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RESEARCH ARTICLE

In vitro Tolerance of *Staphylococcus haemolyticus* and *Candida albicans* Biofilms to Dalbavancin and Anidulafungin

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Abstract: ***Background*:** Concerns about infections resulting from bacterial biofilm formation in invasive devices such as catheters and prostheses are becoming widespread in the public health domain. *Staphylococcus haemolyticus*, a coagulase-negative bacterium, and *Candida albicans*, a yeast, have become recurrent pathogens of these diseases because their presence in these devices enhances the likelihood of infection. It is believed that these microorganisms produce biofilms, which complicate treatment and slow the patient´s recuperation. Dalbavancin is a semisynthetic, lipoglycopeptide-class antibiotic utilized as an anti-infective agent to break down gram-positive bacteria biofilms. Anidulafungin is an echinocandin class antifungal medication that works very well against resistant yeast strains and removes biofilms.

***Objective*:** This study aims to examine the anti-infective agents´ tolerance to the biofilms of *Staphylococcus haemolyticus* and *Candida albicans*.

***Methods*:** Polymicrobial biofilms were grown in a CDC Biofilm Reactor (CBR) for use in *in vitro* experiments.

***Results*:** When dalbavancin maintained its antibiotic activities against *Staphylococcus haemolyticus* in comparison with their activity against the sessile forms, the antifungal anidulafungin lost efficacy in eliminating *Candida albicans*.

***Conclusion*:** The planktonic forms of microbes are examined in relation to the tolerance to these anti-infective drugs.

Keywords: Anidulafungin, anti-infective agents, biofilms, *Candida albicans*, CDC biofilm reactor, dalbavancin, *Staphylococcus haemolyticus.*

1. INTRODUCTION

Among the most frequent hospital-acquired (nosocomial) infections are two pathogens. The first is the gram-positive, facultative-anaerobic, coagulase-negative, non-motile, non-spore-forming *Staphylococcus haemolyticus*. This bacterium uses glucose, maltose, and fructose as energy sources to develop in all types of culture media [1]. According to Bakthavatchalam [2], *S. haemolyticus* is the second most commonly isolated coagulase-negative *Staphylococcus* (CoNS) species from blood cultures. *Candida* spp., primarily *Candida albicans*, is the second pathogen and the fifth most prevalent cause of hospital-acquired infections [3]. Candidaemia results in a mortality rate of about 40% [4]. This commensal yeast, which is frequently discovered in human skin and the digestive tract, functions like an opportunistic microorganism because it easily takes on a pathogenic profile as a result of the modifications to the patient's immune system or the environment. In actuality, the most common pathogen that colonizes and infects surgical sites where catheters are frequently handled is *C. albicans* [5]. There are two drugs for the treatment of these infections. One of these is dalbavancin, also known as DAL, a newly developed powerful semisynthetic bactericide that is a member of the lipoglycopeptides family and has completely changed the antimicrobial treatment landscape due to its capacity to break down bacterial biofilms [6, 7]. Recently approved by the Food and Drug Administration (FDA), DAL offers a variety of anti-infective properties that are unique to gram-positive bacteria. The other is called anidulafungin, or AFG, a semisynthetic echinocandin that is derived from *Aspergillus nidulan* fermentation products. When used in either a sessile form or as a biofilm, this fungicide is considered to be the first-line treatment for invasive candidiasis [8, 9]. AFG exhibits one of the highest rates of susceptibility to *Candida* [10]. The European Medicines Agency has validated AFG [11].

The purpose of this study was to first characterize the adhesion kinetics and formation of poly-microbial biofilms and then to demonstrate the prophylactic efficacy of a combination of DAL antibiotic and AFG fungicide against *S. haemolyticus* and *C. albicans* biofilms respectively, compared to

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their sessile forms. This was conducted because the tolerance to these anti-infective agents is a matter of clinical concern.

