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ORIGINAL ARTICLE

Staphylococcus aureus biofilm formation and antibiotic susceptibility tests on polystyrene and metal surfaces

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Abstract

Aim: We compared the MBEC™-HTP assay plates made of polystyrene with metal discs composed of TMZF® and CrCo as substrates for biofilm formation.
Methods and Results: *Staphylococcus aureus* was grown on polystyrene and on metal discs made of titanium and chrome–cobalt. Antibiotic susceptibility was assessed by examining the recovery of cells after antibiotic exposure and by measuring the biofilm inhibitory concentration (BIC). The minimal inhibitory concentration (MIC) was assessed with planktonic cells. Bacterial growth was examined by scanning electron microscopy. The antibiotic concentration for biofilm inhibition (BIC) was higher than the MIC for all antibiotics. Microscopic images showed the biofilm structure characterized by groups of cells covered by a film.
Conclusions: All models allowed biofilm formation and testing with several antibiotics *in vitro*. Gentamicin and rifampicin are the most effective inhibitors of *Staph. aureus* biofilm-related infections. We recommend MBEC™-HTP assay for rapid testing of multiple substances and TMZF® and CrCo discs for low-throughput testing of antibiotic susceptibility and for microscopic analysis.
Significance and Impact of the Study: *In vitro* assays can improve the understanding of biofilms and help developing methods to eliminate biofilms from implant surfaces. One advantage of the TMZF® and CrCo discs as biofilm *in vitro* assay is that these metals are commonly used for orthopaedic implants. These models are usable for future periprosthetic joint infection studies.

Introduction

Metal surfaces are widely used in biomedical devices such as dental and orthopaedic implants. For example, titanium alloys and metal-on-metal bearings alloys in total hip arthroplasty are becoming more popular. However, they can also be colonized by biofilms, leading to implant-related infections (Hosman *et al.* 2009), which are a major financial burden and can be fatal (Oduwole *et al.* 2010). The most common bacteria that colonize implant surfaces are *Staphylococcus epidermidis* and *Staphylococcus aureus* (Costerton *et al.* 1995).

Bacteria adhering to implant surfaces evade host defences and become more resistant to antibiotics by forming biofilms (Brady *et al.* 2008; Kristian *et al.* 2008; Otto 2009; Schommer *et al.* 2011). Bacteria and the host defence cells compete for the implant surface. If connective tissue cells colonize first, they form a cell 'lawn' that inhibits bacterial adherence to the surface, protecting the implant from bacterial colonization. If, however, bacteria colonize the surface first, they can form a biofilm that is resistant to host immune cells. This competition between host cells and bacteria is called 'the race for surface' (Gristina 1994).

Improved understanding of the structure of biofilms and how they function will help to develop treatments to eliminate biofilms from implant surfaces. *In vitro* models of biofilms allow the testing of antimicrobial susceptibility

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and the analysis of biofilm architecture and molecular behaviour. Minimum Biofilm Eradication Concentration–High Throughput Plate (MBEC™-HTP) is an *in vitro* model for testing antibiotic susceptibility of biofilms (Moskowitz *et al.* 2004). This system allows several substances to be tested at the same time. Other models have been developed using intraocular lenses (Okajima *et al.* 2006), central venous catheters (Gaonkar and Modak 2003), bone grafts substitutes and metal surfaces (Clauss *et al.* 2010; Wanner *et al.* 2011) as substrates. Because TMZF® (TiMo12Zr6Fe2) and CrCo (Vitallium®-CoCr30Mo5) are used to manufacture knee and hip prosthesis, they could also be used to develop *in vitro* biofilm models, although biofilm growth on TMZF® and CrCo has not been previously examined.

