Knee Surgery, Sports Traumatology, Arthroscopy (2019) 27:1943–1952
https://doi.org/10.1007/s00167-018-5230-x

EXPERIMENTAL STUDY

Bacterial biofilm formation is variably inhibited by different formulations of antibiotic-loaded bone cement in vitro

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Received: 27 June 2018 / Accepted: 17 October 2018 / Published online: 28 October 2018

Abstract

Purpose The aim of the present study was to quantitatively assess biofilm growth on the surface of bone cements discs containing different antibiotics, including colistin and linezolid. Biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* were grown on bone cement discs for 96 h.

Methods Biofilm amounts were measured by confocal laser microscopy using live/dead staining and dedicated software at different time intervals (48, 72, and 96 h).

Results Bone cement containing vancomycin was not effective at reducing MRSA biofilm formation 96 h following bacterial inoculation. At a comparable time interval, linezolid-, clindamycin-, and aminoglycoside-loaded cement was still active against this biofilm. At the 72- and 96-h observations, *S. epidermidis* biofilm was present only on tobramycin and gentamicin discs. *P. aeruginosa* biofilms were present on cement discs loaded with colistin at all time intervals starting from the 48-h observation, whereas no biofilms were detected on tobramycin or gentamicin discs.

Conclusion Bone cements containing different antibiotics have variable and time-dependent windows of activity in inhibiting or reducing surface biofilm formation. The effectiveness of bone cement containing vancomycin against MRSA biofilm is questionable. The present study is clinically relevant, because it suggests that adding the right antibiotic to bone cement could be a promising approach to treat periprosthetic infections. Indeed, the antibiofilm activity of different antibiotic-loaded bone cements could be preoperatively assessed using the current methodology in two-stage exchange procedures.

Keywords Bacterial biofilm · Antibiotic-loaded bone cement · Confocal laser microscopy · *Staphylococcus* · *Pseudomonas*

Abbreviations

ALBC	Antibiotic-loaded bone cement
BHI	Brain–heart infusion
CLSM	Confocal laser scanning microscopy
3D	Three dimensions
MRSA	Methicillin-resistant *Staphylococcus aureus*
PBS	Phosphate-buffered saline
PI	Propidium iodide
PMMA	Polymethylmethacrylate
SCVs	Small colony variants

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00167-018-5230-x) contains supplementary material, which is available to authorized users.

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Introduction

Antibiotic-loaded bone cement (ALBC) is commonly used for the treatment of prosthetic joint infections (PJIs), assuming that it serves as a source for the local delivery of antibiotics [36]. The release of antibiotic from ALBC shows a biphasic profile, with a spike of high local concentrations in the first hours following implantation and a gradual elution thereafter [3, 5, 6, 20, 31]. Despite the release of antibiotics from ALBC implants, over time bacteria may adhere to the

surface, grow in biofilms, and develop antibiotic resistance [9, 16, 22]. Bacteria may survive on ALBC spacers used in two-stage exchange procedures for PJI independent of their former susceptibility to the antibiotics included in the spacer [19, 28, 30], explaining possible clinical re-infections [1]. Thus, the comprehensive evaluation of antibacterial effectiveness of different ALBCs should include their ability to prevent biofilm formation on the surface of bone cement. Confocal laser scanning microscopy (CLSM) is an advanced imaging technique for the direct visualization of biofilms on biomedical implants. This methodology allows non-destructive examination of the bacterial slime and generates high-resolution three-dimensional (3D) images [10, 14]. Few in vitro and in vivo studies have directly assessed the formation of bacterial biofilms on ALBC for orthopaedic uses, yielding inconsistent results [12, 15, 17, 21, 23, 32]. The variability in the findings of these studies may reflect non-standardized experimental designs. Indeed, most previous investigations did not provide any quantitative data regarding the volume of bacterial slime on the surface [10]. Moreover, there is a complete lack of published data on the antibiofilm effectiveness of colistin- and linezolid-loaded bone cements, even though the systemic administration of these antibiotics is largely used for PJIs sustained by resistant microorganisms. The aim of the present study was to obtain quantitative measurements of biofilm growth on polymethylmethacrylate (PMMA) bone cement loaded with different antibiotics, including colistin and linezolid, at different times post-bacterial inoculation. A standardized in vitro model was used to test the hypothesis that the formation of bacterial biofilms on bone cement is a time-dependent process that can be variably inhibited using ALBC containing different antibiotics. The difference in biofilm growth on the ALBCs tested in the present study had not been comparatively assessed using CLSM. This methodology could be a useful starting point to choose the proper antibiotic to add to PMMA, especially in surgical procedures with high doses of ALBC, such as two-stage exchange for PJIs.

