TITLE PAGE

Title:

Antibiotic Efficacy against Methicillin-Susceptible *Staphylococcus aureus* Biofilms on Synthetic and Biological Vascular Grafts

Running title:

Antibiotics vs. Staph Biofilms on Grafts

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MAIN BODY

ABSTRACT

Objective: To compare the efficacy of five antibiotics against methicillin-susceptible *Staphylococcus aureus* (MSSA) biofilms on the surface of four vascular grafts.

Methods: *In vitro* study of two clinical MSSA strains (MSSA2 and MSSA6) and four vascular grafts (Dacron, Dacron-silver-triclosan, Omniflow-II, and bovine pericardium). After a 24-hour incubation period, the graft samples were divided into six groups: growth control (no treatment), ciprofloxacin 4.5mg/L, cloxacillin 100 mg/L, dalbavancin 300 mg/L, daptomycin 140 mg/L, and linezolid 20 mg/L. Quantitative cultures were obtained and results expressed as $\log_{10}$ colony-forming units per milliliter (CFU/mL). Analysis of variance was performed to compare biofilm formation between the different groups.

Results: Mean $\pm$ SD MSSA2 count on the growth control Dacron graft was 10.05 ± 0.31 CFU/mL. Antibiotic treatment achieved a mean reduction of 45%; ciprofloxacin was the most effective antibiotic (64%). Baseline MSSA2 counts were very low on the Dacron-silver-triclosan (0.50 ± 1.03 CFU/mL) and Omniflow-II (0.33 ± 0.78 CFU/mL) grafts. On the bovine pericardium patch, the count was 9.87 ± 0.50 CFU/mL, but this was reduced by a mean of 45% after antibiotic treatment (61% for ciprofloxacin). Mean MSSA6 count on the growth control Dacron graft was 9.63 ± 0.53 CFU/mL. Antibiotics achieved a mean reduction of 48%, with ciprofloxacin performing

best (67% reduction). Baseline MSSA6 count on the Dacron-silver-triclosan graft was 8.54 ± 0.73 CFU/mL. Antibiotics reduced biofilm formation by 72%; cloxacillin was the most effective treatment (86%). The MSSA6 count on the untreated Omniflow-II graft was 1.17 ± 1.52 CFU/mL. For the bovine pericardium patch, it was 8.98 ± 0.67 CFU/mL. Mean reduction after antibiotic treatment was 46%, with cloxacillin achieving the greatest reduction (68%).

Conclusion: In this *in* vitro study, ciprofloxacin and cloxacillin performed best at reducing biofilms formed by clinical MSSA strains on the surface of biological and synthetic vascular grafts.

KEYWORDS

Biofilms, *Staphylococcus aureus*, Vascular graft, Infections, Antibiotics.

1. INTRODUCTION

Vascular grafts are a fundamental part of routine vascular surgery. They are used for arterial reconstruction in different areas of the body and are essential when autologous material is not available. They do, however, carry a risk of infection.[1] Vascular graft infections (VGIs) are multifaceted, complex, and potentially devastating.[2,3] Their management requires a multidisciplinary approach, often entailing tailored surgical removal of prosthetic material and antibiotic therapy, which is the cornerstone of VGI treatment.[4,5] Biofilm formation is one of the greatest challenges encountered in prosthetic graft infections, as these films hamper prevention, diagnosis, and treatment. Understanding the intricate mechanisms underlying biofilm formation[6–10] and associated antibiotic susceptibility is crucial for effective VGI management.

As a follow-up to a previous study by our group in which we quantified the formation of methicillin-susceptible *Staphylococcus aureus* (MSSA) biofilms on the surface of vascular grafts routinely used in vascular surgery,[11] we designed a new experiment to further test the grafts that had shown the greatest resistance to biofilm formation. The aim of the present study was to study the efficacy of five antistaphylococcal antibiotics commonly used for VGIs (ciprofloxacin, cloxacillin, dalbavancin, daptomycin, and linezolid) against biofilms formed on synthetic two biological vascular grafts.

