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The relationship between inhibition of bacterial adhesion to a solid surface by sub-MICs of antibiotics and subsequent development of a biofilm

Nuno Cerca [a], Silvia Martins [a], Gerald B. Pier [b], Rosário Oliveira [a], Joana Azeredo [a,*]

[a] *Centro de Engenharia Biológica, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[b] *Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA*

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Abstract

Many studies have demonstrated that subminimal inhibitory concentrations (sub-MICs) of antibiotics can inhibit initial microbial adherence to medical device surfaces. It has been suggested that, by inhibiting initial adhesion, biofilm formation might be prevented. However, since initial adherence and subsequent biofilm formation may be two distinct phenomena, conclusions regarding the effects of sub-MIC antibiotics on initial adhesion cannot be extrapolated to biofilm formation. In this study, we evaluated the adherence of several clinical isolates of coagulase-negative staphylococci (CoNS) to acrylic and the effect of sub-MICs of vancomycin, cefazolin, dicloxacillin and combinations of these antibiotics on adherence and biofilm formation. Most of the antibiotics used resulted in effective reduction of bacterial adherence to acrylic, in some cases reaching over 70% inhibition of adherence. When strains with a high biofilm-forming capacity were grown in sub-MICs of those antibiotics, there existed combinations of the drugs that significantly inhibited biofilm formation. However, most of the antibiotic combinations that inhibited adherence did not have a profound effect on biofilm formation. When comparing the results of the effect of sub-MIC amounts of antibiotics in inhibiting adherence with their effect on the inhibition of biofilm formation, significant differences were found, mainly when using combinations of antibiotics. In general, the effect on the inhibition of adherence was greater than the effect on inhibiting biofilm formation. These results demonstrate that assays evaluating the inhibition of initial adherence to medical surfaces cannot fully predict the effect on inhibition of biofilm formation.

Keywords: Adhesion; Antibiotics sub-MIC; Biofilm; CoNS; Inhibition

1. Introduction

Staphylococcus epidermidis and other coagulase-negative staphylococci (CoNS) are now recognized to be one of the most common causes of serious nosocomial infections [30]. This is related, in part, to the organism's ability to adhere to indwelling medical devices and form biofilms [31]. A major barrier to the long-term use of medical devices is development of biofilm infection [4]. When growing and surviving in biofilms, CoNS are more resistant to antibiotic agents when compared to planktonic cells [2,15,25,29], and often the antibiotic concentration needed to eradicate the biofilm is above the peak serum concentration of the antibiotic [19], rendering it ineffective in treating biofilm infections. Despite several efforts to find medical therapies to treat biofilm infections, the physical removal of an infected medical device is often necessary [16], which carries an additional economic cost. Therefore, there is great interest in finding methods or strategies to inhibit biofilm formation.

Several strategies have been proposed to inhibit biofilm formation on medical devices, including the administration of sub-MICs of antibiotics [5,12,17], use of furanone compounds [4], anti-inflammatory drugs [3], bacterial extracts

* Corresponding author.
E-mail address: jazeredo@deb.uminho.pt (J. Azeredo).

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[14], development of new anti-adhesive medical surfaces [8,22] and coating medical devices with several different compounds, including antibiotics [16,21,28].

It has been demonstrated that sub-MICs of antibiotics are able to modify the physicochemical properties and the architecture of the outer surface of *S. epidermidis*, affecting overall virulence [26]. Sub-MICs of antibiotics have been successfully used to inhibit bacterial initial adhesion to abiotic substrates [17] and it was suggested that these studies could provide insights into preventing biofilm formation on medical devices [12]. Several studies have already demonstrated that initial adherence to a surface and subsequent biofilm formation can be two independent phenomena [32], and so conclusions drawn regarding an effect of sub-MIC antibiotics on initial bacterial adherence may not be directly extrapolated to biofilm formation.

In this study, we used sub-MICs of antibiotics as a tool to assess the relevance of inhibiting adhesion as a way of preventing subsequent biofilm inhibition. We evaluated the changes in both initial adhesion and in biofilm formation of several strains of CoNS growing on acrylic in the presence of sub-MICs of cefazolin, vancomycin, dicloxacillin, and combinations of these drugs. We were particularly interested in determining if there is a correlation between inhibition of bacterial adherence and subsequent development of a biofilm, as both components of device related infection would need to be inhibited in order for a prophylactic or therapeutic strategy to be effective.

