

## Evaluation of antimicrobial activity of certain combinations of antibiotics against *in vitro* *Staphylococcus epidermidis* biofilms

Fernanda Gomes, Pilar Teixeira, Howard Ceri\* & Rosário Oliveira

IBB-Institute for Biotechnology & Bioengineering, Centre of Biological Engineering, University of Minho, Braga, Portugal & \*Department of Biological Sciences, University of Calgary, Alberta, Canada

Received October 20, 2010

**Background & objectives:** *Staphylococcus epidermidis* is the most common pathogen associated with infections of surgical implants and other prosthetic devices owing to its adhesion and biofilm-forming ability on biomaterials surfaces. The objective of this study was to compare susceptibilities of biofilm-grown cells to single antibiotic and in combination with others to identify those that were effective against *S. epidermidis* biofilms.

**Methods:** Biofilms were grown in the MBEC™ assay system. The use of this methodology allowed a rapid testing of an array of antibiotics alone (eight) and in combination (25 double combinations). The antibacterial effect of all treatments tested was determined by colony forming units (cfu) enumeration method.

**Results:** The MBEC™ assay system produced multiple and reproducible biofilms of *S. epidermidis*. Although none of the antibiotics tested have demonstrated an antimicrobial effect (log reduction >3) against all *S. epidermidis* isolates biofilms, but combinations containing rifampicin showed in general a broader spectrum namely rifampicin-gentamicin and rifampicin-clindamycin. Levofloxacin in combination with rifampicin showed a killing effect against three isolates but failed to attain a bactericidal action against the other two.

**Interpretation & conclusions:** Our findings showed that rifampicin should be a part of any antibiotic therapy directed against *S. epidermidis* biofilms. However, the efficient antibiotics combination might be dependent on *S. epidermidis* isolate being tested.

**Key words** Antibiotics susceptibility - biofilm - MBEC™ assay - *Staphylococcus epidermidis*

Previously regarded as an innocuous commensal microorganism on the human skin, *Staphylococcus epidermidis* is now seen as an important opportunistic pathogen<sup>1-3</sup>. This bacterium has become the leading cause of infections related to indwelling medical devices such as vascular catheters, prosthetic joints and artificial heart valves, mainly due to its capacity to form

biofilms on such materials thus causing persistent or recurrent infections<sup>4,5</sup>. Infections of medical implants material are associated with considerable morbidity and costs<sup>4</sup>. These infections are very difficult to eradicate since bacteria in biofilms can be up to 1,000-fold more resistant to antibiotic treatment than the same organism growing planktonically<sup>6-8</sup>. Another problem is the ability

of bacteria to acquire resistance to antibiotics therapy. This arises from the frequent use of antibiotics and mainly those of broad-spectrum. Only a few antibiotics are relatively active against *S. epidermidis* biofilms, and rifampicin, a transcription inhibitor, is among the most effective molecules for treating biofilm-related infections<sup>9</sup>. However, in a study where the prevalence of drug resistance among clinically significant blood isolates of *S. epidermidis* (n = 464) and consumption of antibiotics at a tertiary care teaching hospital (Meilahti Hospital, Helsinki) were analysed for the period 1983-1994, a remarkable increase was found in resistance to rifampin (from 0 to 23%) despite the low usage of this agent<sup>10</sup>. Accordingly, since rifampicin demonstrated a high risk of rapid development of resistance, it should not be used as monotherapy<sup>1</sup>.

Taking this fact into account, antibiotic combinations are often necessary in the treatment of *S. epidermidis* infections and these combinations are used involving antibiotics like rifampicin to avoid the appearance of antimicrobial resistance<sup>1,11</sup>. Moreover, the combinations can also enhance the effects of individual antimicrobial agents by synergic action. Another alternative to overcome the resistance problem in Staphylococci is the use of novel antibiotics such as linezolid, daptomycin, tigecycline and quinupristin/dalfopristin that have been developed and claimed to be 100 per cent efficient<sup>12</sup>. Some of the newer antimicrobial agents may provide alternatives for monotherapy or combination therapy with rifampicin<sup>1</sup>. However, this new antibiotic generation is very expensive, so the use of conventional antibiotics or antibiotic combinations still represents a valid therapeutic option.

