Influence of sub-inhibitory concentrations of antimicrobial agents on biofilm formation in indwelling medical devices

M. Henriques[1], N. Cerca[1], J. Azeredo[1], R. Oliveira[1*]

[1]Centre of Biological Engineering - CEB, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[*]Corresponding author: Rosário Oliveira

Departamento de Engenharia Biológica

Universidade do Minho, Campus Gualtar

4710-057 Braga, Portugal

Tel: +351 253604419

Fax: +351 253678986

roliveira@deb.uminho.pt

Running head: Sub-MIC effect on biofilms

Abstract

Biofilms of *Staphyloccocus epidermidis* and *Candida* spp. are two of the most frequent factors of infection and pathogenesis associated to the use of indwelling medical devices. Several strategies have been proposed and/or developed to prevent infection. The aim of this study was to compare the effect of sub-inhibitory concentrations of anti-microbial agents on biofilm formation.

Biofilms of three strains of *S. epidermidis* and two of both *Candida albicans* and *Candida dubliniensis* where formed in the presence of three antibiotics and two antifungal agents respectively. Based in the control samples, the percentage of biofilm formation inhibition by the different agents was determined and compared.

The results showed that the influence of the antibacterial and antifungal agents tested is strain dependent, with the effect of the different agents also varying among strains, even if they have the same mechanism of action.

Keywords: antimicrobial agents, biofilm, *Candida albicans*, *Candida dubliniensis*, *Staphylococcuss epidermidis*.

I. Introduction

In order to invade and infect the human body microorganisms need to form resistant and strong structures - the biofilms. Biofilm structures comprise a heterogeneous mosaic that is characterized by a basal layer and stacks of microcolonies extending up into the aqueous phase; a porous biofilm with mushroom-like structures interdispersed with water channels; and a dense-confluent biofilm that appears more tightly packed, often containing multiple species of microorganisms with regions of lower density that may act as transport channels within the biofilm (1). Whatever the structure, the microbial cells are always embedded into an extracellular polymeric matrix. The role of the biofilm is to resist the cleansing action of fluids, which is a mechanism of defence at various anatomical sites, and acting also as a reservoir for nutrients.

Severe problems related to biofilm formation on indwelling medical devices have been reported (2, 3), including dysfunctioning of the implanted device to lethal sepsis of the patient. The eradication of a biofilm is a complicated issue, because microorganisms in a biofilm are more resistant to antimicrobial agents than their planktonic counterparts (4).

The major part of the human body colonizers are bacteria, however some yeast infections are also found. These microorganisms can colonize either the human cells or indwelling devices, being the latter one of the major problems once their use is increasing in the last years.

Staphylococcus epidermidis and related coagulase-negative staphylococci (CoNS) are now considered among the major nosocomial pathogens associated to the use of indwelling medical devices and their ability to form biofilms is one of the most significant factors of virulence (5).

Candida albicans and *Candida dubliniensis* are two pathogenic yeasts that are well known for colonization of the oral cavity, and prosthetic devices and responsible for Candidiasis, that can be fatal in immunosupressed individuals.

A successful clinical response to therapy typically not only depends on the susceptibility of the pathogenic organism but also relies heavily on the host immune system, drug penetration and distribution, patient compliance and absence of a protected or persistent focus of infection. Microbial resistance of a given pathogen must be regarded as a quantifiable variable, determined by measurement of drug susceptibility and must be defined with respect to a reference population (6). This quantifiable variable can be the minimal inhibitory concentration (MIC), which is the lowest drug concentration that avoids visible growth of the microbial pathogen.

The use of antimicrobial agents has been fully studied in the last years. Several therapies have been developed to improve the results obtained with the normal therapies (antimicrobial uses in single doses), as controlled release of drugs using coated polymers (7). Occasionally, as for example in immunosupressed individuals, drugs must be applied in longer treatments in order to prevent any infection. In this case, the dose of these agents is typically low, which may result in blood levels that are either clinically subinhibitory or less than the in vitro MIC (8). The exposure of patients to subinhibitory concentrations of antimicrobial agents may induce various

changes in bacterial and yeast properties, including morphological or ultrastructure changes and inhibition or stimulation of enzyme and toxin production (8, 9).

In the mouth, the diluent effect of saliva and the cleansing action of the oral musculature often tend to reduce the viability of the agents to below that of the effective therapeutic concentration (10). Thus, microbial cells in the oral cavity experience only a limited exposure to the antimicrobial agent during treatment and the concentration of the drug may vary in different niches of the mouth (11).