2. MATERIALS AND METHODS

2.1. Microorganism Isolation

S. haemolyticus (ID: B2004000008089) and *C. albicans* (ID: P2015000152), which were isolated from blood cultures of patients referred to the University of Navarra Clinic (Pamplona, SPAIN), were tested for their ability to produce biofilms. In order to confirm the identity and purity of each isolate, *C. albicans* was cultivated on BBL CHROMagar *Candida* medium (CHROMagar, Paris, FRANCE) and then verified using VITEK-2™ YST ID colorimetric assays. Regarding *S. haemolyticus*, it was cultured in lamb blood agar cultures and then confirmed using the VITEK-2™ GP ID colorimetric assays for gram-positive cocci.

2.2. CDC Biofilm Reactor (CBR)

The biofilm was grown using a CDC biofilm reactor (BIOSURFACE TECHNOLOGIES CORPORATION, MN, USA), a well-recognized device that generates microbial biofilms under high shear and continuous flow [12]. This method, established by the American Society for Testing and Materials, makes it possible to study a wide range of biomaterials with different compositions as well as diverse physicochemical and structural characteristics, such as plastic polymers, metals, and ceramics, among others [13]. The biomaterial needed to be placed in a disk form or coupon measuring 1.27 cm in diameter and 0.3 cm in height in order to be inserted into a CBR.

2.3. Inoculum

To guarantee the strain´s purity and viability, they were cultured on blood agar at least two times for a total of 24 hours at 35.5°C. The suspension that was collected from this culture had its cell density increased to 0.5 McFarland or roughly 1.5 x 10^8 CFU/mL.

2.4. Biofilm Generation

The CBR was loaded with 450 mL of Brain Heart Infusion (BHI) culture medium and 1 mL of the inoculum of each strain. It was also equipped with 24 PTFE/silicon™ reaction disks or coupons as a fixation base for the sessile form. The ultimate concentration of microorganisms in the CBR varied between 8 ×10^4 and 2.7 ×10^5 CFU/mL.

2.5. Preparation of Stock Solutions of Antimicrobials

Based on the standard procedures specified by the Clinical and Laboratory Standards Institute (CLSI) in its manual M07-A9 [14] for antimicrobial dilution, 4 mg of either DAL or AFG were weighed and dissolved in 4 mL of sterile distilled water, resulting in a final concentration of 1,000 μg/mL for each antimicrobial.

2.6. Biofilm Handling and Analysis

To detach the biofilm adhered to the surface of a biomaterial, each tube containing a disc was vortexed for 30 s at 2,500 rpm, then sonicated for 5 min at 50 kHz in a sonicator bath (P-Selecta, Ultrasons H, Barcelona, SPAIN), and vortexed once more for an additional 30 seconds. The broth obtained after sonication was diluted (1:10, 1: 100, 1: 1,000, and 1: 10,000) in Mueller-Hinton agar (Becton Dickinson, USA), and 0.1 mL of each dilution was cultured on Bushnell Haas agar (Becton Dickinson, USA) in order to calculate the number of viable cells adhered per unit area of the material. The plates were incubated at 37°C for a whole day (24 h). The following calculation [15] converted the sonicated culture data, which were expressed in CFU/mL, into $\log_{10}$ of the adherent cells per unit area (CFU/cm^2; each disk´s surface area was 1.267 cm^2): Log_{10} (CFUcm^2) = Log_{10} (mean CFU/volume plated) • (volume scraped/surface coupon) • (dilution).

2.7. Antimicrobial Susceptibility Testing

A checkerboard approach was utilized to assess the influence on the potency of the combination of antimicrobial drugs (AFG and DAL) in comparison to their activities to detect interaction [16], given the limit of 24 reaction disks for each CBR experiment. After being treated with dilutions of the antimicrobial drugs, microorganisms were examined for their capacity to manifest apparent growth. Accordingly, the microbicide effects of each treatment (either antimicrobial alone or in combination) were expressed as the minimal inhibitory concentration to achieve a 50% effect (MIC_{50}) in the planktonic form, as well as the minimal biofilm-eliminating concentration of 50% effect ($MBEC_{50}$) [17, 18].