The aim of the present work was to investigate the antimicrobial activity of some of the most common antibiotics alone and in combination against *in vitro* *S. epidermidis* biofilms.

### Material & Methods

**Bacterial isolates & antibiotics:** In this study, previously well characterized biofilm-producing *S. epidermidis* isolates were used: 117977, 132034, 150571, 1457 and 9142. The first three were obtained from the Department of Biological Sciences, University of Calgary, Calgary, Canada and the last two were provided by Dr G.B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA. These were clinical isolates (isolated from infected catheters) and were stored at -80°C. All the assays were performed using brain heart

infusion (BHI) medium, tryptic soy broth (TSB) and tryptic soy agar (TSA) [Merck, Germany], prepared according to the manufacturer's instructions. This study was done in Ceri lab, Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada.

Antibiotics tested were vancomycin, tetracycline, rifampicin, gentamicin, cefazolin, cephalotin, levofloxacin and clindamycin. All antibiotics were purchased from Sigma Chem. Co., USA. The concentrations used to test the susceptibility of biofilm-grown *S. epidermidis* to single and double-combinations of antibiotics are presented in Table I.

**Biofilm formation:** Biofilms were grown in a Calgary biofilm device (CBD) [commercially available as the MBEC physiology and genetics assay (Innovotech Inc., Edmonton, AB, Canada)], as originally described by Ceri *et al*<sup>14</sup>. The CBD consists of two-part reaction vessel. The top component of the device is a polystyrene lid with 96 identical pegs. The lid is inserted into the bottom piece of the device- a microtiter plate into which the inoculated growth medium is placed. The CBD is then placed on a gyro rotary shaker in an incubator which provides a shear force against the pegs, this facilitates the formation of 96 statistically equivalent biofilms on the surface of the pegs<sup>14-16</sup>.

In brief, several colonies of the isolates grown on TSA plates were suspended in saline (0.9% NaCl) to a density of 1.0 on the McFarland scale, as indicated by the manufacturer. The bacterial suspension was resuspended in medium to obtain a cellular concentration of *circa*  $1 \times 10^7$  colony forming units (cfu)/ml. This solution was used as inoculum for the MBEC<sup>TM</sup>

**Table I.** Antibiotics' breakpoints and concentrations used to test susceptibility

Antibiotic	Breakpoint (µg/ml)		
	Sensitive	Intermediate	Resistant*
Vancomycin	4	8-16	32
Tetracycline	4	8	16
Rifampicin	1	2	4
Gentamicin	4	8	16
Cefazolin	8	16	32
Cephalothin	8	16	32
Levofloxacin	1	2	4
Clindamycin	0.5	-	2

\*Concentration used in single, double-combination antibiotic susceptibility testing

Source: Ref. 13

**Table II.** Antibiotics used and combinations tested

Antibiotic	Combinations		
VANC	VANC + RIF	TET + CEPH	GENT + CEPH
TET	VANC + GENT	TET + LEVO	GENT + LEVO
RIF	VANC + CEF	TET + CLIND	GENT + CLIND
GENT	VANC + CEPH	RIF + GENT	CEF + CEPH
CEF	VANC + LEVO	RIF + CEF	CEF + LEVO
CEPH	VANC + CLIND	RIF + CEPH	CEF + CLIND
LEVO	TET + RIF	RIF + LEVO	CEPH + LEVO
CLIND	TET + GENT	RIF + CLIND	CEPH + CLIND
VANC + TET	TET + CEF	GENT + CEF	LEVO + CLIND

VANC, vancomycin; TET, tetracycline; RIF, rifampicin; GENT, gentamicin; CEF, cefazolin; CEPH, cephalothin; LEVO, levofloxacin; CLIND, clindamycin

device (MBEC™ Biofilm Technologies Ltd. Calgary, Alberta, Canada). The biofilms were grown during 48 h, at 37°C at 150 rpm and on a rocking platform where the shear force was created against the pegs forming 96 equivalent biofilms. To enumerate the biofilm cfu on individual control pegs, pegs were broken off the MBEC peg lid using sterile forceps, placed into 200 µl of sterile saline and sonicated for 8 min. Bacteria were then enumerated by serial dilution plating. Colony forming units/peg counts were determined from at least three independent experiments.

