Journal of Antimicrobial Chemotherapy (1999) **44**, 43–55

JAC

Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*

Beatriz Amorena[a]*, Elena Gracia[a]†, Marta Monzón[a], José Leiva[b], Concepción Oteiza[b], Marta Pérez[a], José-Luis Alabart[a] and José Hernández-Yago[c]

[a]CSIC Department of Animal Health and Production, Agricultural Research Service (SIA-DGA), PO Box 727, 50080 Zaragoza; [b]Department of Microbiology, University Clinics, 31080 Pamplona; [c]Cytology Research Institute, Amadeo de Saboya,4, 46010 Valencia, Spain

Four slime-producing isolates of *Staphylococcus aureus* were used in an antibiotic susceptibility assay for biofilms developed on 96-well polystyrene tissue culture plates. The study involved 11 antibiotics, two biofilm ages (6 and 48 h), two biofilm growth media (tryptone soy broth (TSB) and delipidated milk) and three antibiotic concentrations (4 × MBC, 100 mg/L and 500 mg/L). ATP-bioluminescence was used for automated bacterial viability determination after a 24 h exposure to antibiotics, to avoid biofilm handling. Under the conditions applied, viability in untreated biofilms (controls) was lower when biofilm growth was attempted in milk rather than in TSB. Various antibiotics had a greater effect on viability when used at higher (≥100 mg/L) antibiotic concentrations and on younger (6 h) biofilms. Increased antibiotic effect was observed in milk-grown rather than TSB-grown biofilms. Phosphomycin and cefuroxime, followed by rifampicin, cefazolin, novobiocin, vancomycin, penicillin, ciprofloxacin and tobramycin significantly affected biofilm cell viability at least under some of the conditions tested. Gentamicin and erythromycin had a non-significant effect on cell viability. Transmission electron microscopy revealed that cells at the inner biofilm layers tend to remain intact after antibiotic treatment and that TSB-grown biofilms favoured a uniformity of cell distribution and increased cell density in comparison with milk-grown biofilms. A reduced matrix distribution and enhanced cell density were observed as the biofilm aged. The *S. aureus* biofilm test discriminated antibiotics requiring shorter (3 h or 6 h) from those requiring longer (24 h) exposure and yielded results which may be complementary to those obtained by conventional tests.

Introduction

The difficulty in eradicating a chronic infection associated with microcolony and biofilm formation lies in the fact that biofilm bacteria are able to resist higher antibiotic concentrations than bacteria in suspension.[1,2] To control a chronic infection, antibiotics are chosen on the basis of conventional in-vitro diffusion and dilution evaluation methods,[3] not involving biofilm formation.

With the aim of determining the antibiotic susceptibility of biofilm bacteria, efforts have been made using animal models, involving an in-vivo formation of microcolonies and biofilms.[4,5] Animal models may shed light on the evolution of infection and antibiotic pharmacodynamics, but they require the killing of animals, are expensive and time-consuming, and allow the study of only a restricted number of antibiotics and therapy schemes. For these reasons, various models have been designed using biofilms developed *in vitro*.[6–11] These models require handling of individual samples, with cell viability being mainly determined by colony plate count (cfu). Viability can also be determined by flow cytometry,[12] radiochemistry[13] and luminometry.[14–16] The last approach, based on bacterial ATP quantification,[17] has been applied to evaluate antibiotic

*Corresponding author. Department of Animal Health, Agricultural Research Service (SIA-DGA), Montañana Road, 176, 50016, Zaragoza, Spain. Tel: +32-976-57-63-36; Fax: +32-976-57-55-01; E-mail: beatriz@mizar.csic.es

†Present address: Exopol S.L., Polígono Río Gállego, Calle D, Parc. 8; S. Mateo de Gállego, 50840 Zaragoza, Spain.

susceptibility and cell viability of bacteria difficult to grow, such as *Mycobacterium* spp.[18] and *Mycoplasma* spp.[19]

Using *Staphylococcus aureus* isolates susceptible to at least 11 antibiotics in the microbroth dilution susceptibility test, an antibiotic susceptibility assay for biofilm bacteria was developed in this work using slime-producing (SP) isolates, live cells being automatically quantified by ATP-bioluminescence. In this study, an effect of the biofilm (its age and growth medium) and the antibiotic (its type, concentration and exposure period) on the antibiotic killing ability is demonstrated using biofilm bacteria.

Materials and methods

Organisms

Four *S. aureus* mastitis isolates were used: 72, 80, 510 and 9213. Two variants were available from each isolate: 72–, 80–, 510– and 9213– (original isolates, all non slime-producing, NSP) and 72+, 80+, 510+ and 9213+ (the SP isolate variant produced in the laboratory from each original NSP isolate). All four SP isolates were used for biofilm formation and subsequent antibiotic study, except in the time-killing (exposure period) susceptibility study, where only isolates 510+ and 9213+ were used. Isolate 510+ was eliminated from all the penicillin studies because it was already resistant to this antibiotic in the microbroth dilution test (MIC = 16). SP variants were produced in our laboratory from the NSP isolates (grown in tryptone soy broth containing 2% glucose; TSB 2%) by selecting for adherent colonies, as previously described.[20] SP isolate variants were distinguished from NSP isolate variants by the ability of SP bacteria to adhere more to different surfaces (plastic, glass, metals, bone, gelatine beads, etc.) as compared with NSP bacteria.[7,20–22] They were also distinguished by the colony morphology in Congo red agar,[21,23] the former having a rough type and the latter a smooth type of colony morphology. The Congo red agar colony morphology was verified in each biofilm test, to confirm the absence of SP to NSP reversions, which occurred at a frequency of 0.5×10^{-4} to 1×10^{-4}.[20] The identity of ribotypes of each NSP isolate and the SP isolate variant was verified using Eco RI as restriction enzyme.[24]

Antibiotics

Eleven antibiotics were chosen for the study according to their common use in research, human medicine and veterinary practice. They belonged to the following groups: penicillins (penicillin G), cephalosporins (cefazolin and cefuroxime), aminoglycosides (gentamicin and tobramycin), macrolides (erythromycin), quinolones (ciprofloxacin), rifampicins (rifampicin), glycopeptides (vancomycin) and miscellaneous (phosphomycin, of epoxidic nature, and novobiocin, of acidic nature). Ciprofloxacin was from Bayer (Barcelona, Spain) and the remaining antibiotics were from Sigma (St Louis, MO, USA). A 0.22 μm filter-sterilized stock solution was prepared wih antibiotic at 2000 μg/mL. MICs and MBCs were obtained for the SP and the NSP variant of each isolate, using the microbroth dilution method for bacterial suspensions.[3] MBC was defined as a 99.99% reduction of cell viability with respect to that of the initial inoculum. To facilitate antibiotic comparisons between the biofilm and the microbroth dilution assays, antibiotics were diluted in all cases in the standard Mueller–Hinton medium, which is recommended for the latter assay.[3] This avoided possible side effects caused by interactions of the various media with the antibiotic or with the ATP-bioluminescence reaction.

Using biofilm assays, an antibiotic concentration study was carried out, involving 11 antibiotics with a 24 h exposure and biofilms of 6 h and 48 h of age. A study on only three concentrations was carried out, to facilitate the detection of some major differences between antibiotics (the minimal concentration required to detect significant killing in biofilms by each particular antibiotic was not determined). The concentrations used represented a wide concentration range: 4 × MBC (calculated from the MBC of the isolate with the highest MBC among the four isolates tested; this concentration purposefully exceeded the MBC value obtained in the microbroth dilution assay, knowing the resistance of biofilm bacteria to killing, observed at 1 × MBC in preliminary experiments and in previous work[25]); 100 mg/L (a high concentration exceeding 4 × MBC in most cases and also used in other biofilm studies[25]); and 500 mg/L (applied to seven antibiotics, to determine whether the killing of biofilm bacteria reached a plateau when it became significant at high concentrations such as 100 mg/L or continued to increase when the antibiotic concentration was further increased). The use of these high concentrations, exceeding the peak values expected in serum during therapy, was a tool to show the inherent inefficiency of particular antibiotics against biofilm cells.