2.8. Statistics

Statistical analysis was conducted using JASP Statistics Software (https://jasp-stats.org/) for Windows. Data were expressed as mean and standard deviation. One-way ANOVA with Tukey´s multi-comparisons as a *post hoc* test was conductedto analyze the results of biofilm eradication experiments. The alpha threshold was set at 0.05.

3. RESULTS

In a series of pilot studies, the isolates in their planktonic form were initially examined for their susceptibility to each of the antimicrobial agents applied separately. When using the macro dilution method established in guidance M07-A9, the interaction of the planktonic forms of both *C. albicans* and *S. haemolyticus* with each of the anti-infective agents investigated matched perfectly with the cutoff values specified by the CLSI in its manual M100-S25. Therefore, only planktonic *S. haemolyticus* alone was sensitive to DAL (threshold concentration = 0.122 μg/mL) and resistant to AFG (MIC_{50} = 125 μg/mL), but *C. albicans* was sensitive to AFG (threshold concentration = 0.122 μg/mL), and resistant to DAL (MIC_{50} = 250 μg/mL). Consequently, for the next experiments, the lowest concentrations of anti-infective drugs, either alone or in combination, were set at 0.244 μg/mL.

Biofilm growth kinetics were determined in a CDC biofilm reactor over a 24-hour period. When it came to testing the ability of DAL to penetrate and destroy the biofilm of *S. haemolyticus*, it was necessary to increase its concentration 16 times between the planktonic (MIC_{50}= 1.953 μg/mL) to the

sessile ($MBEC_{50}$= 31.250 µg/mL) forms to obtain a 50% antibacterial action (Table **1**). Regarding the yeast, a fungicidal impact against *C. albicans* biofilms required four times the dose of AGF ($MBEC_{50}$= 15.625 µg/mL) compared to its planktonic forms (MIC_{50}= 3.906 µg/mL) (Table **1**). It is interesting to note that in the poly-microbial preparation trials, $MBEC_{50}$ (250 µg/mL) exceeded MIC_{50} (7.812 µg/mL) by up to 32 times (Table **1**). The number of viable cells per PTFE reaction disk (log_{10} CFU/cm^2) following antimicrobial treatments with $MBEC_{50}$ concentrations was roughly cut in half in each of the three experimental settings.

Possible pharmacological interactions between anti-infective agents were analyzed using a checkerboard method. The tolerance to the anti-infective agents in either mono-microbial or poly-microbial biofilms is shown in Fig. (**1**). *C. albicans* became much less susceptible to AFG treatment when grown in a poly-microbial phase (see the outlier MIC_{90}). With a Fractional Inhibitory Concentration (FIC) value of 2, the sample was deemed to be "non-interfering".