This protocol was performed with three different biofilm growth media: TSB, TSB + 0.25% glucose and BHI medium. After selecting the medium that allowed the highest biofilm formation (cfu per peg >6 log), the previous procedure was repeated with the selected medium.

**Biofilm challenge and recovery:** The challenge plates were prepared using the antibiotics at break-point concentrations (Table I) alone and in all possible double combinations (Table II). The biofilms formed on the lid of the MBEC™ were rinsed twice with 0.9 per cent saline and placed into the challenge plate overnight at

37°C, at 150 rpm, on a rocking platform and 95 per cent relative humidity. After that the challenged biofilms were rinsed twice in saline and were transferred to a recovery plate that consisted of TSB medium plus (1% v/v) Tween 80. Biofilms were removed from all pegs at once, by sonication for 8 min on high with an Aquasonic sonicator (model 250HT, VWR Scientific, Mississauga, ON, Canada)<sup>14</sup>. The vibration disrupted biofilms from the surface of the 96 pegs into the recovery plate. Then, colony forming units were determined as follows: the recovery medium (containing the sonicated biofilms) was serially diluted. The biofilm cultures (10-fold diluted) were spotted on TSA plates, the plates were incubated for 48 h at 37°C to ensure maximum recovery of the surviving microorganisms and after that the cfu counted.

## Results

In this study, eight antibiotics, commonly used in the treatment of Gram-positive infections, were tested at their breakpoint concentrations. The effect of these antibiotics combined in pairs (Table II) was also assessed. TSB without glucose stimulated more biofilm formation (data not shown), forming at the end of the incubation period (48 h), a biofilm of approximately 6 log cfu per peg. Thus, this medium was selected for the subsequent antibiotic susceptibility tests on *S. epidermidis* biofilms. The amount of glucose (0.25% w/v) used to promote the formation of *S. epidermidis* biofilms in traditional 96-well plates<sup>6,17</sup> was not favourable to biofilm formation in CBD.

The effect of the tested antibiotics alone was evaluated against the biofilms of the five clinical isolates of *S. epidermidis* assayed. The results obtained are expressed as reduction in treated biofilms compared to untreated controls (Table III). In general, none of the antibiotics tested was effective against *S. epidermidis*

**Table III.** Viable cell number reduction in 48 h-biofilms, expressed as log<sub>10</sub> cfu/ml, after overnight exposure to individual antibiotics

<i>S. epidermidis</i> isolate	VANC	TET	RIF	GENT	CEF	CEPH	LEVO	CLIND
117977	0.00 ± 0.24	1.20 ± 0.24	2.37 ± 0.68	0.00 ± 0.29	0.00 ± 0.23	0.00 ± 0.29	2.86 ± 0.48	1.68 ± 0.35
132034	0.26 ± 0.73	2.70 ± 0.48	4.01 ± 0.47	0.56 ± 0.30	0.29 ± 0.46	2.39 ± 0.38	0.32 ± 0.19	0.15 ± 0.32
150271	0.36 ± 0.44	0.31 ± 0.40	3.15 ± 0.18	0.14 ± 0.42	0.46 ± 0.36	1.84 ± 0.51	1.87 ± 0.36	2.20 ± 0.32
1457	0.00 ± 0.51	2.02 ± 0.20	1.58 ± 0.22	0.00 ± 0.19	1.63 ± 0.47	2.40 ± 0.31	2.18 ± 0.35	1.72 ± 0.20
9142	0.57 ± 0.53	1.54 ± 0.42	2.42 ± 0.44	0.19 ± 0.38	0.14 ± 0.40	0.26 ± 0.54	3.63 ± 0.12	0.28 ± 0.51