In this study, we investigated whether biofilms grow *in vitro* on TMZF® and CrCo discs and on the MBEC™-HTP assay. The evaluation of the biofilms formed on different surfaces was assessed by comparing the antibiotic susceptibility of *Staph. aureus* and by examining the structure of *Staph. aureus* biofilms grown by scanning electron microscopy (SEM). We used *Staph. aureus* ATCC 29213 in this study because it has been previously shown to be a good biofilm former and produces mature biofilms after only 24 h (Ceri *et al.* 1999; Clauss *et al.* 2010).

Materials and methods

Bacterial strains

American Type Culture Collection *Staph. aureus* (ATCC 29213) was used in this study (Ceri *et al.* 1999; DeRyke *et al.* 2006). For the preparation of the inoculums, the lyophilized strains were freshly grown overnight on Müller–Hinton (MH) agar plates (Sigma-Aldrich, Hamburg, Germany). Discrete colonies were obtained from MH agar plates and resuspended in MH broth to a McFarland turbidity of 0·5.

Substrates for biofilm formation

MBEC™-HTP assay plates (Ceri *et al.* 1999) made of polystyrene were purchased from Innovotech (Edmonton, Alberta, Canada). TMZF® a titanium alloy (TiMo12Zr6Fe2) discs (Koban *et al.* 2011) and CrCo a chrome–cobalt–molybdenum alloy (Vitallium®-CoCr30Mo5) discs (Hosman *et al.* 2009), usually employed for joint replacement implants confection, were purchased from Stryker GmbH & Co KG (Duisburg, Germany). Each peg of the MBEC™-HTP plates had an area of 130 mm^2, while each TMZF® and CrCo disc had an area of 157 mm^2.

Antibiotics

Gentamicin and rifampicin were purchased from SERVA GmbH (Heidelberg, Germany), vancomycin from Astro Pharma (Vienna, Austria), fosfomycin from BioChemika-Fluka (Buchs, Switzerland), and clindamycin as well as linezolid from Pfizer (Vienna, Austria). For the antibiotic susceptibility tests, we chose the substances usually indicated for treatment of periprosthetic joint infections or for *Staph. aureus* infections (Raad *et al.* 1995; Anguita-Alonso *et al.* 2005; Buttaro *et al.* 2005; Trampuz and Zimmerli 2005; Colli *et al.* 2007; Teller *et al.* 2007; Ensing *et al.* 2008; Fujimura *et al.* 2008; Schiefer *et al.* 2008; Swieringa *et al.* 2008; Michalopoulos *et al.* 2011; Nadrah and Strle 2011; Coraça-Huber *et al.* 2012; Tang *et al.* 2012).

Minimum inhibitory concentration (MIC)

The MIC was determined for each antibiotic by placing Etest strips (Biomérieux, Marcy L'Etoile, France) (Rennie *et al.* 2012) on MH agar plates inoculated with *Staph. aureus* (ATCC 29213) for 24 h at 37°C. Assays were carried out in triplicate.

Biofilm formation and antibiotic susceptibility tests for *Staphylococcus aureus* grown in the MBEC™-HTP assay

Two millilitres of 2×10^5 CFU ml^{-1} of *Staph. aureus* was added to each MBEC™-HTP plate. The plates were covered and incubated at 37°C for 48 h on a rocking table at 12 cycles min^{-1}. For antibiotic susceptibility tests, after 48 h, the lids were removed from the MBEC™-HTP plates, rinsed in saline solution for 1 min to remove the planktonic cells, added to challenge plate containing 100 μl per well of antibiotics dilutions in MH broth (1, 32 or 256 $\mu g\ ml^{-1}$) and incubated at 37°C for 24 h. The challenge plates were removed from the incubator, and the lids containing biofilms were rinsed in saline solution for 1 min and placed on a new 96-well plate containing MH broth without antibiotics (recovery plate). To detach the biofilms from the pegs in the lid, the recovery plates were placed in a sonicator (Transsonic 570 Elma®, Singen, Germany) and sonicated for 5 min on the high setting. The MBEC™-HTP lids were removed and replaced with a conventional microtitre plate lid, and the recovery plates were incubated at 37°C for 24 h. After 2 and 5 h (Coraça-Huber *et al.* 2012), 10 μl of suspended cells was transferred from each well to individual MH agar plates. After 24 h at 37°C, colony-forming units (CFU) were counted. Results from 5 h were used to determine the biofilm inhibitory concentration (BIC), which was considered the lowest concentration of antibiotic that resulted in no

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bacterial growth. All experiments were carried out in triplicate, and all antibiotic concentrations were tested in triplicate.

