy of treatment detected when the age of the biofilm decreased and the exposure period and the antibiotic concentration increased has been described in individual[9,15,32] and combination[25,26] antibiotic studies on *S. epidermidis* and *S. aureus.* Furthermore, in agreement with previous findings,[25] it was observed that increased antibiotic concentrations allow earlier detection of significant killing. Altogether, these observations may be related to *in vivo* findings, in which chronic infections involving old biofilms are overcome by prolonging the period of sustained antibiotic levels and applying combined therapy.[24]

Synergy has been demonstrated against staphylococci, including coagulase-negative species,[6,25,33,34] and methicillin-resistant isolates.[8] The synergic effect observed in this study in the combinations of tetracycline with clindamycin, vancomycin or rifampicin allowed the detection of significant bactericidal effects, even when the concentration was decreased to 0.5 × MIC in 6 h biofilms or when applying 2 × MIC on 48 h biofilms. It is possible that the degree of effectiveness of combinations involving tetracycline is related to the evident bactericidal effect of this antibiotic already shown in the individual antibiotic study. Because its killing effect is still significant at low concentrations when used in combination, this antibiotic could potentially be considered as a candidate for future studies of *in vivo* combination therapy, especially in clinical infections involving biofilm bacteria of low accessibility. In fact, the combination minocycline (tetracycline)–rifampicin has been found to have an efficacy of 70% in a rabbit model.[5]

There are many antibiotic combination studies involving rifampicin, vancomycin and different cephalosporins that have been associated with a substantial bactericidal effect.[7,25,26] In spite of the known tendency of rifampicin to trigger the appearance of resistance, its efficacy against bacteria adhered to biomaterials has been widely demonstrated.[24,35–37] In agreement with these findings, the results obtained in this study reveal that rifampicin improves its efficacy in combination with cefalothin or tetracycline. More specifically, the combination rifampicin–cefalothin was more advantageous than the combination rifampicin–tetracycline in young (6 h) biofilms and at high concentrations (⩾4 × MIC), whereas the opposite occurred at lower concentrations in aged biofilms (⩾24 h), tetracycline being more efficient than cefalothin under these conditions. These differences suggest that different therapeutic approaches could be applied according to the stage of biofilm formation throughout the infection process. Evidence for efficacy has also been obtained when rifampicin has been combined with other antibiotics.[1,3,4,6,38] However, in this work, when combined with clindamycin (Table 2), rifampicin did not seem to produce a significant effect, as also observed by Rybak & McGrath.[31] A possible explanation for this exception could be the low antibiotic concentration chosen in this work (4 × MIC = 2 mg/L for both antibiotics).

Vancomycin, the last resource in many methicillin-resistant staphylococcal infections, has been proposed in combination for the treatment of *S. epidermidis* infections.[39–41] Although it was effective in combination with tetracycline or rifampicin, in agreement with previous studies,[26,32] in this study vancomycin did not show high bactericidal activity when combined with cefalothin and specially with clindamycin (interestingly, the combination of vanco-