VANC, vancomycin; TET, tetracycline; RIF, rifampicin; GENT, gentamicin; CEF, cefazolin; CEPH, cephalothin; LEVO, levofloxacin; CLIND, clindamycin  
Values are mean ± standard deviation. (n=4)

biofilm. Only rifampicin was effective against *S. epidermidis* isolates 132034 and 150271 as well as levofloxacin against isolate 9142 (Table III) because the log<sub>10</sub> cfu reduction observed was higher than 3

log. Although the reduction caused by rifampicin and levofloxacin is mostly inferior to 3 log, these were the antibiotics having the broadest and highest antimicrobial effect against all *S. epidermidis* isolates tested.

**Table IV.** Viable cell number reduction in 48 h-biofilms, expressed as log<sub>10</sub> cfu/ml, after overnight exposure to double combinations of antibiotics

Isolates	VANC + TET	VANC + RIF	VANC + GENT	VANC + CEF
117977	1.33 ± 0.30	2.32 ± 0.36	0.00 ± 0.38	0.00 ± 0.18
132034	2.89 ± 0.49	4.22 ± 0.42	0.58 ± 0.24	0.25 ± 0.36
150271	0.11 ± 0.24	2.91 ± 0.12	0.28 ± 0.28	0.93 ± 0.78
1457	1.89 ± 0.63	2.47 ± 0.60	1.45 ± 0.59	2.21 ± 0.57
9142	1.72 ± 0.17	2.75 ± 0.43	0.47 ± 0.43	0.40 ± 0.37
Isolates	VANC + CEPH	VANC + LEVO	VANC + CLIND	TET + RIF
117977	0.00 ± 0.15	2.56 ± 0.38	1.75 ± 0.37	2.49 ± 0.49
132034	1.02 ± 0.40	0.53 ± 0.23	0.26 ± 0.50	3.37 ± 0.43
150271	1.75 ± 0.39	2.60 ± 0.35	2.16 ± 0.20	2.03 ± 0.24
1457	2.35 ± 0.44	2.13 ± 0.42	1.24 ± 0.35	2.09 ± 0.49
9142	0.39 ± 0.49	2.50 ± 0.37	0.68 ± 0.29	2.28 ± 0.40
Isolates	TET + GENT	TET + CEF	TET + CEPH	TET + LEVO
117977	1.43 ± 0.41	1.29 ± 0.48	1.07 ± 0.31	2.62 ± 0.43
132034	2.77 ± 0.35	2.41 ± 0.26	1.92 ± 0.48	3.14 ± 0.17
150271	0.23 ± 0.35	1.12 ± 0.45	1.78 ± 0.13	2.63 ± 0.15
1457	2.21 ± 0.48	1.84 ± 0.46	1.71 ± 0.83	2.07 ± 0.44
9142	2.01 ± 0.27	1.58 ± 0.49	1.51 ± 0.59	1.81 ± 0.29
Isolates	TET + CLIND	RIF + GENT	RIF + CEF	RIF + CEPH
117977	1.72 ± 0.26	2.46 ± 0.63	1.82 ± 0.24	1.76 ± 0.43
132034	2.86 ± 0.51	3.11 ± 0.56	3.45 ± 0.28	3.08 ± 0.32
150271	2.23 ± 0.52	2.49 ± 0.22	2.69 ± 0.45	2.73 ± 0.24
1457	1.96 ± 0.46	2.06 ± 0.16	1.85 ± 0.37	2.11 ± 0.55
9142	1.84 ± 0.18	3.14 ± 0.56	1.90 ± 0.20	1.41 ± 0.69
Isolates	RIF + LEVO	RIF + CLIND	GENT + CEF	GENT + CEPH
117977	3.49 ± 0.44	2.65 ± 0.33	0.00 ± 0.44	0.00 ± 0.39
132034	3.18 ± 0.56	3.68 ± 0.23	0.22 ± 0.13	0.79 ± 0.65
150271	2.83 ± 0.21	2.73 ± 0.49	0.06 ± 0.51	1.04 ± 0.42
1457	3.24 ± 0.42	2.46 ± 0.31	2.19 ± 0.35	2.49 ± 0.67
9142	1.71 ± 0.58	2.64 ± 0.27	0.39 ± 0.18	1.03 ± 0.44
Isolates	GENT + LEVO	GENT + CLIND	CEF + CEPH	CEF + LEVO
117977	3.76 ± 0.57	1.48 ± 0.31	0.00 ± 0.28	3.90 ± 0.30
132034	0.31 ± 0.37	0.14 ± 0.23	1.03 ± 0.43	0.11 ± 0.42
150271	2.91 ± 0.43	2.15 ± 0.25	1.82 ± 0.24	3.06 ± 0.55
1457	2.28 ± 0.43	1.98 ± 0.49	2.40 ± 0.47	2.55 ± 0.26
9142	1.83 ± 0.24	0.16 ± 0.38	1.92 ± 0.65	2.14 ± 0.58
Isolates	CEF + CLIND	CEPH + LEVO	CEPH + CLIND	LEVO + CLIND
117977	1.11 ± 0.25	3.38 ± 0.42	0.86 ± 0.32	2.68 ± 0.28
132034	0.29 ± 0.16	0.64 ± 0.19	0.87 ± 0.61	0.07 ± 0.30
150271	1.50 ± 0.25	2.74 ± 0.16	1.48 ± 0.27	2.54 ± 0.32
1457	1.44 ± 0.36	2.28 ± 0.50	1.75 ± 0.36	2.25 ± 0.42
9142	0.31 ± 0.43	2.72 ± 0.51	1.13 ± 0.45	2.33 ± 0.61