2. MATERIALS AND METHODS

2.1. Setting and grafts

This study was conducted in the antimicrobial resistance laboratory at Vall d'Hebron Research Institute (VHIR) as a follow-up to a previous study by our team[11] where almost all manufactured grafts available for vascular reconstruction were included. For the present study we chose the grafts that had shown greater resistance to biofilm formation, in order to study how they could interfere on biofilm formation in combination with antibiotics. We studied two synthetic grafts—a standard Dacron polyester vascular graft (Intergard, Le Ciotat, Cedex, France) and a Dacron graft combining silver acetate and triclosan (Dacron-Silver-Triclosan) (Intergard Synergy, Maquet, Orleans, France)—and two biological grafts—a vascular prosthesis combining a polyester mesh and denatured ovine collagen (Omniflow II; LeMaitre Vascular, Inc., Burlington, Massachusetts, USA) and a bovine pericardium patch (XenoSure; LeMaitre Vascular, Inc.). The synthetic grafts were sterilized with gas at a low temperature, while the biological grafts were prepared with 70% alcohol under sterile conditions.

Using the standardized method described in the previous study,[11] the grafts were cut into circular pieces under sterile conditions to fit the diameter of a 10-mL calibrated loop (Deltalab SL, Barcelona, Spain). Each piece was attached to a loop using three stitches of nonabsorbable monofilament polypropylene (Prolene 6-0; Ethicon Inc.; Johnson & Johnson, Somerville, New Jersey, USA). The cap of a 10-mL tube was then pierced, and a handle fitted so that the graft could be suspended within the tube, 1.5 cm from the bottom, in order to submerge the samples in the inoculum and prevent them from floating.

2.2. Strains

We studied two clinical MSSA strains (MSSA2 and MSSA6) obtained from patients with a catheter-related bacteriemia at Vall d'Hebron University Hospital. The strains were selected because they were the strongest biofilm producers in a previous study by our group[11].

2.3. Biofilm formation on vascular graft surfaces

A modification of the protocol developed by Chandra et al[12] was used for biofilm formation. First, the MSSA strains were grown overnight in tryptic soy broth (TSB; Becton, Dickinson, and Company, Le Pont-de-Claix, France) at 37°C with agitation at 60 rpm for 24 hours. Then, the suspension was washed three times with sterile phosphate-buffered saline (PBS), pH 7.2 (Merck, Germany) and centrifuged at 2500 rpm for 5 minutes at 4°C and resuspended with 10 mL of PBS Ph 7.2. An inoculum of $1x10^7$ colony-forming units per milliliter (CFU/mL) was prepared with PBS pH 7.2. The graft was submerged in a 10-mL tube previously filled with 4 mL of the inoculum containing $1x10^7$ CFU/mL. The tubes were incubated for 90 minutes at 37°C (adhesion step). Then, the vascular graft was gently transferred to a new tube containing 4 mL of TSB, taking care not to add any drops containing unattached microorganisms. The grafts were incubated for 24 hours at 37°C with agitation at 60 rpm (growth step).

2.4. Microbiological assays

At 24 hours, once the biofilms had formed on the vascular grafts, the grafts were randomly assigned to six groups: 1) no treatment (growth control), 2) ciprofloxacin 4.5 mg/L, 3) cloxacillin 100 mg/L, 4) dalbavancin 300 mg/L, 5) daptomycin 140 mg/L, and 6) linezolid 20 mg/L. The grafts were transferred to new tubes prepared with 4 mL

of TSB medium containing the corresponding antibiotic at the test concentration (no antibiotics were added to the growth control tubes) and incubated overnight at 37ºC without agitation. The experiments were carried out in triplicate (three graft pieces for each group). Sterility controls were used in all experiments.

After 24 hours of incubation, the suture attaching the graft piece to the loop handle outside the tube was cut in a sterile field, with care taken not to transfer any bacterial cells from the loop. The graft was then gently transferred to a sterile 12-well plate containing 1 mL of TSB using a sterile needle, again with care not to add any drops containing unattached microorganisms. Finally, both surfaces of the graft samples were gently scraped into the 12-well plate using the blunt edge of a no. 11 scalpel (Swann-Morton Ltd., Sheffield, UK). The suspension and graft were transferred to new tubes, vortexed for 1 minute, and sonicated for 10 minutes at 59 MHz and vortexed for another minute. The samples were then serially diluted in physiological serum; 50 µL of this serum were then plated in tryptic soy agar (TSA; bioMérieux SA, Marcy l'Etoile, France) for quantitative culture. Finally, the bacterial cells were quantified and expressed as $\log_{10}$ CFU/mL. The biofilm quantification method is depicted in Figure 1.