2. Materials and methods

2.1. Bacterial strains

Five *S. epidermidis* strains and 2 *S. haemolyticus* strains were used. *S. epidermidis* 9142 is a known producer of the major surface polysaccharide promoting CoNS adherence and biofilm formation, poly-*N*-acetyl glucosamine (PNAG). *S. epidermidis* IE75, *S. epidermidis* IE186 and *S. haemolyticus* IE246 were isolated from infective endocarditis patients; *S. epidermidis* M129, *S. haemolyticus* M176 and *S. epidermidis* M187 were isolated from patients with peritonitis associated with renal dialysis.

2.2. Substrate preparation

Acrylic was cut into 20 × 20 mm squares that were immersed in a 0.2% solution of a commercial detergent overnight, after which they were transferred to a new solution of 0.2% of a commercial detergent and washed at 40 °C with strong agitation for 5 min. The squares and plates were then rinsed thoroughly with distilled water followed by rinsing with ultra-pure water and dried at 60 °C, overnight. For biofilm assays, surfaces were heat-sterilized by immersion in distilled water and autoclaving at 121 °C for 15 min.

2.3. Antibiotics and determination of the MIC value

The antibiotics used in this study were cefazolin, vancomycin and dicloxacillin, which act as inhibitors of cell wall synthesis and are routinely used to treat staphylococcal infections [11,18,24]. Determination of the MIC range for each strain was carried out according to NCCLS standards [20]. The sub-MIC used was $\frac{1}{2}$ of the lowest MIC value, whenever just one antibiotic was added to the bacterial cell suspension, and $\frac{1}{4}$ of the MIC value, whenever combinations of two antibiotics were added to the bacterial cell suspension. These concentrations were not high enough to inhibit bacterial growth, except in a few specific cases of synergism, well indicated in Section 3.

2.4. Inhibition of initial adhesion

2.4.1. Growth conditions

Tryptic soy broth (TSB) and tryptic soy agar (TSA) were prepared according to the manufacturer's instructions. All strains were inoculated into 15 ml of TSB from TSA plates not older than 2 days. Liquid cultures were grown for 24 (±2) h at 37 °C in an orbital shaker at 130 rpm. The cells were harvested by centrifugation (for 5 min at 10 500 *g* at 4 °C), then washed and resuspended in a saline solution (0.9% NaCl prepared in distilled water) to an optical density equivalent to 1×10^9 cells ml^{-1}. This suspension was used in the biofilm assays. For adherence assays, 1 ml of this cell suspension was transferred to 30 ml of fresh TSB containing sub-MICs of antibiotics, and incubated for 18 (±2) h at 37 °C with shaking at 130 rpm. After being harvested by centrifugation (for 5 min at 10 500 *g* at 4 °C), cells were washed twice and resuspended in a saline solution (0.9% NaCl prepared in distilled water) and adjusted to an optical density equivalent to 1×10^9 cells ml^{-1} and used in the adherence assays.

2.4.2. Static adherence

Static adherence was performed as described previously [6]. Briefly, squares of acrylic were placed in 6-well tissue-culture plates containing 6 ml of a cell suspension grown in the presence of sub-MICs of antibiotics and adjusted to an optical density equivalent to 1×10^9 cells ml^{-1}. Initial adhesion to acrylic was allowed to occur for 2 h at 37 °C in a shaker at 120 rpm. Negative controls were obtained by placing acrylic in a saline solution without bacterial cells. The squares were then carefully washed by immersion. The acrylic squares with adherent bacterial cells were dried at 37 °C. All experiments were done in triplicate, with 4 repeats.

2.4.3. Image analysis

For image observation and enumeration of adherent bacterial cells, the acrylic squares were stained with a 0.2% safranin solution, for contrast. Direct bacterial counts were done using a phase contrast microscope coupled to a 3CCD

video camera that acquires images with 820 × 560 pixel resolution at a magnification of 400×. With this magnification 1 cm^2 is equivalent to 1.823×10^4 captured images (as determined with a Neubauer chamber). For each surface analyzed, 20 images were taken. Cells were counted using automated enumeration software.

2.5. Biofilm formation

2.5.1. Biofilm assays

Formation of bacterial biofilms was performed as described previously [7]. Briefly, sterilized acrylic squares were placed in 6-well tissue culture plates containing 6 ml of TSB supplemented with 0.25% of glucose and the respective amount of antibiotic. Then 200 µl of a 0.9% NaCl solution containing 1×10^9 cells ml^{-1} were added and growth was allowed to occur for 48 h at 37 °C in a shaker at 120 rpm. Every 8 h the TSB medium containing suspended bacterial cells was removed and an equal volume of fresh TSB with 0.25% glucose and antibiotic were added. Negative controls were obtained by incubating the surfaces in TSB supplemented with 0.25% glucose and antibiotics without adding any bacterial cells. All experiments were done in quadruplicate with three repeats.