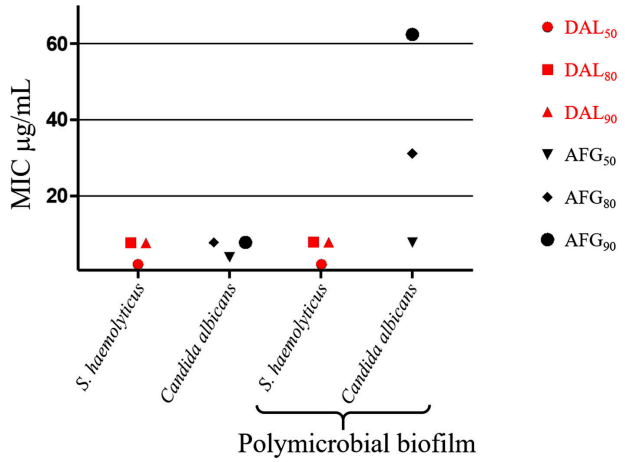

Fig. (1). Graphical representation of the antimicrobial susceptibility of *S. haemolyticus* and *C. albicans* in mono-microbial *versus* poly-microbial biofilm phases. Anidulafungin (AFG) was used against *C. albicans* and dalbavancin (DAL) to treat *S. haemolyticus*. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Fig. (**2**) represents the effects of antimicrobials in biofilm eradication experiments. The ANOVAs demonstrated a significant anidulafungin and dalbanvancin microbiocide impact on fully grown *C. albicans*, ($p < 0.001$; Fig. **2A**) and *S. haemoliticus*, ($p < 0.001$; Fig. **2B**) biofilms respectively. Lethal concentrations (250 µg/mL) of AFG and DAL caused almost the elimination of C. albicans (80%) and S. haemolyticus (91%) biofilms. When the polymicrobial biofilm was produced by a mix of *S. haemolyticus* and *C. albicans*, the combination of antimicrobials led to a smaller reduction of CFUs ($p < 0.001$; Fig. **2C**). Only 46.5% of the poly-microbial biofilm formation was arrested at the maximum concentration (250 µg/mL).

Comparing the planktonic and individual forms of two microorganisms revealed that there was an increase in the tolerance to the anti-infective agents DAL (antibiotic) and AFG (fungicide). It was noteworthy that *C. albicans* developed a stronger tolerance to AFG through the creation of a poly-microbial biofilm, compared to *S. haemolyticus*, against the antibiotic (DAL).

Table **1** shows a 16-fold increase in the $MBEC_{50}$ of DAL to destroy *S. haemolyticus* biofilms compared to the MIC_{50} required to treat bacteria´s planktonic form. This is consistent with research from the past [19], which found that coagulase-negative *Staphylococcus spp* required a higher DAL concentration, ranging from 2 µg/mL to 16 µg/mL. DAL concentrations necessary to attain a bactericidal effect against a *Staphylococcus* biofilm *in vitro* are likely achievable *in vivo*, indicating a potent, successful remedy against biofilm formation in catheters and other invasive devices [19]. AFG was found to be four times more concentrated than necessary for a *C. albicans* biofilm (Table **1**), which is in line with the findings of Valentin [20] and Fernández-Rivero [21] that AFG was the only antimicrobial agent that could effectively destroy *C. albicans* increasing its concentration from 0.016 µg/mL to 0.060 µg/mL and from 0.016 µg/mL to >16 µg/mL, respectively. The significant rise in the minimum inhibitory concentrations of AFG observed in the treatment of poly-microbial planktonic combination is also noteworthy (Fig. **1**). These concentrations ranged from a MIC_{50} of 7.812 µg/mL to a MIC_{90} of 62.50 µg/mL, making it challenging to achieve these concentrations in plasma.

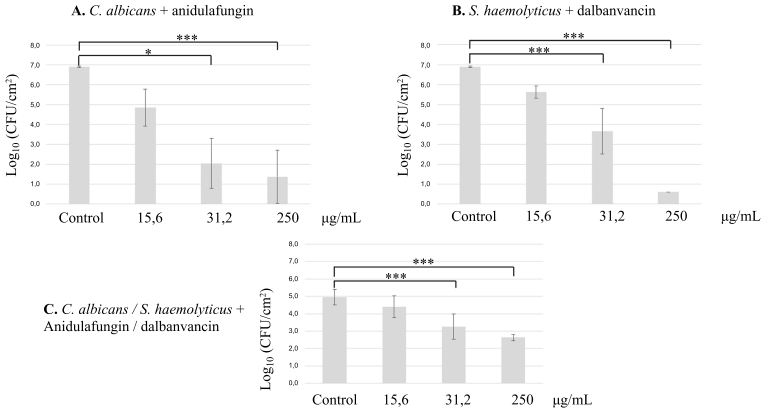

Fig. (2). (**A-C**) Eradication of the numbers of viable biofilm-forming cells (expressed as CFU) at different concentrations of the antimicrobials. Biofilms were grown in dynamic conditions in a CDC reactor. Experiments were run in triplicate. Data shown as average ± SD. One-way ANOVA followed by Tukey´s multi-comparisons test. *$p < 0.05$, ***$p < 0.001$.