2.5. Statistical analysis

The untreated growth control biofilms were used to establish a baseline for comparing the efficacy of the antibiotic agents tested. Analysis of variance was used to compare biofilm formation on each graft according to the antibiotic used and biofilm formation for each treatment between all grafts. Bar graphs with error bars were built to represent the means and standard deviation for each graft and treatment. A *P* value of

211 < .05 was considered significant. For the comparison between pairs of grafts or
212 treatments, a post-hoc analysis of multiple comparisons was carried out using the
213 Tukey test. IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, N.Y.,
214 USA) was used for the statistical analyses.
215
216 **3. RESULTS**
217 The biofilm quantification results are summarized in Tables I (MSSA2) and Table II
218 (MSSA6) and for each graft show the counts for the growth control group (baseline
219 growth) and the five antibiotic groups. These results are described in more detail
220 below. Tables with statistical analyses can be found in Supplementary Material 1.
221
222 **3.1. MSSA2**
223 Mean ± SD MSSA2 count on the Dacron graft was 10.05 ± 0.31 CFU/mL. Using this as a
224 baseline value, biofilm formation was significantly reduced by all the antibiotics tested
225 ($P < .001$), with a mean reduction of 45% (Supplementary Material 1). The greatest
226 reduction was observed for ciprofloxacin (64%), which was the only antibiotic to show
227 significant differences with the other treatments. Mean MSSA2 count was very low on
228 the Dacron-silver-triclosan graft (0.50 $\pm$ 1.03 CFU/mL). All the antibiotics eradicated the
229 biofilms on this graft, but given the low baseline growth, no significant differences
230 were observed. The count was also low on the Omniflow-II graft (0.33 $\pm$ 0.78 CFU/mL),
231 and in this case, all the antibiotics except cloxacillin eradicated the biofilms. Mean
232 MSSA2 count on the bovine pericardium patch was 9.87 $\pm$ 0.50 CFU/mL. All the
233 antibiotics achieved a significant reduction in biofilm formation ($P < .001$). The mean
234 reduction, 45%, was the same as that observed with the Dacron graft. Ciprofloxacin

was the most effective antibiotic (61% reduction) and showed the strongest differences in relation to the other treatments. Biofilm formation overall was significantly less extensive on the Dacron-silver-triclosan and Omniflow-II grafts than on the Dacron and bovine pericardium grafts ($P < .001$).

3.2. MSSA6

Mean MSSA6 count on the growth control Dacron graft was 9.63 ± 0.53 CFU/mL. A significant reduction in biofilm formation was achieved by all the antibiotic treatments ($P < .001$), with a mean reduction of 48%. Similarly to with the MSSA2 strain, ciprofloxacin achieved the greatest reduction (67%) and was significantly more effective than all the other antibiotics except cloxacillin, which achieved a mean reduction of 57.5%. Mean MSSA6 count on the Dacron-silver-triclosan graft was 8.54 ± 0.73 CFU/mL, and compared with baseline growth, all five antibiotics effectively reduced the biofilms formed ($P < .001$), with a mean reduction of 72%. Cloxacillin was the most effective treatment, with a reduction of 86%. The only significant differences between treatments were observed for cloxacillin and ciprofloxacin. Baseline biofilm growth on the Omniflow-II graft was very low (1.17 ± 1.52 CFU/mL). All the antibiotics eradicated the biofilms formed, but given the low baseline count, no significant differences were found. Mean MSSA6 count on the bovine pericardium patch was 8.98 ± 0.67 CFU/mL. The antibiotics achieved a mean reduction of 46% ($P < .001$ vs. growth control). Cloxacillin was the most effective antibiotic (reduction of 68%). The MSSA6 biofilm formation was significantly reduced on the Omniflow-II graft compared with all the other grafts ($P < .001$). The combined results of the experiments, with SD values, are shown in Figure 2 and Figure 3.

4. DISCUSSION

The main findings of this experimental study indicate that ciprofloxacin and cloxacillin were the most effective antibiotics for reducing MSSA2 and MSSA6 biofilm formation on the vascular grafts tested. The antibiotics reduced MSSA2 and MSSA6 biofilms on the Omniflow-II graft and MSSA2 biofilms on the Dacron-silver-triclosan graft. None of the antibiotics, all applied as monotherapy for 24 hours, was able to completely eliminate the biofilms formed on the Dacron or bovine pericardium grafts.