2.5.2. Biofilm quantification

Biofilms were quantified by dry-weight determinations, as previously described [1] with some modifications. Briefly, the colonized acrylic surfaces were removed from the plates and placed at 80 °C overnight. Then the weight of the surface was determined on a digital scale. Surfaces were placed again at 80 °C for 2 more h and weighed again, to check the stability of the dry weight. Then, the biofilm was mechanically removed from the surface, and the surfaces were thoroughly cleaned with 0.2% commercial detergent solution. Cleaned surfaces were kept overnight at 80 °C prior to a third weight determination. The difference in the weight of the surface with and without the biomass attached is the biofilm dry-weight.

2.6. Statistical analysis

The data from the assays were compared using one-way analysis of variance (ANOVA) by applying Levene's test of homogeneity of variances and the Tukey multiple comparisons test. Where appropriate for paired samples, *t*-tests were use, with all calculations carried out using SPSS software (Statistical Package for the Social Sciences). Differences achieving a confidence level of 95% were considered significant.

3. Results

3.1. Determination of the sub-MIC value of antibiotics

The results from antimicrobial susceptibility testing of all CoNS strains are summarized in Table 1. MIC values were generally higher when using cefazolin or dicloxacillin, compared to vancomycin. *S. epidermidis* 9142, *S. epidermidis* M187 and *S. haemolyticus* M176 were found to be the most antibiotic-resistant strains. Table 1 also presents the concentration of antibiotics used in the assays employing sub-MIC of antibiotics.

3.2. Inhibition of adherence

Results studying the effects of growth with sub-MICs of antibiotics on bacterial adherence to acrylic are presented in Table 2. Dicloxacillin was the antibiotic that prevented initial adherence to the greatest extent when only one antibiotic at $\frac{1}{2}$ of the MIC was used (average reduction per strain of $54 \pm 11\%$). Vancomycin was the least effective antibiotic in this regard (average reduction per strain of $25 \pm 7\%$). When using combinations of two antibiotics, each at $\frac{1}{4}$ of the MIC, the combinations where dicloxacillin was present were generally the highest inhibitors, reaching in some cases nearly 80% inhibition (*S. epidermidis* IE186 with dicloxacillin and vancomycin or *S. epidermidis* M129 with dicloxacillin and cefazolin). Some combinations of antibiotics, even at the lowest concentrations tested, could inhibit initial adhesion at fairly high percentages. Some of the combinations had a synergistic effect and were able to inhibit bacterial growth, as in the case of *S. epidermidis* M187 and *S. haemolyticus* IE246 when grown in the presence of cefazolin and dicloxacillin.

Table 1
Determination of the MIC range and sub-MICs used in adherence and biofilm formation assays (in µg/ml). C, cefazolin; V, vancomycin; D, dicloxacillin

Strain	MIC range			Sub-MIC		
	C	V	D	C	V	D
S. epidermidis 9142	64–128	8–16	64–128	32	4	32
S. epidermidis IE75	8–32	4–8	0.5–16	4	2	0.25
S. epidermidis IE186	2–16	8	0.5–4	1	4	0.25
S. epidermidis M129	4–32	8	4–16	2	4	2
S. epidermidis M187	64–128	8	16–64	32	4	8
S. haemolyticus IE246	0.5–2	2–4	0.25–2	0.25	1	0.125
S. haemolyticus M176	32–128	2–4	16–128	16	1	8

Table 2
Inhibition of initial adhesion (in percentage) to acrylic due to growth in sub-MICs of antibiotics (C) cefazolin, (V) vancomycin and (D) dicloxacillin at the sub-MIC concentration

Strain	C	V	D	$\frac{1}{2}$C + $\frac{1}{2}$V	$\frac{1}{2}$V + $\frac{1}{2}$D	$\frac{1}{2}$C + $\frac{1}{2}$D
S. epidermidis 9142	49 (±8)	34 (±4)	66 (±3)	36 (±9)	44 (±3)	40 (±3)
S. epidermidis IE75	13 (±6)	20 (±6)	36 (±9)	10 (±6)	16 (±7)	46 (±6)
S. epidermidis IE186	44 (±11)	30 (±9)	66 (±6)	58 (±4)	79 (±3)	66 (±8)
S. epidermidis M129	42 (±10)	29 (±3)	48 (±4)	23 (±9)	52 (±9)	77 (±3)
S. epidermidis M187	2 (±5)	26 (±5)	58 (±4)	42 (±4)	*	*
S. haemolyticus IE246	21 (±3)	17 (±4)	46 (±9)	*	55 (±5)	*
S. haemolyticus M176	28 (±6)	19 (±3)	59 (±3)	16 (±4)	19 (±3)	12 (±4)

* This combination of antibiotics did not allow the cells to grow, working as a bactericidal concentration and demonstrating a synergistic effect.