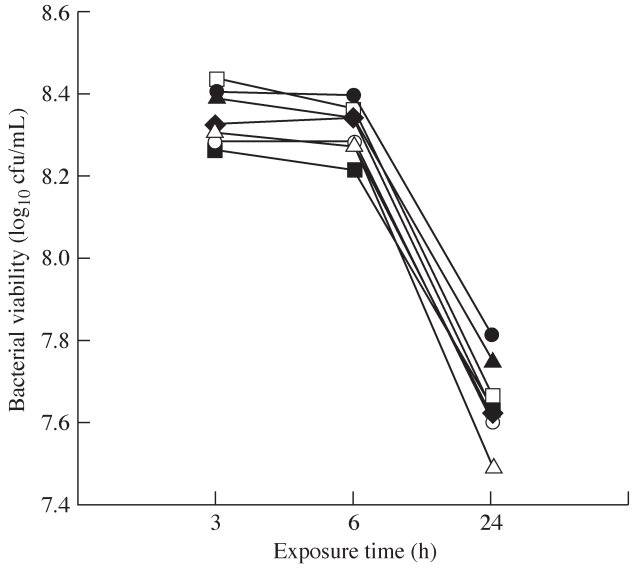


**Figure.** The effect of different antibiotic combinations on 24 h biofilms according to the exposure period and the antibiotic concentrations used: 4 × MIC was used unlesss otherwise indicated; ■, tetracycline–clindamycin; □, trimethroprim–sulfamethoxazole–rifampicin; ●, vancomycin–rifampicin; ○, tetracycline–rifampicin; ▲, tetracycline–rifampicin (MIC); △, tetracycline–vancomycin; ◆, tetracycline–vancomycin (MIC).

mycin with $\beta$-lactam antibiotics appears to be efficient for bacteria in suspension[7]). This observation jeopardizes the high expectations created for this antibiotic.[26,32] The sudden efficacy decrease of vancomycin in aged biofilms (also observed in an individual vancomycin test and in previous studies[39]) was evident for the combination vancomycin–rifampicin (as occurred in young biofilms for the combination cefalothin–rifampicin). Although similar effects have been observed previously for this combination against *S. epidermidis* in suspension when comparing vancomycin–rifampicin versus rifampicin–$\beta$-lactam antibiotics,[6] in some clinical studies[42,43] the first combination (vancomycin–rifampicin) has been shown to be more efficient.

The inefficacy of antibiotics such as vancomycin with increased age of the biofilm may be due to the slow growth of biofilm bacteria, which may render the microorganism less susceptible to antibiotic attack.[38,44] It may also be due to the difficulty of antibiotic penetration to the inner biofilm layers.[39] Although vancomycin is inefficient in aged biofilms, it may be complemented by rifampicin, leading to a better performance of the combination in clinical studies.[32] Synergy involving both antibiotics may be due to several reasons: growth is required for the killing activity of vancomycin but not of rifampicin.[9] Vancomycin is of high molecular weight (1485 Da), highly soluble in water and appears to accumulate in the biofilm, but it may not reach or affect the deep biofilm bacteria. In contrast, rifampicin, with a lower molecular weight (about one-quarter that of vancomycin) and only very slightly soluble in water, does not accumulate in the biofilm; rather, it appears to diffuse and kill the bacterial cell.

The differences observed between isolates in this work (isolate ATCC 12228 showed a lower susceptibility than the other isolates), suggest the need for an individual study of the isolate responsible when aiming at eradicating a particular infection. However, the results obtained in this work also indicate that the general tendency of a particular antibiotic combination when tested against biofilm bacteria can be determined by studying data on a group of isolates, as in previous studies on *S. aureus* using individual antibiotics.[15] A question could be proposed as to whether the procedures used in this work to enrich adherent cells may artificially lead to biofilm formation. Fortunately, clinical isolates may form biofilms without this previous enrichment process (M. Monzón *et al.*, unpublished). Subculturing further increases the difficulty of the antibiotic in killing biofilm bacteria. This has implications for clinical practice and also for time considerations when treatment of a particular patient may not be able to wait for results of susceptibility testing of isolates until several rounds of enrichment have been performed in the laboratory.

Although the synergy observed in this work has not been verified *in vivo*, the concordance of *in vitro* and *in vivo* findings observed in *S. aureus*[15,45] using this biofilm test methodology is encouraging. This concordance suggests that the test could be useful in clinical practice and extensive studies on synergy, especially needed to eradicate the increasingly frequent chronic staphylococcal infections and in particular those that develop on implants and prostheses.

## Acknowledgements

Funded by FIS (96/0230), CONSID (PCM 1694) and Echebano Foundation (337/97), and CICYT (Bio 99-0285).

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*Received 2 January 2001; returned 22 June 2001; revised 31 July 2001; accepted 3 September 2001*