Materials and methods

Bone cement specimens

Experimental samples were manufactured by Tecres SPA (Sommacampagna, Verona, Italy). Specimens of ALBC were prepared from PMMA bone cement with a mould under aseptic conditions. The low viscosity bone cement had a powder-to-liquid ratio of 2:1. Antibiotic salts in powder form were mixed with PMMA copolymer powder and subsequently added to the liquid monomer. Discs measuring 15 mm in diameter and 7 mm in height were produced. The average weight of each specimen was 75 mg; 67.5 mg of which were cement and 7.5 mg antibiotic. Nine experimental groups were created with the following antibiotic content: gentamicin, clindamycin, tobramycin, vancomycin, colistin, gentamicin/vancomycin, gentamicin/clindamycin, tobramycin/vancomycin, and linezolid. Linezolid was provided by Pfizer Inc. (Peapack, NJ, USA).

Bacterial strains

Three bacterial strains originally isolated from patients with clinical PJI were used: (1) methicillin-resistant *Staphylococcus aureus* (MRSA), (2) *Pseudomonas aeruginosa*, and (3) methicillin-susceptible *Staphylococcus epidermidis*. Bacterial strains were identified on the basis of their biochemical profile using automatic systems (Vitek 2, bioMérieux, Marcy l'Étoile, France; Phoenix, Becton DickinsonDiagnostic Systems, Sparks, Md, USA) and the proteomic profile by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). In vitro assays were performed to test the antibiotic sensitivity by automatic (Vitek2; Phoenix) and disc diffusion (Kirby Bauer) antibiotic sensitivity testing. Bacteria were grown at 37 °C for 24 h in Brain–Heart Infusion (BHI) broth (Becton Dickinson Diagnostic Systems, Sparks, MD, USA), and aliquots were frozen in BHI glycerol at − 80 °C until use. All strains had been screened before beginning the study (data not shown) to test their ability to produce biofilms using the spectrophotometric microtiter-plate assay described by Christensen et al. [8]. Based on optical densities of bacterial biofilms, all strains were classified as strong biofilm producers. Bacteria were cultured from cryopreserved beads onto blood-agar plates at 37 °C under aerobic conditions for 18–20 h. From these plates, a single colony was subcultured in BHI broth. Thereafter, cultures were diluted, and the turbidity of the bacterial suspension adjusted to 0.5 McFarland (corresponding to approximately 10^8 CFU/mL).

Biofilm growth

According to the sensitivity patterns of the bacteria, ALBC discs including all available antibiotics, except colistin, were used to assess the biofilm formation in MRSA and *S. epidermidis* cultures. Discs loaded with colistin, gentamicin, and tobramycin were used in *Pseudomonas aeruginosa* cultures. For each antibiotic (or combination) to be tested, six discs of ALBC and three negative controls were placed singly in a Petri flask containing 3 mL of BHI broth with 10% (w/v) glucose. The medium was inoculated with 200 µL (0.5 McFarland) of bacteria and incubated in aerobic conditions. After 24 h at 37 °C, each specimen was removed from the flask and placed in another Petri flask containing 3 mL of BHI broth with 10% (w/v) glucose and 200 µL (0.5 McFarland) of bacteria and incubated in aerobic conditions at 37 °C for 24 h. Two specimens of ALBC and one negative