VANC, vancomycin; TET, tetracycline; RIF, rifampicin; GENT, gentamicin; CEF, cefazolin; CEPH, cephalothin; LEVO, levofloxacin; CLIND, clindamycin

Values are mean ± standard deviation. (n=4)

The results presented in Table IV show the reduction in biofilms  $\log_{10}$  cfu for all combinations of antibiotics tested. Most combinations tested did not promote a 3 log reduction in bacterial counts. Nevertheless, and as it could be expected, most of those containing rifampicin were able to reach at reasonable levels of bactericidal effect. Examples are rifampicin-clindamycin and rifampicin-gentamicin, the former promoting reductions above 2.5 log in biofilm cell counts for all isolates tested. Notably, the combination rifampicin-levofloxacin displayed a high killing effect specifically against three isolates but against 9142 the log reduction was below 2.0.

### Discussion

Standard antibiotic therapy is only able to eliminate planktonic cells, leaving the sessile forms to propagate within the biofilm and to continue to disseminate when therapy is terminated. In biofilms, microbes are protected from antimicrobial agents and the host immune system<sup>18</sup>. In fact, increasingly microorganisms have the ability to withstanding the effect of antibiotics and individual antibiotics are generally ineffective against bacteria biofilms. To overcome such problems, combination of antibiotics is a possible alternative to treat staphylococcal biofilm infections. Previous studies have also demonstrated impressive results with rifampicin, however, the risk of rapid development of resistance is a major problem, and rifampicin should not be used as monotherapy<sup>1,19,20</sup>. It has been considered that combinations of rifampicin with other anti-staphylococcal agents such as quinolones or fusidic acid could prevent the emergence of rifampicin resistance during therapy<sup>19,21</sup>.