Biofilm formation and antibiotic susceptibility tests for cells grown for *Staphylococcus aureus* grown on TMZF® and CrCo discs

TMZF® and CrCo discs were sterilized in an autoclave and placed in 15-ml Falcon tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing MH broth inoculated with 2×10^5 CFU ml^{-1} of *Staph. aureus* (ATCC 29213). The tubes containing the discs were incubated at 37°C for 48 h on a rocking table at 12 cycles min^{-1}. After 48 h, the discs were removed from the original tubes, rinsed in saline solution for 1 min to remove the planktonic cells, added to tubes containing 2 ml of antibiotic (1, 32 or 256 μg ml^{-1}) and incubated at 37°C for 24 h.

For antibiotic susceptibility assays, after 24 h, the discs were removed from the tubes containing antibiotics, rinsed in saline solution for 1 min and added to new tubes containing MH broth without antibiotics. To detach the biofilms from the discs, the recovery tubes were placed in a sonicator (Transsonic 570 Elma®) and sonicated for 5 min on the high setting. The discs were then removed, and the tubes were incubated at 37°C. After 2 and 5 h, 10 μl of each sample was transferred to a MH agar plate. After 24 h at 37°C, CFU were counted. Results from 5 h were used to determine the BIC, which was considered the lowest concentration of antibiotic that resulted in no bacterial growth. All experiments were carried out in triplicate, and all antibiotic concentrations were tested in triplicate.

Scanning electron microscopy (SEM)

For bacteria grown in the MBEC™-HTP assay, the pegs were removed from the lids with flamed pliers and rinsed in saline solution for 1 min to remove planktonic cells. For bacteria grown on TMZF® and CrCo discs, the discs were rinsed in saline solution for 1 min. Pegs and discs were fixed with 2·5% glutaraldehyde (BioChemika Fluka) in 0·1 mol l^{-1} phosphate buffer (pH 7·4) before and after antibiotic treatment. After a brief wash in phosphate buffer, followed by postfixation for 1 h with 1% aqueous osmium tetroxide (ReagentPlus®; Sigma-Aldrich), samples were gradually dehydrated with ethanol. After critical-point drying (CPD 030, Bal-Tec), specimens were mounted on aluminium stubs with double-sided adhesive tape, sputter-coated with 10-nm Au/Pd (Bal-Tec) and examined with a field emission scanning electron microscope (Gemini 982; Zeiss, Goettingen, Germany).

Statistical analysis

Statistical comparisons were made by Spearman nonparametric correlations. A *P*-value below 0·05 was considered statistically significant. All calculations were made using SPSS version 17 (SPSS Inc., Chicago, IL, USA).

Results

Biofilm formation on MBEC™-HTP, TMZF® and CrCo surfaces

The CFU counting from biofilms formed on the different surfaces without antibiotic treatment showed the presence of more cells on the TMZF® and CrCo discs compared to MBEC™-HTP after 2-h incubation. After 5 h, the number of CFU was equal on both surfaces. On the MBEC™-HTP peg surfaces, the number of CFU was $3{\cdot}9_{log10}$ after 2-h incubation and $7{\cdot}0_{log10}$ after 5-h incubation. The CFU counting from biofilms formed on TMZF® and CrCo discs were similar for both surfaces with $6{\cdot}0_{log10}$ after 2 h and $7{\cdot}0_{log10}$ after 5 h (Figs 1 and 2).