Table 1. Micro-biocidal effects of dalbavancin (DAL) and anidulafungin (AFG) against biofilm formation. Note that mono-microbial preparations were treated with its specific anti-infective agent, while poly-microbial preparations were simultaneously treated with both compounds.

-	Planktonic			Biofilm		
	MIC (µg/mL)			Log_{10} CFU/cm^2		
	50	80	90	$MBEC_{50}$ (µg/mL)	Test	Control
S. haemolyticus	1.953	7.812	7.812	31.250	3.44	6.89
C. albicans	3.906	7.812	7.812	15.625	3.41	6.87
S. haemolyticus + *C. albicans*	7.812	31.25	62.50	250.00	2.63	4.93

Abbreviations: MIC: minimal inhibitory concentration to achieve a 50%, 80%, and 90% micro-biocidal effect in planktonic phase; $MBEC^{50}$: minimal inhibitory biofilm elimination concentration of 50% effect; CFU: colony formation units.

4. DISCUSSION

When poly-microbial biofilms formed by *C. albicans* together with *S. haemolyticus* were examined (Table **1**), the number of viable biofilm-forming cells in the reaction disk of control coupons decreased from 6.88 log_{10} CFU/cm^2 (mono-microbial biofilms) to 4.93 log_{10} CFU/cm^2 (poly-microbial biofilm). This decline may result from interactions between microorganisms and their competition for nutrients, as well as from the two species´ limited amount of space. More intriguingly, according to reports elsewhere [22], when *C. albicans* formed a poly-microbial biofilm, the yeast showed a stronger tolerance to AFG than it did in a mono-microbial structure. However, the MIC of DAL needed to eradicate *S. haemolyticus* stayed the same as it did in a mono-microbial biofilm (Fig. **1**). The structural shape of the biofilm itself, in which the yeast grows at the base of the structure beneath *Staphylococcus*, may be the cause of the increase in the resistance of *C. albicans* [23]. As a result, the lipoglycopeptide antibiotic (DAL) is able to carry out its bactericidal action, while the echinocandin fungicide (AFG) is unable to break through this barrier, indicating the ineffectiveness of the antifungal agent, leading to an increased tolerance of *C. albicans* in the poly-microbial biofilm [24].

No interference was found between DAL and AFG (FICI = 2) in the checkerboard test when both drugs were combined in mixed regimes. The activity of DAL against *S. haemolyticus* growth remained intact in a poly-microbial biofilm, indicating that the decrease in the antifungal activity of AFG against *C. albicans* in a poly-microbial biofilm was not caused by drug antagonistic interactions (Fig. **1**). The structural shape of the biofilm itself, where the degree of antimicrobial penetration was probably hampered, may be the cause of tolerance to AFG.

CONCLUSION

In summary, it is likely that the low penetration of AFG resulted in a greater fungicide tolerance of *C. albicans*, which in turn contributed to a statistically significant rise in the antimicrobial tolerance of poly-microbial biofilms (Fig. **2**). This result may indicate that the fungicide is not working as a therapeutic agent when combined with antibiotics. On the other hand, the DAL antibiotic is the preferred treatment for *S. haemolyticus* infections and may be regarded as a destructive antimicrobial for biofilms generated by the pathogen.

AUTHORS' CONTRIBUTION

F.M. designed and performed the experiments, S.B, writers and E.F-M. analyzed the data. I.C. and H.R. assisted with wrote the manuscript in consultation with S.B., and E.F.-M. All the authors have approved the final version of the manuscript to be published and are responsible for its content.

LIST OF ABBREVIATIONS

BHI	=	Brain Heart Infusion
CLSI	=	Clinical and Laboratory Standards Institute
CoNS	=	Coagulase-negative *Staphylococcus*
FDA	=	Food and Drug Administration
FIC	=	Fractional Inhibitory Concentration
MIC	=	Minimal Inhibitory Concentration

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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