One of the main goals of the present work was the comparison of the influence of subinhibitory concentrations of antimicrobial agents in the formation of biofilms by two distinct types of microorganisms, *Staphylococcus epidermidis* and *Candida* species.

II. Materials and Methods

II.A. Bacteria

II.A.1. Growing conditions

Three *S. epidermidis* 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* IE186 was isolated from infective endocarditis patients; and *S. epidermidis* M187 was isolated from patients with peritonitis associated with renal dialysis. The strains were kindly provided by Dr. G.B. Pier from the Harvard Medical School, Boston, USA.

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 ºC in an orbital shaker at 130 rpm. The cells were harvested by centrifugation (for 5 min at 10000 rpm at 4 ºC), 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.

II.A.2. Antibiotics

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 (12, 13). The sub-MIC used was 1/2 of the lowest MIC value (14).

II.A.3. Biofilm formation

Formation of bacterial biofilms was performed as described previously (15). 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 ºC 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 was 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.

II.A.4. Biofilm quantification

Bacterial biofilms were quantified by dry-weight determinations, as previously described (16) with some modifications. Briefly, the colonized acrylic surfaces were removed from the plates and placed at 80 ºC overnight. Then the weight of the surface was determined on a digital scale. Surfaces were placed again at 80 ºC for 2 more hours 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 ºC 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.

II.B. Yeasts

II.B.1. Yeasts growing conditions

Two strains of *Candida albicans* and two strains of *Candida dubliniensis* were used in this work. In the case of *Candida albicans* one strain was from the American Type Culture Collection, ATCC 32354 (*Candida albicans* B311) and the other was a clinical isolate (*Candida albicans* 12A), kindly provided by the Biology Department, University of Minho, Portugal. In the case of *Candida dubliniensis*, the two strains were obtained from Centraalbureau voor Schimmelcultures, the Netherlands, CBS (*Candida dubliniensis* 7987 and *Candida dubliniensis* 7988).

Yeast cells were grown for 24 h in SDA at 37 ºC, for all the assays. The cells were then inoculated in SDB for 18 h at 37 ºC and 150 rpm. After this, 10^8 cell ml^{-1} were diluted 1:10 in artificial saliva growth medium, used as control and in the same medium supplemented with subinhibitory concentrations of both antifungal agents. Cells were grown in these media for 24 h at 37 ºC and 150 rpm. The cells were then harvested by centrifugation for 10 min at 5000 rpm and 4 ºC and washed twice with ultrapure sterile water. Saliva growth medium was prepared supplementing artificial saliva (in mg l^{-1}: 125.6 NaCl, 963.9 KCl, 189.2 KSCN, 654.5 KH_2PO_4, 200.0 Urea, 763.2 $Na_2SO_4.10H_2O$, 178.0 NH_4Cl, 227.8 $CaCl_2.2H_2O$, 630.8 $NaHCO_3$) with 2 g l^{-1} of glucose, 2 g l^{-1} of yeast extract and 5 g l^{-1} of peptone.

II.B.2. Antifungal agents

The antifungal agents used were: fluconazole (Pentafarma) and amphotericin B (ICN). Fluconazole was diluted in water until a subinhibitory concentration (1/2 MIC) of 0.25 μg ml^{-1}. In the case of amphotericin B, the first dilution was in DMSO, once it is not water soluble, and the further dilutions were made in water to reach a

subinhibitory concentration of 0.01 μg ml^{-1}. The antifungal solutions were prepared immediately before each experiment.

II.B.3. Biofilm formation

The biofilms were formed on acrylic coupons (8×8 mm^2), that were prepared as described previously (17) and placed in the bottom of 24 well microtiter plates. An inoculum of 2 ml yeast cell suspension (10^7 cell ml^{-1}) was added to each well and biofilm was formed in artificial saliva growth medium with and without fluconazole and amphotericin B. The media were changed each 12 h and the biofilms analysed after 78 h of formation. The experiments were performed in triplicate and repeated twice.

II.B.4. Biofilm quantification

After biofilm formation, the coupons were removed from each well and immersed in a new microtiter plate containing 1 ml of methanol in each well. Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. After that, 600 μl of crystal violet were added to each well and incubated for 5 min. The coupons were then gently washed in water and immersed in 1 ml of acetic acid (33 %) to release and dissolve the stain. The absorbance of the obtained solution was read at 570 nm.