Antibiotic susceptibility of *Staphylococcus aureus* grown on MBEC™-HTP plates and on TMZF® and CrCo discs

We first examined the antibiotic sensitivity of *Staph. aureus* grown on MBEC™-HTP and on TMZF® and CrCo discs by assessing the recovery after exposure to several antibiotics. For cells grown in MBEC™-HTP assays, recovery was the slowest following exposure to gentamicin and rifampicin. The concentrations of 32 and 256 μg ml^{-1} of gentamicin and rifampicin showed better efficacy when compared with 1 μg ml^{-1} after 2 and 5 h. For cells grown on TMZF® discs, recovery was the slowest following exposure to vancomycin, and for cells grown on CrCo discs, recovery was the slowest following exposure to both gentamicin and vancomycin. In these cases, the concentrations of 32 and 256 μg ml^{-1} also showed better efficacy after 2- and 5-h incubation when compared with 1 μg ml^{-1}. These findings indicate that gentamicin and rifampicin are the most potent antibiotics against *Staph. aureus* grown on MBEC™-HTP, vancomycin against cells grown on TMZF®, and gentamicin and vancomycin against cells grown on CrCo discs (Figs 1 and 2).

Scanning electron microscopy

SEM revealed extensive cell attachment on the MBEC™-HTP peg surface (Fig. 3a). A three-dimensional structure is visible with deep layers of bacteria surrounded by an amorphous matrix in some areas (Fig. 3b). Similar biofilm structures can be observed on the TMZF® surface.

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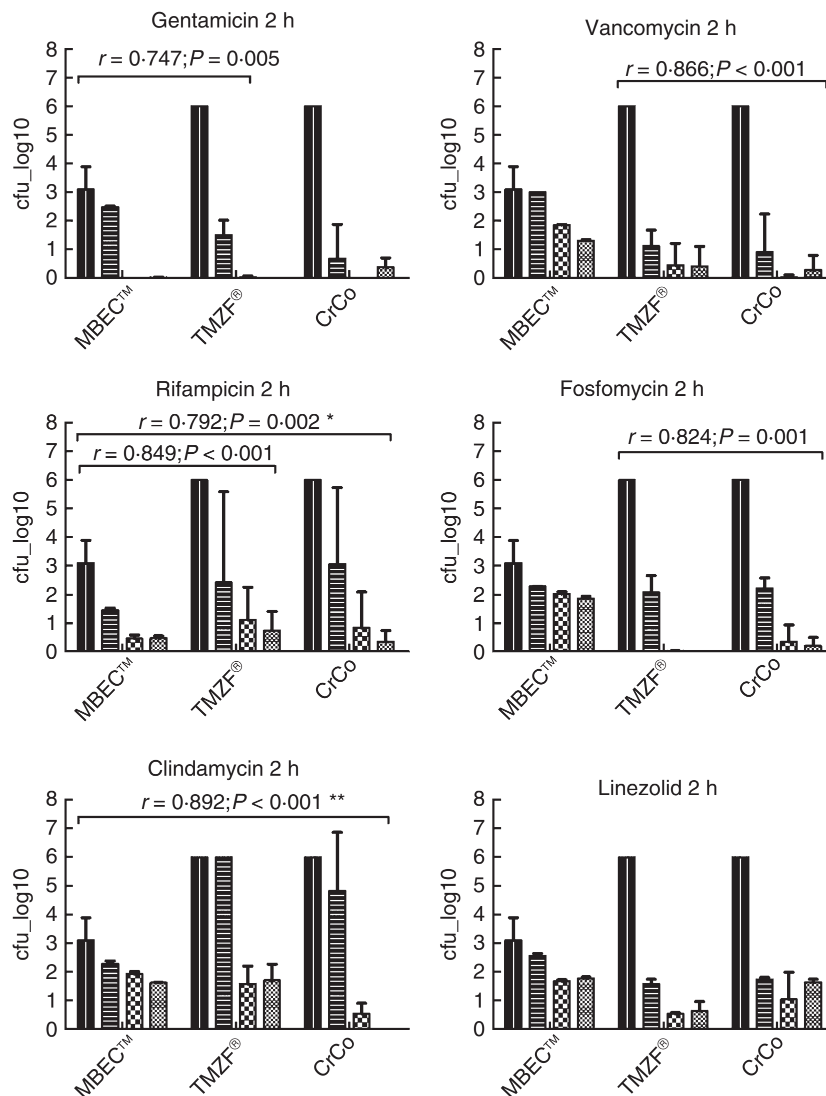