To investigate whether the biofilm test was sensitive enough to distinguish antibiotics requiring shorter (≤6 h) from those requiring longer (≥24 h) exposure, the effect of seven antibiotics (penicillin G, cefazolin, cefuroxime, tobramycin, rifampicin, vancomycin and novobiocin) was determined by decreasing the exposure period (from 24 h to 6 h or 3 h), while keeping biofilm age (24 h) and antibiotic concentration (100 mg/L) constant. This concentration was over 12 × MBC in all cases.

Adherent biofilm formation for antibiotic tests in 96-well plates

To develop biofilms, 25 μL of stationary growth phase SP bacterial culture (requiring about 18 h growth at 37°C in TSB 2% and containing about 2×10^9 cells/mL) were

added under aseptic conditions to a well of a tissue culture-treated polystyrene 96-well plate (cell well tissue culture treated polystyrene plates; Corning, Rochester, NY, USA), containing 175 μL of growth medium (either TSB 2% or filter-sterilized delipidated milk). Biofilms were developed (at 37°C) for 6 or 48 h, the growth medium being discarded and freshly added every 12 h. Each well was washed three times with phosphate-buffered saline (PBS) under aseptic conditions to eliminate unbound bacteria, and 200 μL of the particular antibiotic dilution in Mueller– Hinton broth were added, the mixture being maintained at 37°C. After antibiotic exposures of 3, 6 or 24 h for time killing studies and of 24 h for antibiotic concentration studies, antibiotic solutions were discarded and wells were filled (200 μL) with undiluted dimethyl sulphoxide (DMSO; Panreac, Barcelona, Spain), which was used as ATP extractant. Plates were wrapped in plastic and placed in a sonicator bath (P-Selecta, Barcelona, Spain) for 15 min (in the case of 6 h biofilms) or 30 min (in the case of 48 h biofilms) at 40 Hz and 22–24°C to favour the disintegration of bacterial clumps (viable bacteria were not detected at this stage, as verified by plate count after transferring100 μL of the mixture to a trypticase–soy agar plate). The number of viable bacteria was estimated by measuring the amount of ATP present in the sample using ATP-bioluminescence.

ATP-bioluminescence method

For the assay, opaque well plates (from Bio-Orbit, Helsinki, Finland) were introduced into the luminometer (Luminoskan RS 1.0; Labsystems, Helsinki, Finland), each well containing 40 μL of the sample–extractant mixture.[26] A total of 150 μL of Tris–acetate buffer (0.1 M Tris–acetate and 1 mM EDTA, pH 7.75; Bio-Orbit, Turku, Finland), 25 μL of an enzymatic luciferin–luciferase system (ATP-monitoring reagent; Bio-Orbit) and 10 μL of an ATP standard solution (ATP standard; Bio-Orbit) were added, in that order. Light emission was determined after the addition of each of these compounds (thus providing data per well on buffer control, sample–extractant, and ATP reaction control). Counts were recorded as relative light units (RLU) produced as a result of ATP hydrolysis in each of these steps (the luminometer allows the RLU to ATP conversion).

A calibration curve (bacterial ATP versus cfu/mL of sample) was produced[14] before the antibiotic study to allow the conversion of ATP fmoles to conventional cfu when using *S. aureus* and DMSO as extractant. 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 3.5×10^5 cfu/mL and 3.5×10^9 cfu/mL, with a high correlation being observed between both variables ($r = 0.98$). For this reason, cell viability results within this interval are provided in cfu/well. Tests were carried out in duplicate and on five dates.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) studies were carried out for illustrative purposes, to allow the amplification of images of 6 h and 48 h biofilms grown in TSB and delipidated milk and the visualization of the effect of a 24 h treatment with either cefuroxime or tobramycin on a young (6 h) TSB-grown biofilm. Bacterial growth, biofilm formation and antibiotic application were carried out as described above for the well plate biofilm test, with the following specifications. Five hundred microlitres of stationary growth phase SP bacterial cultures of isolate 9213+ were added under aseptic conditions, together with 4.5 mL of growth medium (TSB 2% or delipidated milk, as specified) to a 60 mm tissue culture-treated Petri dish (Corning). Biofilms were formed in 6 or 48 h. After the three washes, the antibiotic (cefuroxime or tobramycin) was applied at a single concentration (500 mg/L of Mueller–Hinton broth). Controls with Mueller–Hinton broth in the absence of antibiotics were also included. After a 24 h incubation at 37°C and removal of antibiotics or medium, 2.5 mL of 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7), containing 0.05% ruthenium red, were added for TEM analysis. After 2 h at 22–24°C, the sample was washed several times with PBS. Samples were dehydrated in a sequential series of 50%, 70%, 80% and 100% ethanol (10 min each). To liberate preparations from the support material, 100% ethanol was added and biofilms were sectioned into 3 mm × 3 mm square pieces with the help of a scalpel. After discarding the ethanol and adding propylene oxide, a mild agitation followed and the sample squares were placed for inclusion in a mixture of propylene oxide and resin (Sigma, Madrid, Spain) at a proportion of 1/1 (v/v) for 12 h at 22–24°C. Samples were then transferred to 100% resin. After 2–3 days at 22–24°C, samples were placed into newly made resin for 16 h at 70°C and subsequently sectioned for TEM examination.

Statistical analysis

A three-way analysis of variance (ANOVA using the SAS procedure[27]) was applied to study the effect of antibiotic concentration and biofilm age on viability ($\log_{10}$ cfu/well; data from Tables II and III, below) and to determine the effect of exposure time on viability (data from Table IV, below), using in both studies two different biofilm growth media (TSB, Tables II and IV; and filter-sterilized delipidated milk, Tables III and IV). The significance of differences between antibiotics and their controls was assessed by linear contrasts, using the Bonferroni correction for multiple comparisons. An antibiotic or group of antibiotics was considered to have a significant bactericidal effect (killing value) when the difference (decrease) in bacterial viability ($\log_{10}$ cfu) between the treated and the control (untreated) samples was statistically significant ($P < 0.05$).

Results

MICs and MBCs for cells in suspension

The MIC and the MBC for SP versus NSP variants of each isolate obtained with the microbroth dilution method for cells in suspension were similar (differences not exceeding one dilution) and therefore a single value is provided per isolate–antibiotic combination (Table I). The isolates were susceptible to the antibiotics, with the exception of isolate 510, which was resistant to penicillin, with an MIC of 16 mg/L. Therefore, this isolate was discarded from the biofilm studies of penicillin. The 4 × MBC values (corresponding to the isolate having the highest MBC) calculated in Table I are those used in biofilm studies (Tables II and III). These 4 × MBC values exceeded the peak concentration in serum in five cases (penicillin, gentamicin, erythromycin, ciprofloxacin and vancomycin; Table I), but they were lower than 100 mg/L, except in the case of phosphomycin.

Transmission electron microscopy (TEM) of untreated biofilms

Before determining the antibiotic effect on biofilm bacteria, TEM was used to visualize and compare the effects of the growth medium on the characteristics of biofilms. It was observed that TSB-grown biofilms had a relatively uniform thickness throughout the well (Figure 1a), whereas milk-grown biofilms were associated with the presence of clumps or aggregates of different sizes, bacteria being unevenly distributed throughout an extensive biofilm matrix which contained abundant inclusion material (Figure 1b).

Antibiotic concentration study on 96-well plate biofilms after 24 h of exposure

The effect of the antibiotics on cell viability in biofilms using TSB or delipidated milk was determined by ATP-bioluminescence using the pool of data on the four SP isolates under study (Tables II and III). The biofilm test applied had a high repeatability in both cases, with non-significant differences between replicate wells, both within and between test dates.

Study on the bactericidal activity of each antibiotic at different concentrations.