The five antibiotics tested—ciprofloxacin, cloxacillin, dalbavancin, daptomycin, and linezolid—are commonly used to treat VGIs. Cloxacillin has shown *in vivo* bactericidal activity against staphylococcal infections.[13,20] Ciprofloxacin is a broad-spectrum fluoroquinolone with good *in vitro* activity against *S. aureus* (including methicillin-resistant *S. aureus* [MRSA]) and other staphylococcal species.[15] It has also produced good clinical responses in patients with prosthetic joint infections.[16] In our study, ciprofloxacin monotherapy effectively reduced biofilms formed by both MSSA2 and MSSA6, supporting previous findings.[18] Daptomycin and linezolid both have gram-positive coverage.[4] Daptomycin has been described as an effective treatment for the prevention and eradication of biofilm formation in an *in vivo* staphylococcal infection model,[20] and has shown particularly promising results in the treatment of VGIs.[21] Nonetheless, like us, other authors have found that daptomycin alone is ineffective at eradicating biofilms in *S. aureus* prosthetic VGIs.[2] Dalbavancin is a novel, long-acting lipoglycopeptide that is active against gram-positive pathogens.[23,24] It has proven

283 effective at reducing MRSA biofilm formation *in vitro*.[27,28] We, however, did not observe
284 any striking effects for this antibiotic on MSSA2 or MSSA6 biofilm formation. In line
285 with reports by Žiemyte et al.,[29] who also studied immature biofilms (produced by
286 MRSA), we found that dalbavancin reduced but did not completely eradicate biofilm
287 growth. Other studies have highlighted the need to combine dalbavancin with other
288 antibiotics to improve MRSA eradication.[30,31]
289
290 Although the main aim of the present study was to evaluate the efficacy of five
291 commonly used antibiotics against biofilm formation, our experiments also provided
292 insights into biofilm growth on synthetic and biological grafts before the administration
293 of antibiotics. The CFU counts for MSSA2 and MSSA6 on the Omniflow-II graft were so
294 low that it was not possible to analyze the efficacy of the different antibiotics tested.
295 Few *in vitro* studies have been performed using Omniflow-II. Most have focused on the
296 clinical use of this graft for aortic reconstruction in infected fields[32,33] as it has shown
297 strong resistance to reinfection.[34] As far as we know, there is just one *in vivo* study,
298 performed in rats, where MRSA counts were higher on Omniflow-II than
299 polytetrafluoroethylene grafts.[35] These results are in stark contrast to the clinical
300 experience with Omniflow-II and the results of this and another recent study by our
301 group.[11]
302
303 DST was the other graft that showed the greatest resistance to biofilm formation prior
304 to antibiotic treatment, and its protective effect was particularly evident in the case of
305 MSSA2. This finding is consistent with previous findings by our group[11] and other
306 recent studies.[36] Dacron-silver-triclosan grafts have been shown to have superior

antiseptic properties to grafts coated with silver only.[37,38] In our study, all the antibiotics eradicated the scant MSSA2 biofilms formed on the Dacron-silver-triclosan graft. The results for this graft were also better for MSSA6 reduction.

The findings of this study have relevant implications for both the prevention and treatment of VGIs. Although MSSA biofilms adhered weakly to Omniflow-II, this graft has some technical and commercial limitations. Preparation and rinsing protocols must be carefully followed, and in addition, the Omniflow-II graft is currently only available as a straight prosthesis (in varying sizes). When chosen for aortic reconstruction, it may be necessary to manually create a bifurcated prosthesis. If it is decided to use a more available synthetic graft (for emergency repairs, for example), it should be noted that the Dacron-silver-triclosan graft appears to offer greater resistance to biofilm formation. In addition, it was associated with greater reductions in bacterial counts after antibiotic treatment than either the Dacron or bovine pericardium graft. The Dacron-silver-triclosan graft thus may be useful for the prevention and treatment of VGIs, as it seems to be associated with enhanced antibiotic susceptibility after biofilm formation.

This study has some limitations. First, we investigated the efficacy of antibiotics against MSSA biofilm formation using the conventional CFU counting method, which only measures cultivable cells in the biofilm system. In future studies, it would be beneficial to use microscopy techniques, such as confocal laser scanning microscopy, to gain a more thorough understanding of the role of biofilms in antimicrobial resistance. Second, we used just two clinical strains of MSSA. Although *S. aureus* is the most common cause of graft infections, future studies should evaluate other gram-positive

and gram-negative microorganisms and fungi. Also, it should be recalled that we performed *in vitro* experiments with immature biofilms (formed within a 24-hour incubation period), and that these models may not reproduce what occurs in clinical practice. In addition, the antibiotics were administered for just 24 hours. Much remains to be learned about which grafts offer the greatest protection against infection.[39] More *in vivo* and clinical studies are needed to better understand VGIs and to guide recommendations on the use of certain materials over others.