control were gently washed twice with sterile phosphate-buffered saline (PBS) to remove exclusively non-adherent cells. The adherent biofilm was fixed (dried) and observed under CLSM (48-h acquisition). The remaining four samples of ALBC and two negative controls were removed from the flask, placed in another Petri flask containing 3 mL of BHI with 10% (w/v) glucose and 200 µL (0.5 McFarland) of bacteria, and incubated in aerobic conditions at 37° for 24 h. Subsequently, two samples from the ALBC group and one control were gently washed twice with sterile PBS, and dried and observed under CLSM (72-h acquisition). The last two ALBC discs and one control specimen were removed from the flask, placed in another Petri flask containing 3 mL of BHI broth with 10% (w/v) glucose and 200 µL (0.5 McFarland) of bacteria, and incubated in aerobic conditions. After 24 h at 37 °C, the sample was gently washed twice with sterile PBS, stained, and observed under CLSM (96-h acquisition). In addition, six discs of ALBC for each antibiotic (or combination) and three negative controls were subjected to a fresh bacterial challenge every 24 h as described above to perform viable cell counts.

Biofilm analysis

Biofilms were evaluated using the LIVE/DEAD® BacLight Bacteria Viability stains (Life Technologies, Monza, Italy), which consist of a mixture of green-fluorescent nucleic acid stain SYTO® 9 and the red-fluorescent nucleic acid stain propidium iodide (PI). The first green-fluorescent dye (Syto9) crosses all bacterial membranes and binds to the DNA of both Gram-positive and Gram-negative bacteria. The second dye is red-fluorescent PI that only crosses damaged bacterial membranes (dead bacteria). Stained biofilms on ALBC discs and controls were visualized by CSLM (Zeiss, Arese, Milano, Italy) using a 20× dry objective (HC PL FLUOTAR 40.0 immersion oil). A 488-nm laser line was used to excite Syto9, and its fluorescent emission was detected from 500 to 540 nm. PI was excited with a 561 nm laser line, and its fluorescent emission was detected from 600 to 695 nm. Images from three randomly selected areas were acquired for each disc. Sequential optical sections of 1 µm were collected in sequence along the *z*-axis over the complete thickness of the sample. Quantification of biomasses was performed using the IMARISv7.0 software package (BITPLANE, Belfast, UK). Each image was analyzed, quantified, and rendered into a 3D image. Volume rendering was obtained by initially selecting only the colour channel corresponding to the cement and coverslip surfaces and setting them as the lower and upper sides of the image. Therefore, the green and red channels, corresponding to the cell volume of living and dead microorganisms within the biofilm, respectively, were added to the rendering. The elaboration of acquired images provided the visualization of the surface of the disc, biofilm matrix, and biofilm-embedded bacteria. The quantification of total biomass volume was performed by calculating the mean value of the biofilm thickness multiplied by the total surface of the acquired portions, as described by Drago et al. [10]. Finally, using the LIVE/DEAD staining mixture, it was possible to quantify the volume occupied by bacterial cells (live and dead) into the biomass.

Viable cell counts

Viable cell counts of surface-attached bacteria were performed at 48, 72, and 96 h post-inoculation to confirm the CLSM data. At each time interval, two ALBC discs and a negative control were removed from the flask and gently washed twice with PBS to remove non-adherent cells. The cement discs were then vortexed for 10 s, sonicated for 60 s at 40 kHz, and finally vortexed again for 10 s. Tenfold serial dilutions of the sonication fluid were plated onto blood-agar plates and incubated at 37 °C under aerobic conditions for 18–20 h. The number of colony-forming units (CFU) on each cement disc was expressed as CFU/cm^2. The percentage of viable cells on antibiotic-loaded bone cement was calculated relative to viable cells on the unloaded cements. Kirby–Bauer disc diffusion susceptibility tests for antibiotics were performed at 96-h post-inoculation.

Statistical analysis

Differences in the biomass volume were evaluated for statistical significance using an independent sample *t* test. An analysis of variance was used to test the differences among multiple groups. Possible differences were checked by Bonferroni tests. A *p* value of less than 0.05 was considered significant. IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA) was used for database construction and statistical analysis. A post hoc power analysis to assess the smallest difference (in biofilm volume) that could be detected keeping the study power equal to or more than 80% was carried out using G*Power software (Heinrich Heine University, Dusseldorf, Germany). With our sample size (2 specimens/antibiotic), 2.8 $\mu m^3 \times 10^6$ (standard deviation = 0.5) was the minimum difference in average biofilm volume between different cement specimens that could have been detected with 80% confidence.