There was commonly a higher degree of killing in milk-grown biofilms than in TSB-grown biofilms (Tables II and III), as a result of the higher number of significant differences and increased killing value found in the former, with a few exceptions (for example, novobiocin at 100 mg/L). The killing values in Tables II and III allowing the detection of significance ($P < 0.05$) ranged between 0.36 $\log_{10}$ cfu (see ciprofloxacin in Table II) and 2.50 $\log_{10}$ cfu (see

Table I. MIC and MBC[a] of 11 antibiotics determined by a microbroth dilution method, using cell suspensions of four *S. aureus* isolates; the expected peak serum values and the 4 × MBC values (calculated with the MBC for the isolate with the highest MBC) are provided (data are expressed in mg/L)

	Isolate 72		Isolate 80		Isolate 510		Isolate 9213		Peak value expected in serum and route used[b]	4 × MBC value used in biofilm tests[c]
Antibiotic	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC		
Cefuroxime	1	2	1	2	1	2	1	2	40 im	8
Cefazolin	0.12	0.25	0.12	0.25	0.5	4	0.5	2	64 im	16
Penicillin	0.031	1	0.016	1	16	64	1	8	16 iv	32[d]
Gentamicin	2	4	2	4	2	8	0.5	4	5–7 im	32
Tobramycin	0.064	2	0.064	0.12	0.25	2	0.25	2	4–10 im	8
Erythromycin	1	8	2	8	0.5	8	0.5	8	1 po/8 iv	32
Ciprofloxacin	0.25	4	0.25	4	0.5	8	0.25	8	2 po	32
Rifampicin	<0.016	0.01	<0.016	0.01	<0.016	0.01	<0.016	0.01	10 po	0.04
Vancomycin	0.5	4	0.5	1	0.5	1	0.5	1	4–10 im	16
Phosphomycin	16	32	64	128	64	128	8	8	ND	512
Novobiocin	0.031	4	0.016	2	0.03	2	0.031	0.12	ND	16

[a]Because the difference found in the MIC or the MBC for slime-producing (SP) vs non-slime-producing (NSP) comparisons within each particular isolate was non-significant, data are provided per isolate and represent the average of both (SP/NSP) variants of each isolate.
[b]The expected values provided by the National Committee for Clinical Laboratory Standards (1990) are indicated. Routes: iv, intravenous; im, intramuscular; po, oral administration. ND, no data found in literature.
[c]4 × MBC values used in Tables II and III.
[d]Because isolate 510 was resistant to penicillin (MIC = 16), it was removed from the analysis of penicillin in the biofilm study. Thus, the MBC for isolate 9213 was chosen to calculate the 4 × MBC value of penicillin to be used in this study (Tables II and III).

Table II. Effect of 24 h antibiotic treatments on viability decrease (Δ for differences between control and treated samples in $\log_{10}$ cfu[a]) of TSB-grown *S. aureus* biofilm cells, using three antibiotic concentrations and two biofilm ages

	4 × MBC[b]		100 mg/L		500 mg/L	
Antibiotic	6 h	48 h	6 h	48 h	6 h	48 h
Cefuroxime	0.76[c]	0.07	0.69[c]	0.60[c]	0.98[c]	0.66[c]
Cefazolin	0.49[c]	0.07	0.75[c]	0.63[c]	1.07[c]	0.82[c]
Penicillin	NT	NT	0.68[c]	0.13	0.77[c]	0.28
Gentamicin	–0.23	0.12	0.14	–0.01	NT	NT
Tobramycin	NT	NT	0.23	0.17	NT	NT
Erythromycin	–0.05	0.04	0.05	–0.04	NT	NT
Ciprofloxacin	0.05	0.12	0.36[c]	–0.13	NT	NT
Rifampicin	0.57[c]	0.03	1.58[c]	1.47[c]	2.06[c]	2.03[c]
Vancomycin	0.06	0.12	0.66[c]	0.44[c]	1.19[c]	0.71[c]
Phosphomycin	0.89[c]	0.93[c]	0.85[c]	0.66[c]	0.89[c]	0.93[c]
Novobiocin	0.08	–0.10	1.44[c]	1.74[c]	2.24[c]	2.50[c]

[a]Standard errors of these differences did not exceed 0.13 $\log_{10}$ cfu.
[b]See 4 × MBC values in Table I. Phosphomycin had a 4 × MBC value of 512 mg/L (very close to 500 mg/L) and was therefore only tested at this concentration and at 100 mg/L. This explains the identity of data shown on 4 × MBC and 500 mg/L for this antibiotic.
[c]Significant viability decrease ($P < 0.01$) found in the treated versus the untreated control samples in an analysis of variance (four SP isolates were used, except in the case of penicillin, where isolate 510+ was omitted because it was resistant to this antibiotic). NT, not tested.

Table III. Effect of 24 h antibiotic treatment on viability decrease (Δ for differences between control and treated samples in $\log_{10}$ cfu[a]) of delipidated milk-grown *S. aureus* biofilm cells, using three antibiotic concentrations and two biofilm ages

	4 × MBC		100 mg/L		500 mg/L	
Antibiotic	6 h	48 h	6 h	48 h	6 h	48 h
Cefuroxime	1.43[b]	0.39[b]	1.13[b]	0.61[b]	1.51[b]	0.74[b]
Cefazolin	1.07[b]	0.31	1.16[b]	0.51[b]	1.72[b]	0.61[b]
Penicillin	0.86[b]	0.10	0.95[b]	0.28	1.42[b]	0.36
Gentamicin	0.31	0.13	0.30	0.08	NT	NT
Tobramycin	NT	NT	0.79[b]	0.38	NT	NT
Erythromycin	0.29	–0.03	0.36	–0.05	NT	NT
Ciprofloxacin	0.92[b]	0.30	0.97[b]	0.16	NT	NT
Rifampicin	0.87[b]	0.31	1.68[b]	1.35[b]	1.91[b]	1.61[b]
Vancomycin	0.66[b]	0.03	1.78[b]	0.64[b]	1.27[b]	0.62[b]
Phosphomycin	1.48[b]	0.79[b]	1.88[b]	0.95[b]	1.48[b]	0.79[b]
Novobiocin	0.70[b]	–0.03	1.19[b]	0.48[b]	1.65[b]	1.02[b]

[a]Standard error values of these differences did not exceed 0.16 $\log_{10}$ cfu, except in the case of penicillin with a value of 0.26 $\log_{10}$ cfu.
[b]Significant viability decrease (with $P < 0.01$, except in the case of cefuroxime at 4 × MBC for 48 h biofilms, with $P < 0.05$) in the treated versus the untreated control samples in an analysis of variance (four SP isolates were used, except in the case of penicillin, where isolate 510+ was omitted because it was resistant to this antibiotic). NT, not tested.
(See Table II footnotes for meaning of 4 × MBC values.)

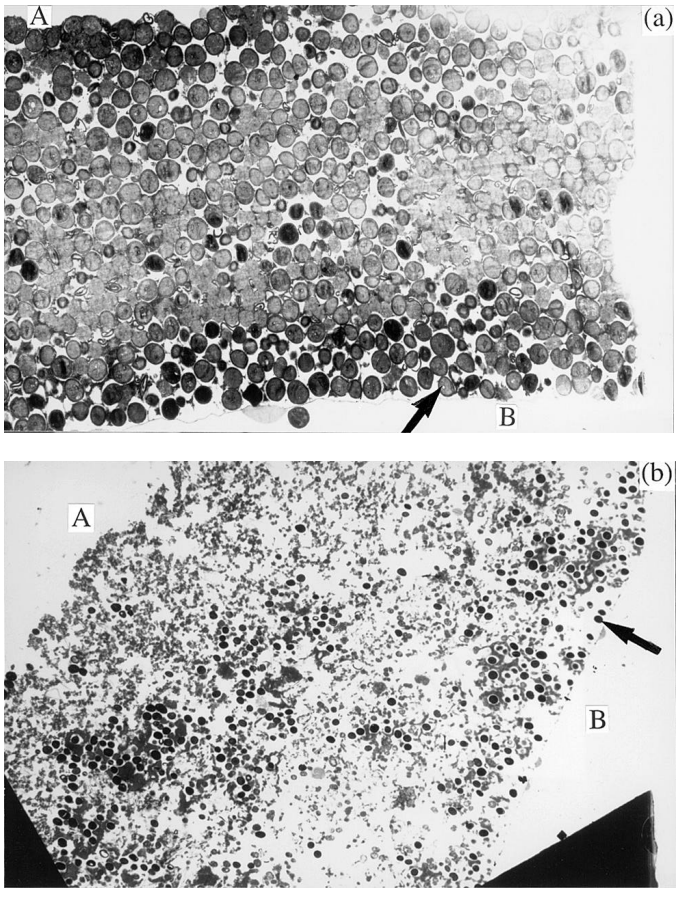

Figure 1. Transmission electron microscopy (TEM) micrographs showing full sections of 48 h biofilms. Growth media were (a) TSB (full biofilm section; magnification, ×3800) and (b) filter-sterilized delipidated milk (full biofilm section; magnification, ×1450). Biofilms were formed with isolate 9213+. A indicates the biofilm limit line in contact with the culture medium. B indicates the biofilm limit line in contact with the support surface. Arrows indicate bacteria. The diameter of a staphylococcal cell is between 0.5 and 1.5 μm.

rifampicin and novobiocin in Table II). The majority (94.3%) of killing values, among those that were significant in Tables II and III (64.2 %), were ⩾0.60 $\log_{10}$ cfu, whereas 42.9% of them exceeded 1 $\log_{10}$ cfu. At the lowest concentration tested (4 × MBC), a killing value ⩾1 $\log_{10}$ cfu was only observed when biofilms were grown in delipidated milk (in the case of cefuroxime, cefazolin and phosphomycin; Table III) but not in TSB (Table II).