Since antibiotics alone were generally not effective against *S. epidermidis* biofilms and taking into consideration the strategy of combined therapy to avoid resistance, the double combinations of the antibiotics were tested against the same biofilms. In a previous study<sup>22</sup>, where some double and triple combinations of antibiotics were studied, several triple combinations, all containing rifampicin were active against *S. epidermidis* and only one double combination vancomycin - rifampicin was reported to be active. In this study, 17 *S. epidermidis* isolates were assessed and the susceptibility to antibiotics was tested in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). However, a triple combination may be an overload of antibiotics and more prone to the development of secondary effects.

Monzón *et al*<sup>11</sup> also tested some double combinations of antibiotics against four *S. epidermidis* isolates and the highest reduction they observed was 2.19 log obtained with the combination vancomycin-rifampicin and only against one specific isolate, using both antibiotics at 4 x MIC.

In this study, we tested the effect of traditional antibiotics alone and in combination, in *S. epidermidis* biofilm cells, using breakpoint concentrations<sup>13,22</sup>. The Calgary Biofilm Device (CBD) was used for biofilm formation because, despite being expensive, it is a high-throughput methodology with several advantages, namely its multiple equivalent biofilms that can be used for testing. Its use can avoid the need to repeat assays, which is usually necessary when using standard plates. An array of antimicrobial compounds with varying concentrations can easily be assessed. This allows rapid testing of compounds for antibiofilm activity. These advantages can overcome the cost associated problems of this methodology.

Our results demonstrated the advantage of using antibiotic combinations in the treatment of *S. epidermidis* infections. However, the effect of these combinations is highly strain-dependent. Alternative agents are novel antibiotics such as linezolid, tigecycline and daptomycin claimed to be highly effective against biofilms but these agents have some disadvantages. Apart from their high cost, these have been in clinical use for a short time only and the extent of their toxicity is yet to be experienced. Hajdu *et al*<sup>4</sup> observed no significant reduction in *S. epidermidis* biofilms cfu with daptomycin and tigecycline, not even at the highest concentrations tested ( $128 \times \text{MIC}$ ). Generally these concentrations are far beyond any concentration that can be achieved after administration of standard therapeutic doses<sup>4</sup>. Aslam *et al*<sup>23</sup> also tested the effect of tigecycline and after 12 h of treatment only a mean reduction of the bacterial growth by  $2\log_{10}$  counts was obtained, notably using a concentration of 1 mg/ml (1,000 fold higher than its MIC for the organisms tested in the planktonic phase). In this case, the concentration of tigecycline expected to be in human serum after standard dosing is 2 mg/l<sup>4</sup>. Utilizing high doses of antimicrobials to eradicate biofilm has had limited success in the clinical setting<sup>23</sup>.

In conclusion, there are some combinations of more traditional antibiotics that can be strongly considered as therapeutic strategies for an efficient control of *S. epidermidis* biofilms associated infections. Rifampicin combined with clindamycin or with gentamicin showed

to have the broadest range of action, although rifampicin in combination with levofloxacin displayed a higher killing effect against three out of the five isolates. As an alternative to monotherapy, these combinations can be advantageous avoiding the likelihood of resistance development.