Figure 1 Antibiotic susceptibility of *Staphylococcus aureus* grown on MBEC™-HTP plates, TMZF® and CrCo discs. *Staphylococcus aureus* were grown on MBEC™-HTP plates and on TMZF® and CrCo discs for 48 h and incubated with 1, 32 or 256 μg ml^{-1} of the indicated antibiotics for 24 h. The media were replaced with antibiotic-free media, and CFU were counted after 2 h. The correlation between most potent antibiotics (all concentrations) for each method used for biofilm formation is indicated as *r* = (correlation coefficient) and *P* = (significance). Threshold: min = 1 - CFU_{log10} and max = 7 CFU_{log10}. Each test was carried out in triplicate. (▥) Control; (▤) 1 μg ml^{-1}; (▩) 32 μg ml^{-1} and (▦) 256 μg ml^{-1}.

Also, an agglomeration of cells is visible in some areas of the biofilm (Fig. 3c), and some of these are covered by a film or granule-like elements (Fig. 3d). A well-structured biofilm can be seen on the CrCo disc surface (Fig. 3e), with a three-dimensional structure covered by a matrix-like film and occasional channels between the cells (Fig. 3f). The biofilm structure observed on MBEC™-HTP was similar to the structure observed on TMZF® surfaces. On CrCo discs, the biofilm structure was more island-like when compared to MBEC™-HTP and TMZF® surfaces that showed biofilms covering bigger areas.

Comparison of antibiotic sensitivity of *Staphylococcus aureus* grown on the MBEC™-HTP assay, TMZF® and CrCo discs and *Staphylococcus aureus* grown in planktonic cultures

We next examined the sensitivity of *Staph. aureus* grown in the biofilm systems and in planktonic cultures to gentamicin, vancomycin, rifampicin, fosfomycin, clindamycin and linezolid (Table 1). Irrespective of the antibiotic tested, the MICs for planktonic cultures were ≤2 μg ml^{-1}. In contrast, the BICs were above the maximum concentration tested for all assays (>32 μg ml^{-1} gentamicin on