Antibiotics had frequently a significantly greater effect on viability at higher antibiotic concentrations and/or in younger biofilms (Tables II and III).

With regard to the specific behaviour of each antibiotic on biofilm cells, generally (in both biofilm types), phosphomycin significantly affected cell viability, whereas gentamicin and erythromycin had non-significant effects on cell viability.

At the lowest concentration studied (4 × MBC), phosphomycin was the only antibiotic having a significant effect on viability in aged (48 h) TSB-grown biofilms (in this exceptional case, the 4 × MBC value exceeds 500 mg/L), whereas in milk-grown biofilms of this age, this antibiotic as well as cefuroxime had significant effects (Tables II and III).

At 4 × MBC in younger (6 h) TSB-grown biofilms, a significant effect on viability was observed in the case of cefuroxime, cefazolin and rifampicin, whereas in milk-grown biofilms, additional antibiotics (penicillin, ciprofoxacin, vancomycin and novobiocin) had this significant effect.

At 100 mg/L, cefuroxime, cefazolin, rifampicin, vancomycin, phosphomycin and novobiocin significantly affected viability in 6 h and 48 h biofilms, grown either in TSB or in delipidated milk. A significant effect was also found against cells of young (6 h) biofilms in the case of penicillin and ciprofloxacin (in both TSB-grown and milk-grown biofilms) as well as in tobramycin (in milk-grown biofilms; Tables II and III). These three antibiotics had a non-significant effect on 48 h biofilms.

Seven of the antibiotics with a significant degree of killing at <100 mg/L in both TSB-grown as well as in milk-grown biofilms (at least in 6 h biofilms) were also studied at a higher concentration (500 mg/L) to determine whether they would continue to increase their effect on viability on further increasing the concentration. The results shown in Tables II and III indicate that in most cases, the killing values obtained at 500 mg/L were higher than those obtained at 100 mg/L.

Study on overall bactericidal activity of the group of antibiotics used.

The overall effect on biofilm bacterial viability ($\log_{10}$ cfu/well) and general trend observed in the group of antibiotics under study (all of which were efficient in the microbroth dilution test for cells in suspension) were determined, according to variations in the age of the biofilm, the antibiotic concentration or the culture medium used. The effect was studied using the same raw data as in Tables II and III. For analysis, data on all the antibiotics under study (from results obtained after a 24 h exposure) and on all the isolates studied were pooled. Significant differences ($P < 0.01$) were found when comparing biofilm ages (after combining data on different antibiotic concentrations as well as data on different culture media; Figure 2a), antibiotic concentrations (after grouping data on different biofilm ages as well as data on different culture media; Figure 2b) and culture media (after pooling data on different biofilm ages as well as data on different antibiotic concentrations; Figure 2c). Significance ($P < 0.01$) was also found for the simple (for example, concentration × medium) and double (concentration × age × medium) interactions. Specifically, the number of viable bacteria recovered from biofilms submitted to antibiotic treatment increased with respect to controls with increase in the age of the biofilm (Figure 2a) and decreased when higher

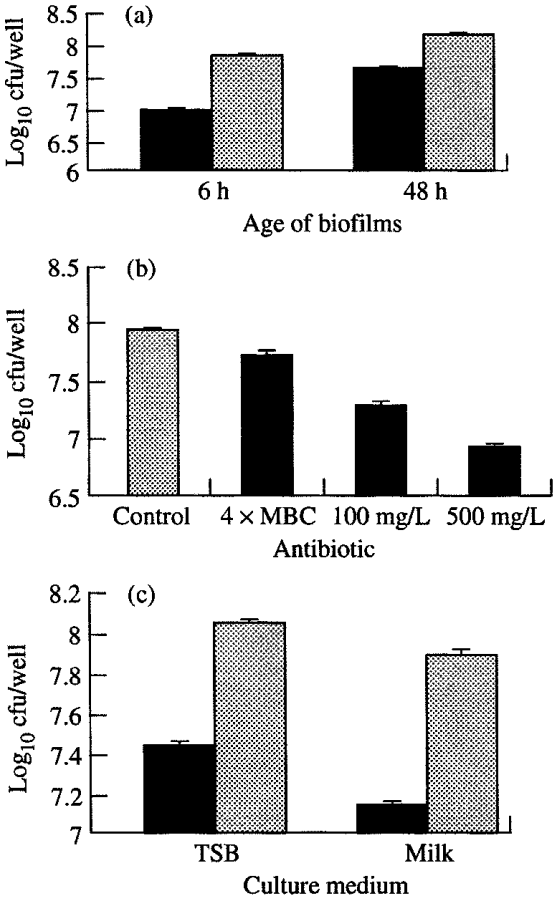

Figure 2. Effect of antibiotic treatment on bacterial viability (mean $\log_{10}$ cfu/well ± S.E.) in relation to the age of biofilms (a), antibiotic concentration (b) and growth medium used (c). Black rectangle, antibiotics; grey rectangle, control.

antibiotic concentrations were used (Figure 2b), or when milk rather than TSB was used as growth medium (Figure 2c). However, the latter comparison (between media) could be considered biased because the cell numbers obtained in biofilms of the same age in the absence of antibiotics were lower ($P < 0.001$) in the case of biofilms grown in delipidated milk as compared with TSB medium. These lower cell numbers associated with milk-grown biofilm assays may at least partially explain the apparently greater effect of the antibiotics when using milk as growth medium instead of TSB. The overall effects of the 11 antibiotics studied as a group are shown in Figure 2. Only some of the antibiotics involved in this overall study, and only under particular conditions, significantly affected biofilm cell viability (Tables II and III), explaining the reduced nature (in $\log_{10}$ cfu units) of some of the differences observed in Figure 2.

Antibiotic exposure periods required for significant bactericidal activity of antibiotics in 96-well plate biofilms

A study was performed to determine whether the biofilm test applied allowed the detection of differences between antibiotics in the exposure periods required to detect significant effects on viability of biofilm cells grown either in TSB or delipidated milk. For this study, seven antibiotics and the pool of data on two SP isolates (one isolate in the case of penicillin) were used. As shown in Table IV, the test on *S. aureus* biofilm bacteria discriminated antibiotics requiring a shorter (6 h) from those requiring a longer

Table IV. Influence of the exposure period applied on cell viability decrease (Δ for differences between control and treated samples in $\log_{10}$ cfu[a]) in TSB-grown and delipidated milk-grown *S. aureus* biofilms of 24 h of age, which had been treated with antibiotics (at 100 mg/L)

	Exposure of TSB-grown biofilms			Exposure of milk-grown biofilms		
Antibiotic	3 h	6 h	24 h	3 h	6 h	24 h
Cefuroxime	–0.07	0.03	0.67[b]	–0.12	0.00	0.51[b]
Cefazolin	–0.02	0.13	0.77[b]	0.02	0.27	0.64[b]
Penicillin	–0.08	0.08	–0.18	–0.16	0.16	–0.10
Tobramycin	–0.05	0.20	0.06	0.00	0.15	0.16
Rifampicin	0.37[b]	0.68[b]	1.59[b]	0.28	0.99[b]	1.72[b]
Vancomycin	–0.23	0.28	0.40[b]	0.04	0.55[b]	0.51[b]
Novobiocin	0.44[b]	0.90[b]	2.01[b]	0.16	0.45[b]	0.44[b]

[a]Standard error values did not exceed 0.17 $\log_{10}$ cfu.
[b]Significant viability decrease (with $P < 0.01$ except in the case of rifampicin, with $P < 0.05$ at 3 h in TSB-grown biofilms) in the treated versus the untreated control samples in an analysis of variance. Two isolates (510+ and 9213+) were used in this study, except in the case of penicillin, where isolate 510+ was omitted because it was resistant to this antibiotic.