### References

- Hellmark B, Unemo M, Nilsson-Augustinsson Å, Söderquist B. Antibiotic susceptibility among *Staphylococcus epidermidis* isolated from prosthetic joint infections with special focus on rifampicin and variability of the *rpoB* gene. *Clin Microbiol Infect* 2009; 15 : 238-44.
- Otto M. *Staphylococcus epidermidis*- the "accidental" pathogen. *Microbiology* 2009; 7 : 555-67.
- Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M. Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J Infect Dis* 2003; 188 : 706-18.
- Hajdu S, Lassnigg A, Graninger W, Hirschl AM, Presterl E. Effects of vancomycin, daptomycin, fosfomycin, tigecycline, and ceftriaxone on *Staphylococcus epidermidis* biofilms. *J Orthop Res* 2009; 27 : 1361-5.
- Knobloch JK-M, von Osten H, Horstkotte MA, Rohde H, Mack D. Minimal attachment killing (MAK): a versatile method for susceptibility testing of attached biofilm-positive and -negative *Staphylococcus epidermidis*. *Med Microbiol Immunol* 2002; 191 : 107-14.
- Cargill JS, Upton M. Low concentration of vancomycin stimulate biofilm formation in some clinical isolates of *Staphylococcus epidermidis*. *J Clin Pathol* 2010; 62 : 1112-6.
- Gilbert P, Das J, Foley I. Biofilm susceptibility to antimicrobials. *Adv Dent Res* 1997; 11 : 160-7.
- Mah T-FC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobials agents. *Trends Microbiol* 2001; 9 : 34-9.
- Gualtieri M, Bastide L, Villain-Guillot P, Michaux-Charachon S, Latouche J, Leonetti J. *In vitro* activity of a new antibacterial rhodanine derivative against *Staphylococcus epidermidis* biofilms. *J Antimicrob Chemother* 2006; 58 : 778-83.
- Lyytikäinen O, Vaara M, Jfirviluoma E, Rosenqvist K, Tiittanen L, Valtonen V. Increased resistance among *Staphylococcus epidermidis* isolates in a large teaching hospital over a 12-year period. *Eur J Clin Microbiol Infect Dis* 1996; 15 : 133-8.
- Monzón M, Oteiza C, Leiva J, Amorena B. Synergy of different antibiotic combinations in biofilms of *Staphylococcus epidermidis*. *J Antimicrob Chemother* 2001; 48 : 793-801.
- Piette A, Verschraegen G. Role of coagulase-negative staphylococci in human disease. *Vet Microbiol* 2009; 134 : 45-54.
- Clinical and Laboratory Standards Institute. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*, 8<sup>th</sup> ed. Approved Standard M7-A8. CLSI, Wayne, PA, USA; 2010.
- Ceri H, Olson ME, Stremick C, Read RR, Morck DW, Buret AG. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities in bacterial biofilms. *J Clin Microbiol* 1999; 37 : 1771-6.
- Bardouniotis E, Huddleston W, Ceri H, Olson ME. Characterization of biofilm growth and biocide susceptibility testing of *Mycobacterium phlei* using MBEC™ assay system. *FEMS Microbiol Lett* 2001; 203 : 263-7.
- Harrison JJ, Turner RJ, Joo DA, Stan MA, Chan CS, Allan ND, *et al*. Copper and quaternary ammonium cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2008; 52 : 2870-81.
- Cerca N, Martins S, Cerca F, Jefferson KK, Pier GB, Oliveira R, *et al*. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. *J Antimicrob Chemother* 2005; 56 : 331-6.
- Wang X, Yao X, Zhu Z, Tang T, Dai K, Sadovskaya I, *et al*. Effect of berberine on *Staphylococcus epidermidis* biofilm formation. *Int J Antimicrob Agents* 2009; 34 : 60-6.
- Mick V, Domínguez MA, Tubau F, Liñares J, Pujol M, Martín R. Molecular characterization of resistance to rifampicin in an emerging hospital-associated methicillin-resistant *Staphylococcus aureus* clone ST228, Spain. *BMC Microbiol* 2010; 10 : 68.
- Zavasky DM, Sande MA. Reconsideration of rifampin: a unique drug for a unique infection. *JAMA* 1998; 279 : 1575-7.
- Moellering RC. Current treatment options for community-acquired methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* 2008; 46 : 1032-7.
- Saginur R, St. Denis M, Ferris W, Aaron SD, Chan F, Lee C. Multiple combination bactericidal testing of Staphylococcal biofilms from implant-associated infections. *Antimicrob Agents Chemother* 2006; 50 : 55-61.
- Aslam S, Trautner BW, Ramanathan V, Darouiche RO. Combination of tigecycline and *N*-acetylcysteine reduces biofilm-embedded bacterial on vascular catheters. *Antimicrob Agents Chemother* 2007; 51 : 1556-8.

Reprint requests: Dr Rosário Oliveira, IBB-Institute for Biotechnology & Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal  
e-mail: roliveira@deb.uminho.pt