5. CONCLUSIONS

Ciprofloxacin and cloxacillin performed best at reducing biofilms formed by MSSA2 and MSSA6 on the surface of biological and synthetic vascular grafts. The Dacron-silver-triclosan and Omniflow-II grafts were associated with the least extensive MSSA biofilm formation and Dacron-silver-triclosan showed the highest antibiotic susceptibility following formation. None of the monotherapy antibiotics tested over a 24-hour period was able to completely eliminate the biofilms formed on either the Dacron or bovine pericardium grafts.

6. ACKNOWLEDGMENTS

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8. CONFLICTS OF INTEREST

None of the authors have conflicts of interest to declare. The grafts were supplied by the respective companies, but these did not participate in any of the stages of the study.

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10. TABLES

Table I

Quantification of MSSA2 biofilms formed on four vascular grafts before (growth control) and after five antibiotic treatments. Results expressed as mean log_{10} CFU/mL and SD.

MSSA 2

TREATMENT	GRAFT							
	Dacron		DST		Omniflow-II		Bovine pericardium	
	Mean (log_{10} CFU/mL)	SD	Mean (log_{10} CFU/mL)	SD	Mean (log_{10} CFU/mL)	SD	Mean (log_{10} CFU/mL)	SD
Growth control	10.05	0.31	0.50	1.03	0.33	0.78	9.87	0.50
Ciprofloxacin 4.5 mg/l	3.61	0.93	0	0	0	0	3.82	1.26
Cloxacillin 100 mg/l	5.49	1.14	0	0	0.21	0.63	5.31	0.73
Dalbavancin 300 mg/l	6.85	0.66	0	0	0	0	6.75	0.45
Daptomycin 140 mg/l	5.33	2.22	0	0	0	0	5.24	1.63
Linezolid 20 mg/l	6.17	0.93	0	0	0	0	6.03	1.03

CFU, colony-forming units; DST, dacron-silver-triclosan; log, logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*; SD, standard deviation.

555 **Table II**

556 Quantification of MSSA6 biofilms formed on four vascular grafts before (growth
557 control) and after five antibiotic treatments. Results expressed as mean log_{10} CFU/mL
558 and SD.

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MSSA 6

TREATMENT	GRAFT							
	Dacron		DST		Omniflow-II		Bovine pericardium	
	Mean (log_{10} CFU/mL)	SD	Mean (log_{10} CFU/mL)	SD	Mean (log_{10} CFU/mL)	SD	Mean (log_{10} CFU/mL)	SD
Growth control	9.63	0.53	8.54	0.73	1.17	1.52	8.98	0.67
Ciprofloxacin 4.5 mg/l	3.15	0.62	3.30	1.15	0.18	0.53	3.67	0.68
Cloxacillin 100 mg/l	4.10	0.94	1.23	1.37	0.71	1.10	2.87	0.57
Dalbavancin 300 mg/l	7.40	0.36	2.74	2.69	0.20	0.59	7.11	0.97
Daptomycin 140 mg/l	4.73	1.58	2.09	1.69	0	0	5.72	1.36
Linezolid 20 mg/l	5.90	0.64	2.51	1.77	0.71	1.08	4.99	0.84

CFU, colony-forming units; DST, Dacron-silver-triclosan; log, logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*; SD, standard deviation.

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11. LEGENDS FOR FIGURES

Figure 1

Biofilm quantification.

CFU, colony forming units; DST, Dacron-silver-triclosan; MSSA, methicillin-susceptible *Staphylococcus aureus*; PBS, phosphate-buffered solution; TSA, tryptic soy agar; TSB, tryptic soy broth.

Figure 2

Quantification of MSSA2 biofilms formed on four vascular grafts after no treatment (GC) and treatment with five antibiotics. The GC results are used as baseline values to evaluate the efficacy of the tested antibiotics, shown in different colors. The error bars indicate the standard deviation. Results are expressed as mean $\log_{10}$ CFU/mL and SD. CFU, colony-forming units; DST, Dacron-silver-triclosan; GC, growth control; log, logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*.

Figure 3

Quantification of MSSA6 biofilms formed on four vascular grafts after no treatment (GC) and treatment with five antibiotics. The growth control results are used as baseline values to evaluate the efficacy of the tested antibiotics, shown in different colors. The error bars indicate the standard deviation. Results are expressed as mean $\log_{10}$ CFU/mL and SD. CFU, colony-forming units; DST, Dacron-silver-triclosan; log, logarithm; MSSA, methicillin-susceptible *Staphylococcus aureus*.