(24 h) exposure period at a concentration ⩾12 × MBC. Two of the antibiotics had a significant effect on cell viability (at least of TSB-grown biofilms) in 3 h (rifampicin and novobiocin). Cefuroxime, cefazolin and vancomycin had a significant effect after 24 h (at least in TSB-grown biofilms). Thus, they could be classified as 'slower' in relation to others (for example, rifampicin or novobiocin). Tobramycin and penicillin (the latter being studied on isolate 9213, with reduced sensitivity to this antibiotic; Table I) did not affect cell viability significantly in this study and again, novobiocin had a lower effect on milk-grown than on TSB-grown biofilms. Some antibiotics could be equally classified as faster (rifampicin) or slower (cefuroxime) in both types of biofilms (milk grown and TSB grown), whereas others (for example, vancomycin) differed among biofilm types in the exposure time required to have significant effects on viability. Factors other than differences in the MBC among antibiotics affected the 'speed' (exposure time required), as antibiotics with the same MBC (for example, cefazolin, vancomycin and novobiocin; MBC = 4 mg/L) had different exposure patterns (Table IV).

TEM study of biofilms treated with antibiotics

To study TEM biofilms after antibiotic exposure, two antibiotics were studied, one with a significant effect (cefuroxime) and the other with a non-significant effect (tobramycin) on cell viability (at 100 mg/L in TSB-grown 6 h biofilms; Table II, Figure 3). In the case of cefuroxime, bacterial cell walls were severely damaged throughout the biofilm; affected cells were mainly located closer to the external biofilm layer, but they were less common at the deeper biofilm layers (Figure 3a). However, in tobramycin-treated preparations, in which a non-significant degree of killing was observed (Table II; Figure 3b), most cells presented thick walls and were intact like untreated-biofilm control cells, especially at the deeper biofilm layers (Figure 3c).

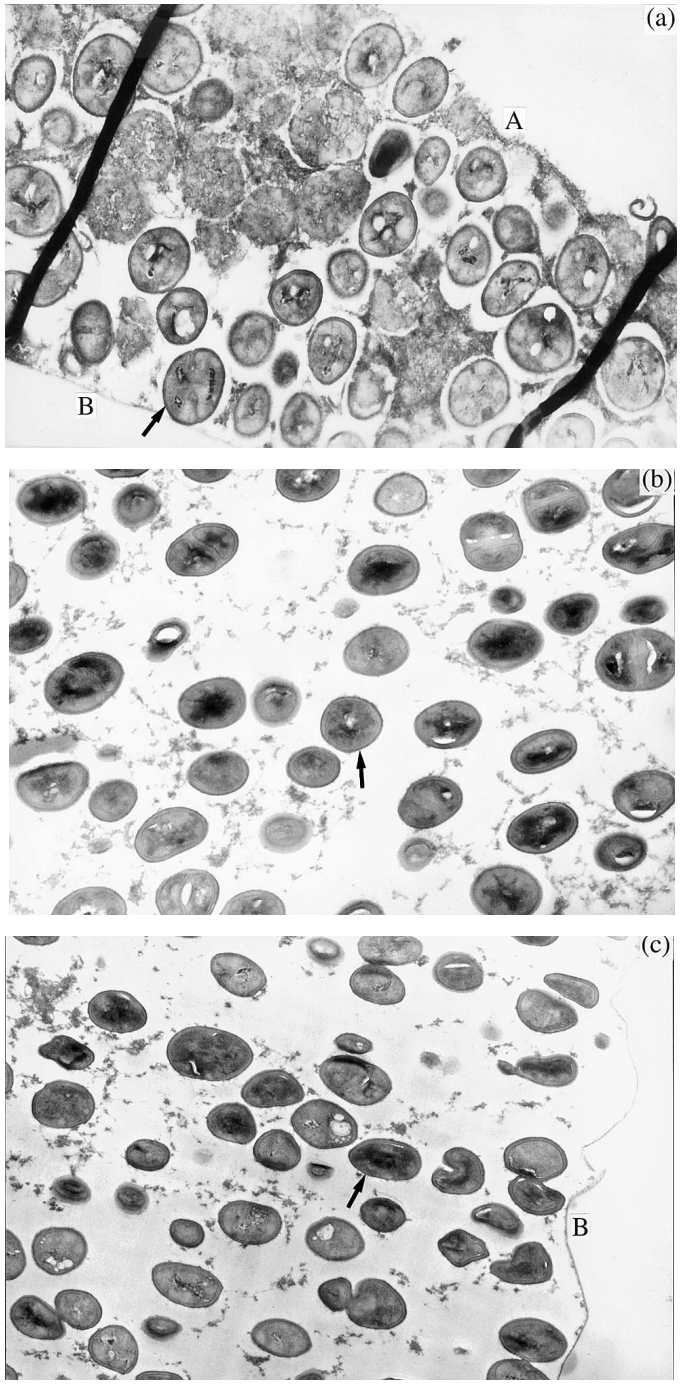

Figure 3. TEM micrographs of a 6 h TSB-grown biofilm following a 24 h incubation with cefuroxime (a), tobramycin (b) or Mueller–Hinton culture medium (control) (c). Biofilms were formed with isolate 9213+. B indicates the biofilm limit line in contact with the support surface. Arrows indicate bacteria. (Magnification, ×9800.)

Discussion

The results obtained in this work show that antibiotic susceptibility in microbroth dilution tests is similar when comparing suspensions of SP versus NSP variants of a given *S. aureus* isolate, but it decreases in the assay of biofilm bacteria developed, especially in aged biofilms and when using the lowest concentrations tested (Figure 2). The observation that the killing value of the antibiotics studied was 100% in the microbroth dilution tests at 1 × MBC but did not reach 1 $\log_{10}$ cfu when the antibiotics were used at 4 × MBC against young (6 h) TSB-grown biofilms may represent a warning signal concerning the exclusive use of classical in-vitro tests. This biofilm-associated antibiotic resistance, also found in previous studies,[10,28–30] can be attributed to a decreased antibiotic diffusion through the extensive biofilm matrix (Figure 1a and b).[1,28] In addition, there may be a decreased antibiotic susceptibility and metabolic activity of bacteria within biofilms (a relatively small difference in ATP production, translated into cfu, is

observed in 48 h versus 6 h biofilms; Figure 2a) and an interaction of the antibiotic with the abundant bacterial products in biofilms, which may affect the antibiotic activity (Figure 3a).[31]

The interest in antibiotic susceptibility tests for biofilm bacteria has increased in the last few years.[9,32–34] A widely used system, the Robbin's device,[9,35,36] allows a continuous growth medium flow, rather than a discontinuous one,[37] but requires expert handling.[9] The 96-well plate assay used in this work follows the present tendency towards time-efficient bacterial viability tests[10,16,38] and, although it has the limitation of involving a discontinuous flow, it facilitates the automation of bacterial viability determination through luminometry,[17] minimizes sample handling, allows the study of different factors within a single test date (different antibiotics and concentrations, biofilm ages, growth media, etc.) and is of potential use in time-killing antibiotic susceptibility studies.[39,40] The application of this test does not appear to be restricted to *S. aureus*; at present, it is being successfully extended in our laboratory to other biofilm-forming bacteria of clinical importance (such as *Staphylococcus epidermidis*, frequently involved in post-surgical contamination of implants and prostheses).

Results presented in this work showed that some antibiotics (such as gentamicin) affecting SP or NSP cells in suspension may not significantly affect the *S. aureus* biofilm cell viability, even when biofilms are exposed to very high concentrations (for example, 100 mg/L). Partial bacterial resistance to antibiotics, as detected in the microbroth dilution test, may be one of the factors contributing to the poor activity in the biofilm test (in the case of gentamicin and erythromycin, against isolate 510+; Table I). However, it cannot be the only factor, because in some cases (tobramycin), all the isolates had MBCs well below the peak value in serum (4–10 mg/L), yet the biofilm cell viability was not significantly affected when the antibiotic concentration was increased to 100 mg/L (TSB-grown biofilms, Table II). The majority (over 80%) of the antibiotics tested did not affect significantly 48 h biofilm cell viability when used at 4 × the MBC obtained by classical microbroth dilution tests. Thus, a further step for improving antibiotic selection after performing a microbroth dilution test may be to determine the in-vitro performance of antibiotics on biofilm bacteria.