III. Results and Discussion

To accomplish the comparison of the influence of subinhibitory conditions of antimicrobial agents on the formation of biofilms by yeasts and bacteria, the pathogenic strains and abiotic surfaces were chosen as to be closely related with typical sites of infection. Although acrylic was used in both assays, concerning *S. epidermidis* the acrylic used was similar to the one used in some indwelling devices, whereas self-polymerised acrylic common in oral prosthetic devices was used with *Candida* species. Biofilms were quantified after 48 h for bacteria and after 78 h for yeasts. This difference is due to the time needed to achieve mature biofilms.

The assessment of the effect of subinhibitory concentrations of both antibacterial and antifungal agents in biofilm formation was determined by the percentage of inhibition of biofilm formation (Tables 1 and 2).

Table 1 –Inhibition of bacterial biofilm formation (in percentage) on acrylic, under sub-MICs (1/2 MIC) of the antibiotics: cefazolin, vancomycin and dicloxacillin

	Percentage of biofilm inhibition using		
	cefazolin	vancomycin	dicloxacillin
S. epidermidis 9142	43 (± 7)	24 (± 9)	54 (± 9)
S. epidermidis IE186	55 (± 4)	24 (± 11)	32 (± 2)
S. epidermidis M187	32 (± 3)	8 (± 3)	60 (± 4)

The effect of sub-inhibitory concentrations of vancomycin on biofilm formation by all the strains of S. epidermidis was the lowest compared to the other two antibiotics used (Table 1).

Table 2 – Inhibition of yeast biofilm formation (in percentage) on acrylic, under sub-MICs (1/2 MIC) of amphotericin B and fluconazole

	Percentage of biofilm inhibition using	
	amphotericin B	fluconazole
Candida albicans B311	20 (± 1)	31 (± 1)
Candida albicans 12A	22 (± 9)	21 (± 5)
Candida dubliniensis 7987	57 (± 4)	38 (± 5)
Candida dubliniensis 7988	29 (± 1)	57 (± 1)

It is interesting to notice that for both antifungal agents the inhibition was higher in the biofilms formed by *Candida dubliniensis* strains (Table 2). When amphotericin B was used the highest effect was on *Candida dubliniensis* 7987, showing that this strain presents a different behaviour compared to the other *Candida dubliniensis* strain. The opposite happened in the presence of subMIC of fluconazole, both strains of *Candida dubliniensis* presented similar reductions on the biofilm amounts, but *Candida albicans* 12A had a low inhibition than *Candida albicans* B311.

These differences among strains were also present in bacterial biofilms, since the percentage of inhibition obtained with the different antibiotics was not similar for the three CoNS assayed (Table 1) and it should be emphasized that they all have the same mechanism of action, being cell wall synthesis inhibitors. Moreover, each antibiotic had a different effect on biofilm inhibition according to the strain targeted.

This is a very important issue since almost all the studies concerning the effect of antimicrobial agents in both bacteria and yeast biofilms tend to generalize the behaviour of a species without concerning the strains differences. It is also very interesting to notice that the effect of the different antimicrobial agents varies depending on the strain. For instances, amphotericin B is a fungicidal agent while fluconazole is fungistatic, but they act differently against the two strains of the two species.

A special remark is due on the method used for biofilm formation. In the present work, both bacteria and yeast biofilms were formed in a fed-batch system in order to avoid the lack of nutrients (18). The type of biofilm formation, static or dynamic can affect considerably the results obtained once the formation of new phenotypes that could be able to subvert the inhibitory effect of the antimicrobial agents can happen when there is no lack of nutrients (19). On the contrary, static model systems may not provide sufficient time for a biofilm variant to develop and the results can be biased.

IV. Conclusions

The main general conclusion from this study is that the effect of sub-inhibitory concentrations of anti-microbial agents is dependent on the microorganism at a strain level and on the type of drug, even for drugs having the same mechanism of action.

If biofilm formation is inhibited by subMIC of an antimicrobial agent, the subsequent clinical results are also affected. In this way, the anti-biofilm effect of a drug may be relevant in assessing the selection of anti-microbial treatment. In the other hand, the failure of a prophylactic strategy based on the administration of subMIC of antimicrobial agents may have important clinical implications, threatening the eradication of the infection and favouring increased resistance against the antimicrobial agent.

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VI. References

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