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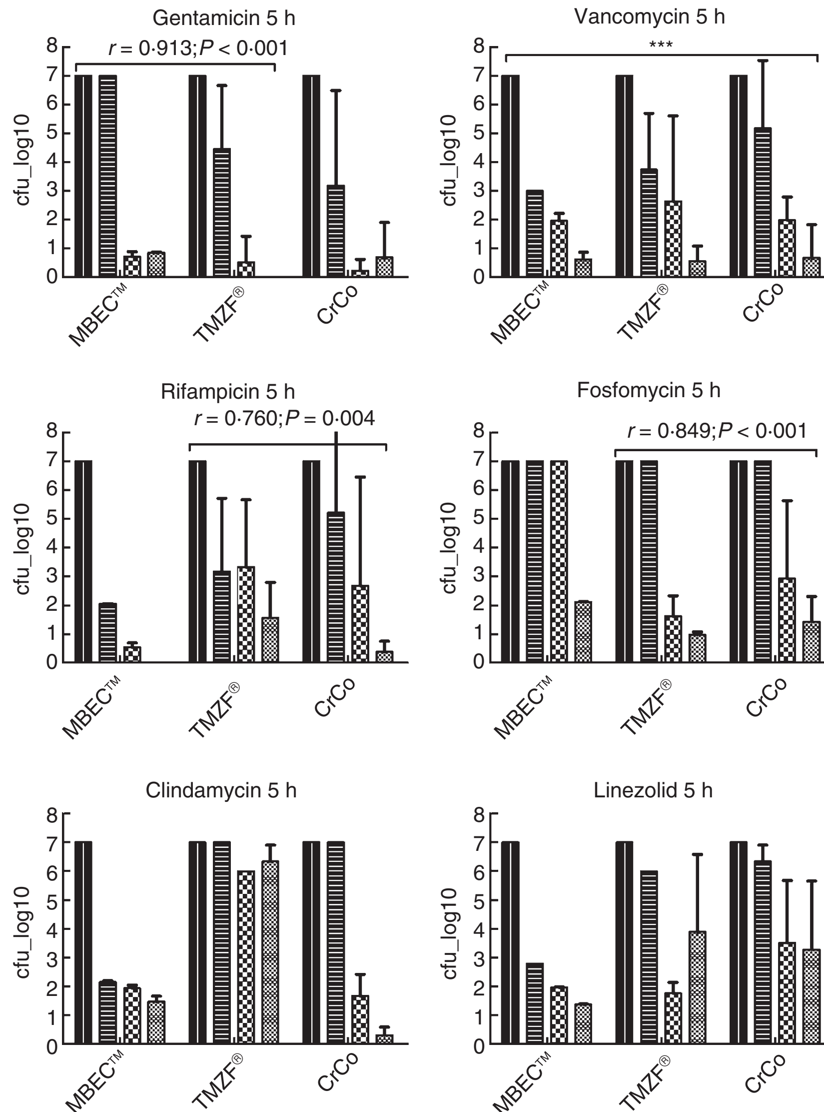

Figure 2 Antibiotic susceptibility of *Staphylococcus aureus* grown on MBEC™-HTP plates, on TMZF® and CrCo discs. *Staphylococcus aureus* were grown on MBEC™-HTP plates and on TMZF® and CrCo discs for 48 h and incubated with 1, 32 or 256 μg ml^{-1} of the indicated antibiotics for 24 h. The media were replaced with antibiotic-free media, and CFU were counted after 5 h. The correlation between most potent antibiotics (all concentrations) for each method used for biofilm formation is indicated as r = (correlation coefficient) and P = (significance). Threshold: min = 1 - CFU_{log10} and max = 7 CFU_{log10}. Each test was carried out in triplicate. ***Correlation between MBEC™-HTP and TMZF® (r = 0·775; P = 0·003), between MBEC™-HTP and CrCo (r = 0·860; P < 0·001) and between TMZF® and CrCo (r = 0·915; P < 0·001). (▥) Control; (▤) 1 μg ml^{-1}; (▩) 32 μg ml^{-1} and (▦) 256 μg ml^{-1}.

the TMZF® disc assay and 256 μg ml^{-1} in all other cases). However, all antibiotics inhibited the bacterial growth to some extent (data not shown). These results show that the BIC was higher than the MIC for all antibiotics.

Discussion

In this study, we compared the MBEC™-HTP assay and TMZF® and CrCo discs as models for biofilm formation *in vitro*. We found that biofilms were formed in all three systems. Each system provided different advantages and disadvantages, although all three models allowed estimation of the BIC.