Comparisons of antibiotic efficiency among different biofilm growth media is a difficult task: the age of the biofilm and the antibiotic concentration must be maintained, but then the bacterial cell number, biofilm characteristics and composition, bacterial metabolic activity and cell surface composition resulting after growth in different media may differ, hampering the comparison. In this work, growth in milk produced significantly fewer bacteria than growth in TSB, giving rise to a discontinuous or lumpy biofilm layer in relation to TSB (Figure 1). This may have positively affected the diffusion or effectiveness of antibiotics (as in the case of novobiocin at 4 × MBC against 6 h biofilms; Tables II and III). A further difficulty may arise when the changing of media implies a change from the in-vitro to the in-vivo situation, where different micro-environments are present and where the capacity for intracellular killing of bacteria in milk cells may vary among antibiotics.[41–43]

In this biofilm model, phosphomycin and cephalosporins (cefuroxime and cefazolin) were active against biofilm bacteria. With regard to phosphomycin, these positive results (Tables II and III), correlate with those obtained in previous studies.[43] However, this antibiotic is not commonly used for therapeutic treatment because it has not been tested for toxicity in some species, has high MICs and MBCs, and causes irritation and pain at the site of injection. The widely applied cephalosporins, which affect cell walls (Figure 3a), may help to improve in biofilms the access of antibiotics such as rifampicin (as observed in coagulase-negative staphylococci),[32] macrolides and aminoglycosides, effective against cells in suspension (Table I).[9,25] Cephalosporin efficacy has also been demonstrated against 2 h biofilms[9] and biofilms of up to 4 days of age.[25] In contrast, penicillin, which also affects the cell wall synthesis, appears to have in this work a weaker effect than cephalosporins, at least in aged (48 h) biofilms. Whether this is due to an increased difficulty of this antibiotic in diffusing[1,28] or maintaining its integrity through the biofilm matrix[31] is unknown.

From the time-killing susceptibility study, rifampicin has the advantage of needing shorter exposure than cephalosporins (Table IV), even though both antibiotic types can be efficient below the peak value in serum (at 4 × MBC in 6 h biofilms; Tables I, II and III). This fast effect is important because rifampicin has a 3 h average lifetime in serum. Although the use of rifampicin to eradicate bacteria within biofilms is controversial,[33,44] two characteristics of this antibiotic (the fast effect described in this work and appearance of resistance[33]), in addition to its well-known intracellular efficiency, may explain why it is of use in combination.[45]

Ciprofloxacin was found to have lower efficiency against biofilm cells than cephalosporins and rifampicin, as expected from previous biofilm studies.[46,47] Calcium and magnesium ions could not have neutralized the quinolones[48] in the biofilm test applied, because they were only added after antibiotic treatment (i.e. for the bioluminescence reaction). Further studies with more recently developed quinolones of high efficiency[49–53] are warranted, considering that quinolones may reach 14 times the serum concentration within phagocytes.[54] This may be advantageous in the therapy against chronic human infections (osteomyelitis, endocarditis, etc.) and ruminant mastitis[41,55,56] (for dry period therapy, to avoid the appearance of antibiotic residues in milk). Overall, these advantages and disadvantages of ciprofloxacin may help to explain why this antibiotic has been successfully used in combination with rifampicin.[57]

Although in this work cephalosporins and rifampicin appear to be good candidates for biofilm bacteria, vancomycin, a potent peptidoglycan synthesis inhibitor,[58] is the most frequently used antibiotic against multi-resistant *S. aureus* (MRSA). Vancomycin produced significant killing of biofilm bacteria, as previously shown,[9,33,59,60] but, unfortunately, it was inefficient at 4 × MBC on aged (48 h) biofilms (Tables II and III). This failure may be attributable to the inhibition of the effect of glycopeptides by the biofilm slime matrix[32] or the lower susceptibility of biofilm bacteria.[61] Efficiency may improve in the biofilm test when vancomycin is replaced by another glycopeptide (teicoplanin)[42] or when used in combinations.[43,62–64] The delayed effect of vancomycin in TSB-grown biofilms (Table IV) is in close agreement with experiments using this antibiotic against bacteria within platelet–fibrin matrices[39] and after early bacterial adherence mediated by fibronectin receptors.[65] This slow antibiotic may be expected to be synergic with fast ones (rifampicin or novobiocin). An analogous synergy has been described for the combination of penicillins (slow) with novobiocin (fast) in drug therapy against mastitis.[66]

TEM work on in-vitro *S. aureus* biofilms submitted to antibiotic treatment is limited, possibly because of the lack of biofilm susceptibility tests or the difficulties in biofilm development (SP variants have only recently become available) and in maintaining the biofilm integrity for TEM studies. The more advanced TEM studies on biofilms developed *in vivo* reveal that the biofilm is analogous to that found in this in-vitro study, although these in-vivo developed biofilms may include host cellular and subcellular components.[10,67–70] This difference indicates that the in-vitro findings must be interpreted with caution before they are considered as predictors of antibiotic performance in live models. We have started a series of antibiotic therapy studies on the correlation between the findings obtained with the in-vitro susceptibility biofilm assay and in rat experimental osteomyelitis models, with positive results.[71] This is encouraging considering the need to improve the selection of antibiotics against chronic infections in humans and animal species.

In conclusion, selection of antibiotics against bacteria within biofilms appears to require the application of both classical tests[3] (as a first screening phase) and biofilm tests (as a second screening–diagnostic phase), a strategy proposed previously.[9] The negative correlation found between biofilm age and antibiotic effect in this work (Table II) and in previous studies[25] suggests the possible usefulness of biofilm tests for selecting suitable prophylactic or treatment measures in live organisms against bacteria within biofilms.[72] The biofilm assay strategy applied in this work may constitute a tool in antimicrobial research and the pharmaceutical industry for initial comparative studies among antibiotics.

Acknowledgements

We gratefully acknowledge R. Baselga, A. Laclériga and R. Gómez-Lus for helpful suggestions. We thank E. Barber for excellent technical help in TEM studies. This work was funded by FIS (No. 96/0230), CONSID (No. PCM1694) and Echebano Foundation (No. 337/97).

References

1. Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. *et al*. (1987). Bacterial biofilms in nature and diseases. *Annual Review of Microbiology* **41**, 435–64.

2. Gristina, A. G., Hobgood, C. D., Webb, L. X. & Myrvik, Q. N. (1987). Adhesive colonization of biomaterials and antibiotic resistance. *Biomaterials* **8**, 423–6.

3. National Committee for Clinical Laboratory Standards. (1990). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Second Edition: Approved Standard M7-A2.* NCCLS, Villanova, PA.

4. Chuard, C., Lucet, J., Rohner, P., Herrmann, M., Auckenthaler, R., Waldvogel, F. *et al.* (1991). Resistance of *Staphylococcus aureus* recovered from infected foreign body *in vivo* to killing by antimicrobials. *Journal of Infectious Diseases* **163**, 1369–73.

5. Kaatz, G., Seo, S., Dorman, N. & Lerner, S. (1990). Emergence of teicoplanin resistance during therapy of *Staphylococcus aureus* endocarditis. *Journal of Infectious Diseases* **162**, 103–8.

6. Anwar, H., Van Biesen, T., Dasgupta, M., Lam, K. & Costerton, J.W. (1989). Interaction of biofilm bacteria with antibiotics in a novel in-vitro chemostat system. *Antimicrobial Agents and Chemotherapy* **33**, 1824–6.

7. Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barret, F. F., Melton, D. M. *et al.* (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical device. *Journal of Clinical Microbiology* **22**, 996–1006.

8. Chuard, C., Vaudaux, P., Waldvogel, F. A. & Lew., D. P. (1993). Susceptibility of *Staphylococcus aureus* growing on fibronectin-coated surfaces to bactericidal antibiotics. *Antimicrobial Agents and Chemotherapy* **37**, 625–32.

9. Domingue, G., Ellis, B., Dasgupta, M. & Costerton, J. W. (1994). Testing antimicrobial susceptibilities of adherent bacteria by a method that incorporates guidelines of the National Committee for Clinical Laboratory Standards. *Journal of Clinical Microbiology* **32**, 2564–8.