Table 3
Inhibition of biofilm formation (in percentage) on acrylic, under sub-MICs of antibiotics (C) cefazolin, (V) vancomycin and (D) dicloxacillin at the sub-MIC concentration

Strain	C	V	D	$\frac{1}{2}$C + $\frac{1}{2}$V	$\frac{1}{2}$V + $\frac{1}{2}$D	$\frac{1}{2}$C + $\frac{1}{2}$D
S. epidermidis 9142	43 (±7)	24 (±9)	54 (±9)	13 (±2)	30 (±3)	10 (±2)
S. epidermidis IE186	55 (±4)	24 (±11)	32 (±2)	40 (±3)	40 (±14)	21 (±4)
S. epidermidis M187	32 (±3)	8 (±3)	60 (±4)	67 (±5)	*	*

* This combination of antibiotics did not allow the cells to grow, working as a bactericidal concentration and demonstrating synergistic effect.

3.3. Inhibition of biofilm formation

Results from testing the effects of growth in the presence of sub-MICs of antibiotics on biofilm formation on acrylic are presented in Table 3. When using only one antibiotic at $\frac{1}{2}$ of the MIC, vancomycin was the antibiotic that was least effective in preventing biofilm formation (average reduction per strain of $21 \pm 10\%$). Dicloxacillin and cefazolin were more effective than vancomycin (average reduction per strain of $51 \pm 12\%$ and $44 \pm 9\%$, respectively).

When using combinations of two antibiotics, each at $\frac{1}{4}$ of the MIC, in most cases the inhibition of biofilm formation was less effective compared with the use of only one antibiotic at $\frac{1}{2}$ of the MIC. The only exception found was for strain *S. epidermidis* M187, for which most combinations of antibiotics had a synergistic effect and were also able to inhibit bacterial growth.

Fig. 1 presents the correlation found between adhesion and biofilm formation inhibition. The correlation coefficient obtained (R) was only 0.48 meaning that these two properties are not very linearly dependent. The main differences were found when using combinations of antibiotics. For instance, when using combinations of vancomycin and dicloxacillin, inhibition of adherence of *S. epidermidis* IE186 was 79% but only 40% of the biofilm formation was inhibited.

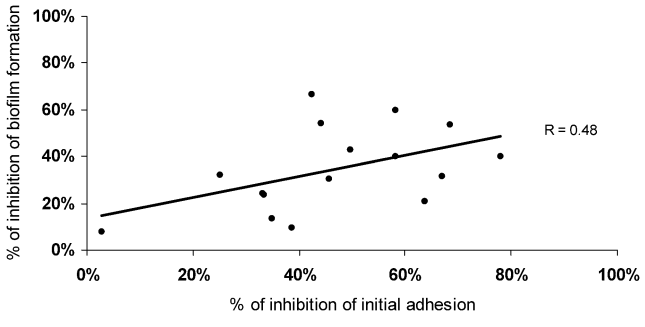

Fig. 1. Correlation between inhibition of adhesion and inhibition of biofilm formation.

4. Discussion

It has been suggested that if a low concentration of antibiotics or other drugs is able to prevent initial adherence of bacteria to surfaces, the subsequent step of biofilm formation would also be inhibited [12]. A similar conclusion might be drawn for other possible interventions being considered to reduce the incidence of device-related infections, such as use of biomaterials with low intrinsic binding of microbes. However, it has previously been demonstrated that the initial adherence and subsequent biofilm formation by staphylococcal strains are two distinct phenomena [9,13]. We therefore undertook this study to determine if growth of CoNS strains in the presence of sub-MICs of antibiotics was equally effective at preventing initial adherence and subsequent biofilm formation on acrylic surfaces. Such results could be relevant to determining the usefulness of an approach targeted at inhibiting bacterial adherence in preventing a biofilm-related infection. Accordingly, antibiotics commonly used for staphylococcal infections were chosen and the effect on either initial adhesion or biofilm formation was evaluated using bacteria grown in low concentrations of such antibiotics. Acrylic was the selected surface because it is a very common polymer used in biomedical applications [10].