For the antibiotic susceptibility tests, we chose the substances usually indicated for treatment of periprosthetic joint infections or for *Staph. aureus* infections. The substances used were gentamicin (Anguita-Alonso *et al.* 2005; Teller *et al.* 2007; Swieringa *et al.* 2008; Nadrah and Strle

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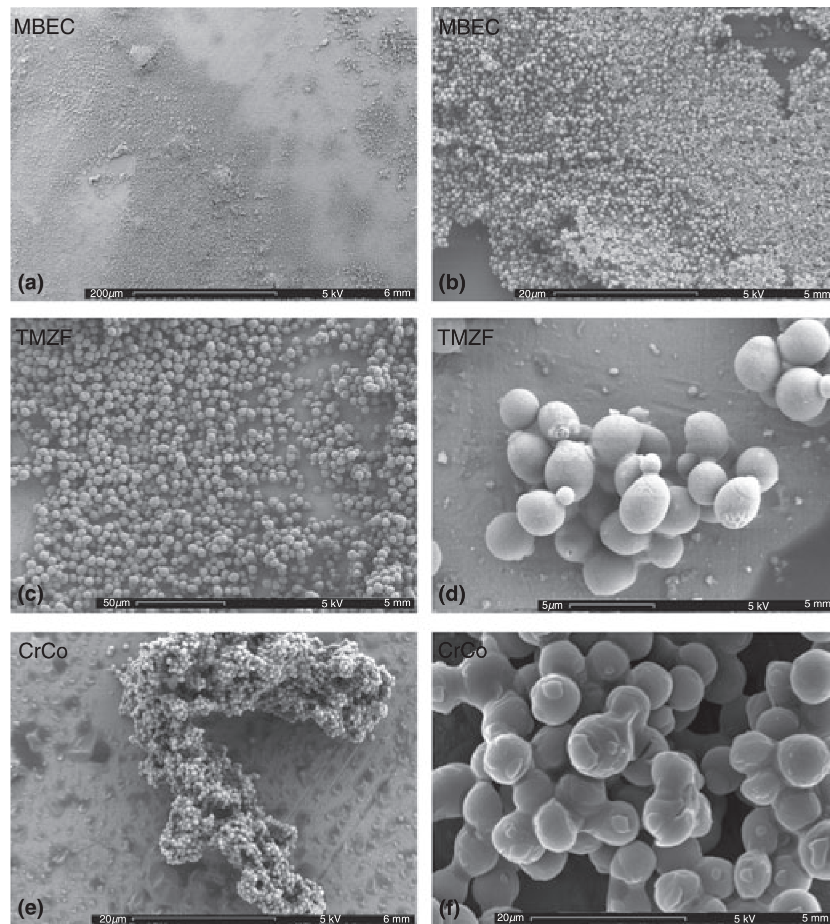

Figure 3 SEM of cells grown on MBEC™-HTP plates (a, b), TMZF® discs (c, d) and CrCo disc (e, f). Cells were grown for 48 h. Magnification = 200× (a), 500× (b, c, e), 5000× (d), and 20 000× (f).

Table 1 Comparison of MICs and BICs for *Staphylococcus aureus*

		BIC (μg ml^{-1})		
Antibiotic	MIC (μg ml^{-1})	MBEC™-HTP	TMZF® discs	CrCo discs
Gentamicin	0·25	>256	>32	>256
Vancomycin	1·00	>256	>256	>256
Rifampicin	<0·016	>256	>256	>256
Fosfomycin	1·00	>256	>256	>256
Clindamycin	0·094	>256	>256	>256
Linezolid	2·00	>256	>256	>256

MICs, minimal inhibitory concentration; BICs, biofilm inhibitory concentration.

2011), vancomycin (Buttaro *et al.* 2005; Fujimura *et al.* 2008), rifampicin (Raad *et al.* 1995; Trampuz and Zimmerli 2005; Coraça-Huber *et al.* 2012), fosfomycin (Michalopoulos *et al.* 2011; Tang *et al.* 2012), clindamycin (Ensing *et al.* 2008; Schiefer *et al.* 2008) and linezolid (Colli *et al.* 2007). The incubation periods chosen to evaluate the efficacy of the antibiotic substances in this study were 2 and 5 h. A previous study carried out by our team showed that the number of CFU after 24 h of incubation with all antibiotics extrapolated the threshold of 7 CFU_{log10}. For this reason, we exclude the period of 24-h incubation from this study (Coraça-Huber *et al.* 2012).