10. Marrie, T. J., Nelligan, J. & Costerton, J. W. (1982). A scanning and transmission electron microscopic study of an infected endocardial pacemaker lead. *Circulation* **66**, 1339–41.

11. Wirtanen, G. & Sandholm, T. M. (1992). Effect of the growth phase of foodborne biofilms on their resistance to a chlorine sanitizer Part II. *Lebensmittel-Wissenschaft + Technologie* **25**, 50–4.

12. Ordóñez, J. V. & Wehman, N. M. (1993). Rapid flow cytometric antibiotic susceptibility assay for *Staphylococcus aureus*. *Cytometry* **14**, 811–18.

13. Hussain, M., Collins, C., Hastings, G. M. & White, P. J. (1992). Radiochemical assay to measure the biofilm produced by coagulase-negative staphylococci on solid surfaces and its use to quantify

the effects of various antibacterial compounds on the formation of the biofilm. *Journal of Medical Microbiology* **37**, 62–69.

14. de Rautlin de la Roy, Y., Messedi, N., Grollier, G. & Grignon, B. (1991). Kinetics of bactericidal activity of antibiotics measured by luciferin–luciferase assay. *Journal of Bioluminescence and Chemiluminescence* **6**, 193–201.

15. Stanley, B. J. & McCarthy, B. J. (1989). Reagents and instruments for assays using ATP and luminescence: present needs and future possibilities in rapid microbiology. In *Rapid Methods in Microbiology. ATP Luminescence* (Stanley, P. E., McCarthy, B. J. & Smither, R., Eds), pp. 73–80. Society for Applied Bacteriology. Technical Series. No. 26. Blackwell, Boston, MA.

16. Wheat, P. F., Hastings, J. G. & Spencer, R. C. (1988). Rapid antibiotic susceptibility tests on Enterobacteriaceae by ATP bioluminescence. *Journal of Medical Microbiology* **25**, 95–9.

17. Vasavada, P. (1993). Rapid methods and automation in dairy microbiology. Symposium: evaluation of milk and dairy products. *Journal of Dairy Science* **76**, 3101–13.

18. Cooksey, R. C., Morlock, G. P., Beggs, M. & Crawford, J. T. (1995). Bioluminescence method to evaluate antimicrobial agents against *Mycobacterium avium*. *Antimicrobial Agents and Chemotherapy* **39**, 754–6.

19. Stemke, W. G. & Robertson, J. (1990). The growth response of *Mycoplasma hyopneumoniae* and *Mycoplasma flocculare* based upon ATP-dependent luminometry. *Veterinary Microbiology* **24**, 135–42.

20. Baselga, R., Albizu, I., De la Cruz, M., Del Cacho, E., Barberán, M. & Amorena, B. (1993). Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. *Infection and Immunity* **61**, 4857–62.

21. Baselga, R., Albizu, I., Aguilar, B., Iturralde, M. & Amorena, B. (1992). Hydrophobicity of ruminant mastitis *Staphylococcus aureus* in relation to bacterial aging and slime production. *Current Microbiology* **25**, 173–9.

22. Gracia, E., Fernández, A., Conchello, P., Laclériga, A., Paniagua, L., Seral, F. *et al.* (1997). Adherence of *Staphylococcus aureus* slime-producing strain variants to biomaterials used in orthopaedic surgery. *International Orthopaedics* **21**, 46–51.

23. Freeman, D. J., Falkiner, F. R. & Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. *Journal of Clinical Pathology* **42**, 872–4.

24. Popovic, T., Bopp, Ch. A., Olsvik, O. & Kiehlbaugh, J. A. (1993). Ribotyping in molecular epidemiology. In *Diagnostic Molecular Microbiology. Principles and Applications* (Persing, D. H., Smith, T. F., Tennover, F. C. & White, T. J., Eds), pp. 573–83. American Society for Microbiology, Washington, DC.

25. Anwar, H., Strap, J. L. & Costerton, J. W. (1992). Eradication of biofilm cells of *Staphylococcus aureus* with tobramycin and cephalexin. *Canadian Journal of Microbiology* **38**, 618–25.

26. Gracia, E. (1997). Test de sensibilidad a antibióticos para *Staphylococcus aureus* formadores de biofilms. Doctoral thesis, University of Zaragoza, Zaragoza, Spain.

27. Statistical Analysis Systems Institute, Inc. (1989). *SAS/STAT User's Guide, Version 6, Fourth Edition, Volume 1*. SAS Institute, Inc., Cary, NC.

28. Anwar, H., Strap, J. L. & Costerton, J. W. (1992). Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. *Antimicrobial Agents and Chemotherapy* **36**, 1347–51.

29. Brown, M. R., Allison, D. G. & Gilbert, P. (1988). Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *Journal of Antimicrobial Chemotherapy* **22**, 777–83.

30. Dall, L. & Herndon, B. (1989). Quantitative assay of glycocalyx produced by viridians group streptococci that cause endocarditis. *Journal of Clinical Microbiology* **279**, 2039–41.

31. Fraber, B. F., Kaplan, M. H. & Clogston, A. (1990). *Staphylococcus epidermidis* extracted slime inhibits the antimicrobial action of glycopeptide antibiotics. *Journal of Infectious Diseases* **161**, 37–40.

32. Brandt, C. M., Rouse, M. S., Tallan, B. M., Laue, N. W., Wilson, W. R. & Steckelberg, J. M. (1995). Effective treatment or cephalosporin–rifampicin combinations against cryptic methicillin-resistant β-lactamase-producing coagulase-negative staphylococcal experimental endocarditis. *Antimicrobial Agents and Chemotherapy* **39**, 1815–19.

33. Dunne, W. M., Mason, E. O. & Kaplan, S. L. (1993). Diffusion of rifampicin and vancomycin through a *Staphylococcus epidermidis* biofilm. *Antimicrobial Agents and Chemotherapy* **37**, 2522–6.

34. Prosser, B. L., Taylor, D., Dix, B. A. & Cleeland, R. (1987). Method of evaluating effects of antibiotics on bacterial biofilm. *Antimicrobial Agents and Chemotherapy* **31**, 1502–6.

35. Vorachit, M., Lam, K., Jayanetra, P. & Costerton, J. W. (1993). Resistance of *Pseudomonas pseudomallei* growing as a biofilm on silastic discs to ceftazimide and co-trimoxazole. *Antimicrobial Agents and Chemotherapy* **37**, 2000–2.

36. Nickel, J. C., Ruseska, I., Wright, J. B. & Costerton, J. W. (1985). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. *Antimicrobial Agents and Chemotherapy* **27**, 619–24.

37. Barth, E., Myrvik, Q. M., Wagner, W. & Gristina, A. G. (1989). In-vitro and in-vivo colonization of *Staphylococcus aureus* and *Staphylococcus epidermidis* on orthopaedic implant materials. *Biomaterials* **10**, 325–8.

38. Pore, R. S. (1994). Antibiotic susceptibility testing by flow cytometry. *Journal of Antimicrobial Chemotherapy* **34**, 613–27.

39. Berthaud, N., Huet, Y., Diallo, N. & Desnotes, J.-F. (1997). Antistaphylococcal activities of quinupristin/dalfopristin *in vitro* across platelet–fibrin matrices and in experimental endocarditis. *Journal of Antimicrobial Chemotherapy* **39**, *Suppl. A*, 93–8.

40. Hamilton-Miller, J. M. T. & Shah, S. (1997). Activity of quinupristin/dalfopristin against *Staphylococcus epidermidis* in biofilms: a comparison with ciprofloxacin. *Journal of Antimicrobial Chemotherapy* **39**, *Suppl. A*, 103–8.

41. Bolourchi, M., Hovareshti, P. & Tabatabayi, A. (1995). Comparison of the effects of local and systemic dry cow therapy for staphylococcal mastitis control. *Preventive Veterinary Medicine* **25**, 63–7.

42. Perdikaris, G. S., Pefanis, A., Giamarellou, H., Nikolopoulos, A., Margaris, E. P., Donta, I. *et al.* (1997). Successful single-dose teicoplanin prophylaxis against experimental streptococcal, enterococcal, and staphylococcal aortic valve endocarditis. *Antimicrobial Agents and Chemotherapy* **41**, 1916–21.