Results

Controls

All tested bacterial strains were able to develop biofilms on plain PMMA bone cement specimens (controls) within the 48-h CLSM observation (Fig. 1). The overall volume

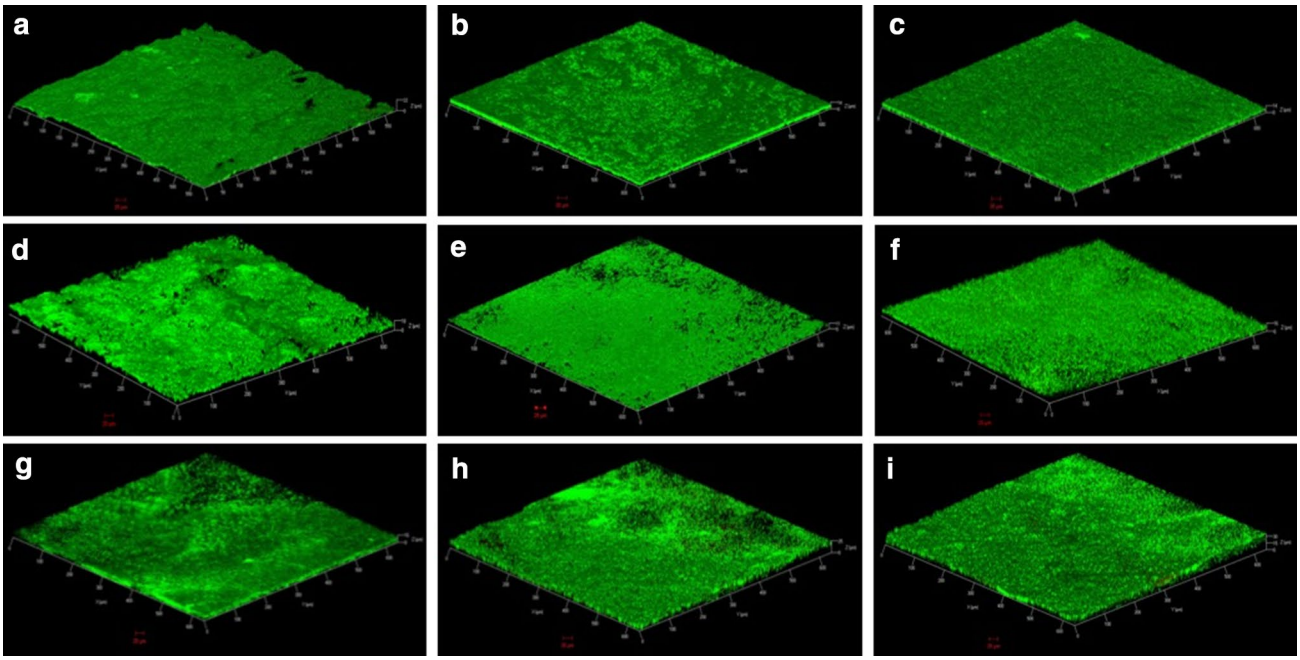

Fig. 1 3D reconstruction of *Staphylococcus aureus* (**a–c**), *Staphylococcus epidermidis* (**d–f**), *and Pseudomonas aeruginosa* (**g–i**) grown on plain PMMA. The left panels (**a, d, g**) represent the biofilms of MRSA (**a**), *S. epidermidis* (**d**), and *P. aeruginosa* (**g**) grown on plain PMMA bone cement specimens (controls) at 48 h. The pictures (**b, e, h**) refer to biofilm formation at 72-h observation of *Staphylococcus aureus* (**b**), *Staphylococcus epidermidis* (**e**), and *Pseudomonas aeruginosa* (**h**), respectively. The right panels (**c, f, i**) represent the biofilms of MRSA (**c**), *S. epidermidis* (**f**), and *P. aeruginosa* (**i**) at 96 h. The green fluorescence is associated with live cells. Scale bar 20 µm

of biomass did not show significant increases over time. The volume occupied by cells (live and dead) embedded into the matrix was less than 2% of the overall biofilm volume in all the experimental samples at any time interval.