SEM images of the biofilms obtained in all three models showed groups of cells embedded in an amorphous substance. In all cases, the cells had a similar organization and an amorphous structure covering the cells. Such an amorphous structure around bacteria and clumps of bacteria embedded in a dense matrix is considered characteristic of a biofilm (Nickel *et al.* 1985; Walsh *et al.* 1986; Henry-Stanley *et al.* 2010). The differences found between BIC and MIC in our study further support the fact that the cells developed some kind of resistance against the antibiotics, which could also indicate biofilm formation. The BIC and MIC would be similar if only cumulative planktonic cells were present, and the lower antibiotic susceptibility of the cells grown on the different surfaces

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suggests the presence of a protective extracellular matrix, in agreement with the SEM findings.

Antibiotic sensitivity tests showed that gentamicin was the most potent substance against *Staph. aureus* biofilms. Independent of model or time of incubation, gentamicin was able to eradicate more bacteria than the other antibiotics tested. For the biofilms grown on MBEC™-HTP plates, in addition to gentamicin, rifampicin showed high potency against biofilms. For the biofilms grown on TMZF® and the CrCo discs, gentamicin, vancomycin and also fosfomycin showed high potency against *Staph. aureus* (ATCC 29213) biofilms. The differences in the efficacy of vancomycin and fosfomycin observed between the methods could be due to the surface area of the different substrates. TMZF® and the CrCo discs have more area for the growth of the biofilms compared to the MBEC™-HTP pegs. Apart from the difference in surface area of the substrates, the surface structure could also be a reason for differences in the efficacy of the antibiotics. Topographical features can influence the arrangement and the resulting behaviour of cells on surfaces and may affect biofilm growth. However, the roles of specific surface structures in modifying bacterial attachment and subsequent behaviour remain unclear (Hochbaum and Aizenberg 2010; Epstein *et al.* 2011).

For the control groups, the number of CFU obtained from biofilms formed on the discs after 2 h of incubation was two times higher than the CFU obtained from MBEC™-HTP plates. This could explain the difference in efficacy between some antibiotics, as observed for vancomycin and fosfomycin. The efficacy of gentamicin, rifampicin, vancomycin (Joosten *et al.* 2005; Kotulova and Slobodnikova 2010; Michalopoulos *et al.* 2011) and fosfomycin (Tang *et al.* 2012) against *Staph. aureus* biofilms was also described by other researchers in previous works.

The MBEC™-HTP plates allowed us to obtain biofilms and to rapidly and easily test several antibiotics. However, we modified the procedure for detaching the biofilms. According to the manufacturer, the plates should be placed inside the sonicator without any water contact, which contradicts the sonication principles which say that water is necessary for the propagation of the acoustic impulse and detachment of the biofilm from the pegs. Contact of the MBEC™-HTP plates with the water of the sonicator, even after sealing the plates, increases the risk of contamination. Therefore, unlike Ceri *et al.* (1999), we think that the MBEC™-HTP plates may have some limitations for studying biofilms *in vitro*. In addition, for SEM, the MBEC™-HTP pegs must be removed from the plates using pliers, which is difficult. This could also disturb the biofilm structure, and it increases the risk of contamination. This could decrease the accuracy of the microscopic analysis. The risk of contamination or damage is less with TMZF® and CrCo discs because less manipulation is needed.

All three models were effective for biofilm formation and testing. The MBEC™-HTP assay has the advantage of high throughput. One disadvantage of this system was observed during the sonication process. This step could increase the risks of contamination since the plates are not adapted to an ultrasonic bath and even after sealing them some water could reach the interior. Therefore, we recommend the MBEC™-HTP assay for tests involving several antibiotics or other substances and the TMZF® and CrCo discs for low-throughput antibiotic susceptibility tests and for microscopic analysis. Finally, the findings suggest that gentamicin and rifampicin are good choices for treating *Staph. aureus* biofilm-related infections.

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