43. Pérez Fernández, P., Herrera, I., Martínez, P., Gómez-Lus, M. L. & Prieto, J. (1995). Enhancement of the susceptibility of *Staphylococcus aureus* to phagocytosis after treatment with phosphomycin

compared with other antimicrobial agents. *Chemotherapy* **41**, 45–49.

44. Ramírez de Arellano, E., Pascual, A., Martínez-Martínez, L. & Perea, E. J. (1994). Activity of eight antibacterial agents on *Staphylococcus epidermidis* attached to Teflon catheters. *Journal of Medical Microbiology* **40**, 43–7.

45. Drancourt, M., Stein, A., Argenson, J. N., Roiron, R., Groulier, P. & Raoult, D. (1997). Oral treatment of *Staphylococcus* spp. infected orthopaedic implants with fusidic acid or ofloxacin in combination with rifampicin. *Journal of Antimicrobial Chemotherapy* **39**, 235–40.

46. Widmer, A. F., Frei, R., Rajacic, Z. & Zimmerli, W. (1990). Correlation between in-vivo and in-vitro efficacy of antimicrobial agents against foreign body infections. *Journal of Infectious Diseases* **162**, 96–102.

47. Gander, S. (1996). Bacterial biofilms: resistance to antimicrobial agents. *Journal of Antimicrobial Agents* **37**, 1047–50.

48. Balows, A., Hausler, W. J., Herrmann, K. L. & Shadomy, H. J. (1991). *Manual of Clinical Microbiology*. American Society for Microbiology, Washington, DC.

49. Baquero, F. & Cantón, R. (1996). In-vitro activity of sparfloxacin in comparison with currently available antimicrobials against respiratory tract pathogens. *Journal of Antimicrobial Chemotherapy* **37**, *Suppl. A*, 1–18.

50. Endtz, H. P., Mouton, J. W., Den Hollander, J. G., van den Braak, N. & Verbrugh, H. A. (1997). Comparative in-vitro activities of trovafloxacin (CP-99,219) against 445 Gram-positive isolates from patients with endocarditis and those with other bloodstream infections. *Antimicrobial Agents and Chemotherapy* **41**, 1146–9.

51. Crémieux, A.-C., Mghir, A. S., Bleton, R., Manteau, M., Belmatoug, N., Massias, L. *et al.* (1996). Efficacy of sparfloxacin and autoradiographic diffusion pattern of [^{14}C]sparfloxacin in experimental *Staphylococcus aureus* joint prosthesis infection. *Antimicrobial Agents and Chemotherapy* **40**, 2111–16.

52. Kaatz, G. W., Seo, S. M., Aeschlimann, J. R., Houlihan, H. H., Mercier, R.-C. & Rybak, M. J. (1998). Efficacy of trovafloxacin against experimental *Staphylococcus aureus* endocarditis. *Antimicrobial Agents and Chemotherapy* **42**, 254–6.

53. Maserati, R., Cagni, A. E. & Segú, C. (1996). Sparfloxacin therapy for experimental endocarditis caused by methicillin-resistant *Staphylococcus aureus*. *Chemotherapy* **42**, 133–9.

54. Pemán, J., Cantón, E., Hernández, M. T. & Gobernado, M. (1994). Intraphagocytic killing of Gram-positive bacteria by ciprofloxacin. *Journal of Antimicrobial Chemotherapy* **34**, 965–74.

55. Sánchez, M. S., Ford, C. W. & Yancey, R. J. (1988). Evaluation of antibiotic effectiveness against *Staphylococcus aureus* surviving within the bovine mammary grand macrophage. *Journal of Antimicrobial Chemotherapy* **21**, 773–86.

56. Sandholm, M., Kaartinen, L. & Pyorala, S. (1990). Bovine mastitis—why does antibiotic therapy not always work? An overview. *Journal of Veterinary Pharmacology and Therapy* **13**, 248–60.

57. Kang, S. L., Rybak, M. J., McGrath, B. J., Kaatz, G. W. & Seo, S. M. (1994). Pharmacodynamics of levofloxacin, ofloxacin, and ciprofloxacin, alone and in combination with rifampicin, against methicillin-susceptible and resistant *Staphylococcus aureus* in an in-vitro infection model. *Antimicrobial Agents and Chemotherapy* **38**, 2702–9.

58. Nieto, M. & Perkins, H. R. (1971). Physicochemical properties of vancomycin and iodovancomycin and their complexes with diacetyl-L-lysyl-D-alanyl-D-alanine. *Biochemical Journal* **123**, 773–87.

59. Daschner, F. D. & Kropec, A. (1995). Glycopeptides in the treatment of staphylococcal infections. *European Journal of Clinical Microbiology and Infectious Diseases* **14**, *Suppl. 1*, S12–17.

60. Evans, R. & Clifford, J. H. (1987). Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. *Antimicrobial Agents and Chemotherapy* **31**, 889–94.

61. Darouiche, R. O., Dhir, A., Miller, A. J., Landon, G. C., Raad, I. I. & Musher, D. M. (1994). Vancomycin penetration into biofilm covering infected prostheses and effect on bacteria. *Journal of Infectious Diseases* **170**, 720–3.

62. Houlihan, H. H., Mercier, R. C. & Ryback, M. J. (1997). Pharmacodynamics of vancomycin alone and in combination with gentamicin at various dosing intervals against methicillin-resistant *Staphylococcus aureus*-infected fibrin–platelet clots in an in-vitro infection model. *Antimicrobial Agents and Chemotherapy* **41**, 2497–501.

63. Schmitz, F.-J. & Jones, M. E. (1997). Antibiotics for treatment of infections caused by MRSA and elimination of MRSA carriage. What are the choices? *International Journal of Antimicrobial Agents* **9**, 1–19.

64. Raymond, J., Vedel, G. & Begeret, M. (1996). In-vitro bactericidal activity of cefpirome in combination with vancomycin against *Staphylococcus aureus* and coagulase-negative staphylococci. *Journal of Antimicrobial Chemotherapy* **38**, 1067–71.

65. Berthaud, N. & Desnotes, J.-F. (1997). In-vitro bactericidal activity of quinupristin/dalfopristin against adherent *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy* **39**, *Suppl. A*, 99–102.

66. Tardáguila, J., Gonzalo, C., Marco, J. C. & San Primitivo, F. (1997). Eficacia al parto de 2 métodos de tratamiento antibiótico de secado en el ovino lechero de raza Churra. *Informe Técnico Económico Agrícola Suppl. 18*, 552–4.

67. Fassel, T. A., Mozdiak, P. E., Sanger, J. R. & Edmiston, C. E. (1997). Paraformaldehyde effect on ruthenium red and lysine preservation and staining of the staphylococcal glycocalyx. *Microscopy Research and Technique* **36**, 422–7.

68. Gristina, A. G., Dobbins, J. J., Giammara, B., Lewis, J. C. & DeVries, W. C. (1988). Biomaterial-centered sepsis and the total artificial heart microbial adhesion vs tissue integration. *Journal of the American Medical Association* **259**, 870–4.

69. Passerini, L., Lam, K., Costerton, J. W. & King, E. G. (1992). Biofilms on indwelling vascular catheters. *Critical Care Medicine* **20**, 665–73.

70. Mayberry-Carson, K. J., Mayberry, W. R., Tober-Meyer, B. K., Costerton, J. W. & Lambe, D. W. (1986). An electron microscopic study on the effect of clindamycin on adherence of *Staphylococcus aureus* to bone surfaces. *Microbios* **45**, 21–32.

71. Gracia, E., Laclériga, A., Monzón, M., Leiva, J., Oteiza, C. & Amorena, B. (1999). Application of a rat osteomyelitis model to compare *in vivo* and *in vitro* the antibiotic efficacy against bacteria with high capacity to form biofilms. *Journal of Surgical Research* **79**, 146–53.

72. Bamberger, D., Fields, M. & Herndon, B. (1991). Efficacies of various antimicrobial agents in treatment of *Staphylococcus aureus* abscess and correlation with in-vitro tests of antimicrobial activity and neutrophil killing. *Antimicrobial Agents and Chemotherapy* **35**, 2335–9.

Received 11 October 1998; returned 14 January 1999; revised 10 February 1999; accepted 24 February 1999