All strains were able to adhere in great extent to acrylic in the absence of antibiotics. The most effective antibiotic

in preventing initial adhesion was dicloxacillin (mean inhibition of $54 \pm 11\%$), and the least effective was vancomycin (mean inhibition of $25 \pm 7\%$). However, for each antibiotic used, a wide variation in inhibition of adherence was found. For instance, when using cefazolin 49% of the adherence of *S. epidermidis* 9142 was inhibited, whereas only 13% of the adherence of *S. epidermidis* IE75 was achieved with this drug. Dicloxacillin inhibited 66% of the initial adherence of *S. epidermidis* 9142, while the effect on *S. epidermidis* IE75 was only 36% inhibition of adherence. Since the clinical strains used in this study had different susceptibilities to antibiotics, the concentration of each antibiotic used in the inhibition assays varied for each strain (see Table 1). In order to determine whether the variation in inhibition of adherence for the different strains was due to the variable antibiotic concentrations used, a linear regression plot was derived for each sub-MIC antibiotic concentration used and the respective percentage of inhibition. The correlation coefficients obtained (R) were 0.13 for cefazolin, 0.92 for vancomycin and 0.54 for dicloxacillin. This means that although a good relationship was found between drug concentration and percentage of inhibition for vancomycin, for the remaining antibiotics, the difference in inhibition could not be attributed to the differences in drug concentration. Probably, other factors intrinsic to an individual strain could contribute to decreasing the susceptibility to the sub-MICs of the antibiotics, such as the expression of surface antigens [9].

When sub-MIC combinations of antibiotics were used, we again saw a wide variation in inhibition of CoNS adherence to acrylic. Notably, combinations where dicloxacillin was present were always more effective than when dicloxacillin was absent. As expected, some synergistic effects on inhibition of growth were found with a combination of the antibiotics used. For instance, when $\frac{1}{4}$ of the MIC of cefazolin plus $\frac{1}{4}$ of the MIC of dicloxacillin were used, *S. epidermidis* M187 and *S. haemolyticus* IE246 were not able to grow.

Some of the CoNS strains used in the adherence assays had a poor ability to form biofilms (data not shown). Thus, only high biofilm-forming bacteria were selected for the assays of biofilm inhibition by sub-MIC antibiotics. As seen in the adherence assays, dicloxacillin was the most effective antibiotic at preventing biofilm formation on acrylic. However, when cefazolin or vancomycin was used, the percent inhibition of biofilm formation was generally lower. The difference between adhesion and biofilm inhibition was even higher when combinations of the antibiotics at $\frac{1}{4}$ of the MIC value were used.

Although it has been suggested that by preventing initial adherence, microbial biofilm formation could be prevented, experimental support for this conclusion is minimal. It has been reported that when testing several antibiotics with different mechanisms of action, after the initial adherence of CONS to either acrylic or silicone, the bacteria became more resistant to some antibiotics compared with non-adherent planktonic cells [2]. In a different study, it was suggested that attached bacteria would have a slower metabolic rate, and that could partially explain the increase in resistance to antibiotics [33]. Pagano et al. evaluated the differences between a prophylactic and therapeutic approach to the CoNS biofilm problem. These authors verified that by adding low concentrations of linezolid or vancomycin before the bacteria could reach the surface, they were able to inhibit biofilm formation. However, if the application of the drug was delayed just by 6 h after initial adherence occurred, the inhibition of biofilm formation was less effective [23].

Rupp and Hamer assessed the inhibition capabilities of some antibiotics on adherence and biofilm formation using a few *S. epidermidis* strains. Although those authors did not search for a relationship between inhibition of adherence and inhibition of biofilm formation, some differences were found between the ability of a given antibiotic to inhibit adherence and biofilm formation [27].

In summary, despite some similarities in the results of adherence and biofilm inhibition assays, adherence inhibition assays cannot fully predict the outcome in terms of biofilm formation. Even so, it seems that dicloxacillin has a significant effect in preventing CoNS adhesion and also biofilm formation to acrylic. Interestingly, standard bacterial susceptibility tests (with planktonic cells) demonstrated higher susceptibility of CoNS to vancomycin, but this antibiotic was the least effective in preventing initial adhesion and biofilm formation. Clearly, standard bacterial susceptibility tests do not reveal the potential of an antibiotic to inhibit biofilm formation.

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