MRSA

Data regarding biofilm formation on ALBC and control specimens incubated in MRSA medium as observed by CLSM are reported in Table 1. Biofilm formation at 96 h was observed on all antibiotic-loaded specimens (Fig. 2). Significantly lower bacterial biomass volume was detected

Table 1 Biofilm volume on antibiotic-loaded bone cement discs in methicillin-resistant *Staphylococcus aureus* culture

Sample	BV (μm^3) ± SD $\times 10^6$	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls
	48 h	72 h		96 h	
Controls	4.4 ± 0.5	3.9 ± 0.2		3.9 ± 0.4	
Gentamicin	–	–		1.2 ± 0.3	0.001
Clindamycin	–	0.5 ± 0.02	< 0.001	1.4 ± 0.2†	0.001
Tobramycin	–	1.2 ± 0.2	< 0.001	1.5 ± 0.9	0.007
Vancomycin	–	–		3.4 ± 0.5	n.s
Linezolid	–	–		1.3 ± 0.2	0.002
Clindamycin + gentamicin	–	–		2.9 ± 0.2	0.01
Tobramycin + vancomycin	–	1.9 ± 1	0.03	3.8 ± 0.5†	n.s
Gentamicin + vancomycin	–	–		4.4 ± 1.4	n.s

BV biofilm volume, *n.s.* non-significant, *SD* standard deviation

†*p* value < 0.05 vs 72-h control

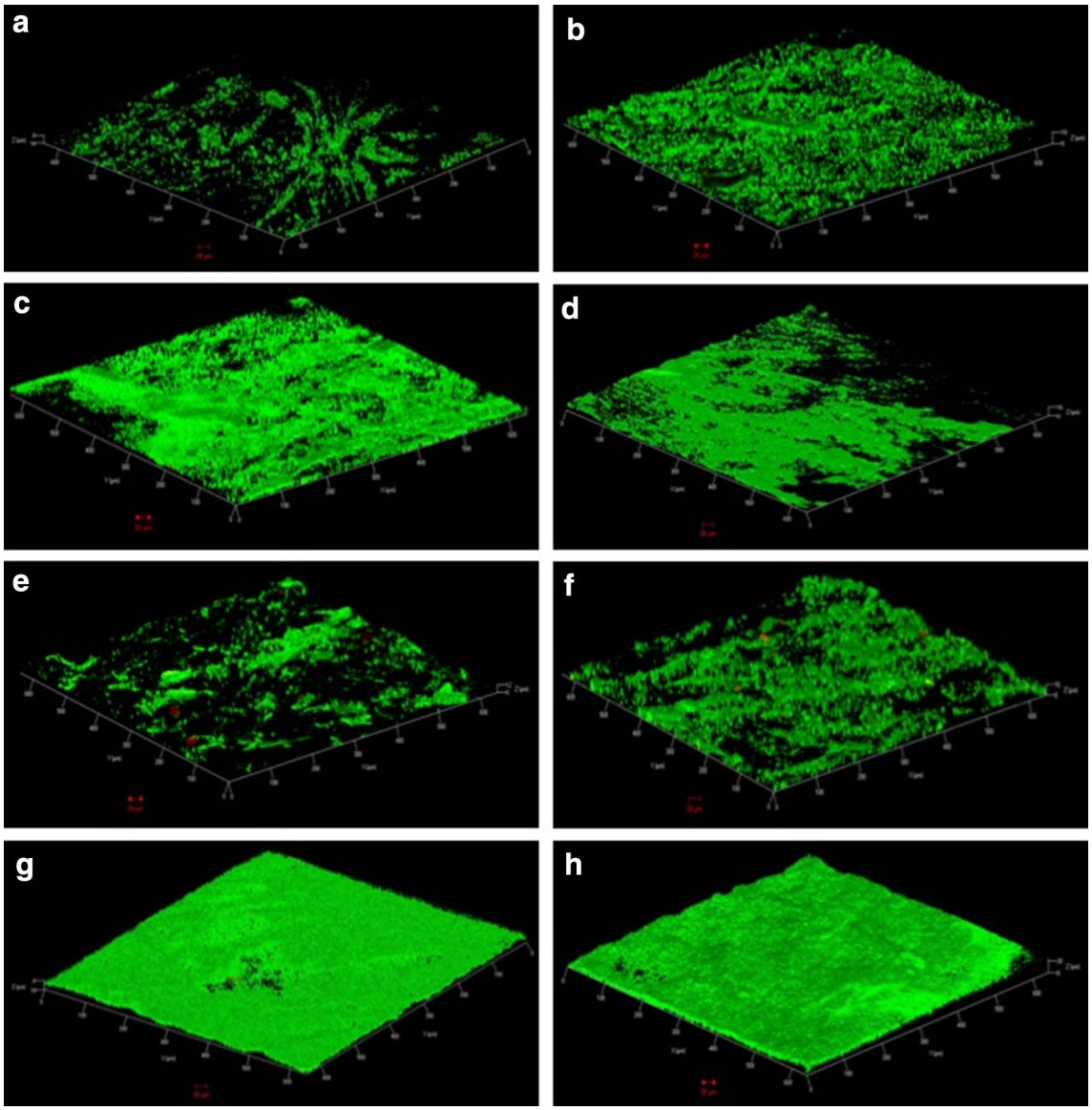

Fig. 2 Methicillin-resistant *Staphylococcus aureus* biofilm on antibiotic-loaded bone cement at 96 h. The panels (**a**–**e**) represent the 3D reconstruction of the biofilm on gentamicin (**a**), clindamycin (**b**), vancomycin (**c**), tobramycin (**d**), and linezolid (**e**) cement discs. The pictures (**f, g, h**) refer to MRSA biofilm formation on ALBC containing the association of two different antibiotics such as clindamycin–gentamicin (**f**), vancomycin–tobramycin (**g**), and gentamicin–vancomycin (**h**). The green fluorescence is associated with live cells. Scale bar 20 µm

on PMMA discs containing clindamycin, aminoglycosides, and linezolid in comparison with discs loaded with vancomycin. At the 96-h observation, the volume occupied by bacterial cells into the overall biomass was less than 1.5% (1.1% live; 0.4% dead) of the overall biofilm volume in all the experimental samples, independent of the antibiotic formulation. The CLSM data were consistent with the viable cell count of surface-attached bacteria (supplement data: Table S1). Bacterial susceptibility to antibiotics at 96-h post-inoculation was not modified in comparison with the baseline testing.

Staphylococcus epidermidis

Data regarding the CLSM analysis of biofilm formation on ALBC and control PMMA specimens incubated in *S. epidermidis* cultures are reported in Table 2. The difference in biofilm formation on ALBC samples containing tobramycin and gentamicin in comparison with the controls (Fig. 3) was significant at 72 h but not at 96 h. No differences in biofilm volume were observed at either time interval between ALBCs containing these two antibiotics. At 72 and 96 h, the volume occupied by cells

Table 2 Biofilm volume on antibiotic-loaded bone cement discs in *Staphylococcus epidermidis* culture

Sample	BV (μm^3) ± SD $\times 10^6$	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls
	48 h	72 h		96 h	
Controls	5 ± 1.4	4.7 ± 0.2		4.5 ± 0.5	
Gentamicin	–	3 ± 0.4	0.006	4.5 ± 0.6[†]	n.s
Tobramycin	–	3.4 ± 0.3	0.003	4 ± 0.4	n.s

BV biofilm volume, *n.s*. non-significant, *SD* standard deviation

[†] *p* value < 0.05 vs 72 h control

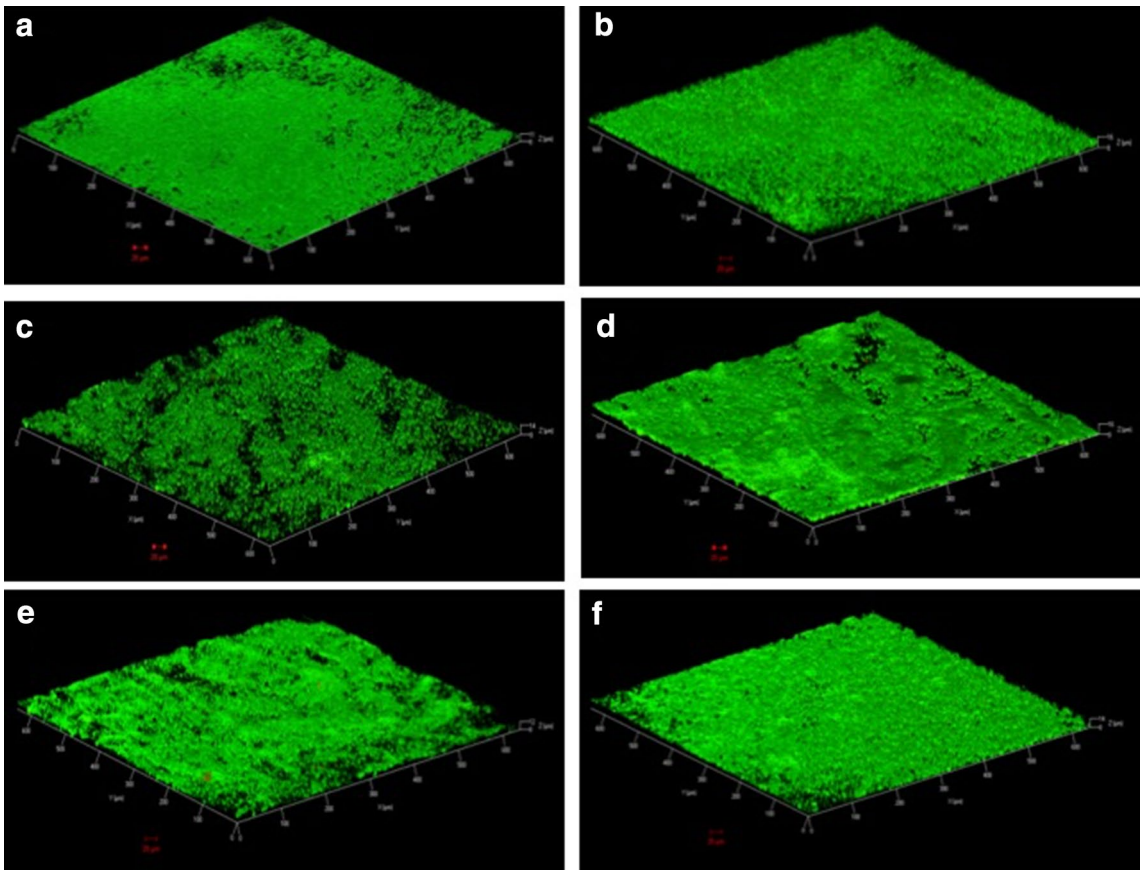

Fig. 3 3D reconstruction of the biofilm of *Staphylococcus epidermidis*. The pictures (**a, b**) refer to bacterial biofilm formation on plain PMMA bone cement specimens (controls) at 72 (**a**) and 96 (**b**) h. The panels (**c, d**) represent the *Staphylococcus epidermidis* biofilm on gentamicin bone cement specimens, at 72-h (**c**) and 96-h (**d**) observation. The pictures (**e, f**) show biofilm formation on tobramycin at 72-h (**e**) and 96-h (**f**) observations. The green fluorescence is associated with live cells. Scale bar 20 µm

with respect to the overall biofilm volume was less than 1.5% (0.9% live; 0.6% dead) on these ALBC discs. The CLSM data were consistent with the viable cell count of surface-attached bacteria (supplement data: Table S2). No changes in susceptibility to antibiotics of the tested strain were detected at 96-h post-inoculation.

Pseudomonas aeruginosa

Data on the development of biofilms on ALBC and control PMMA specimens incubated with *P. aeruginosa* cultures are reported in Table 3. No biofilms were present on tobramycin or gentamicin ALBC discs at any observation. The biofilm

Table 3 Biofilm volume on antibiotic-loaded bone cement discs in *Pseudomonas aeruginosa* culture

Sample	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls	BV (μm^3) ± SD $\times 10^6$	*p* value vs controls
	48 h		72 h		96 h	
Controls	5.3 ± 0.3		9.9 ± 3.7		12.4 ± 4.1	
Colistin	0.8 ± 0.3	< 0.001	2.8 ± 0.7†	0.03	2.5 ± 1.1	0.01

BV biomass volume, *SD* standard deviation

†*p* value ≤ 0.05 vs 48 h control

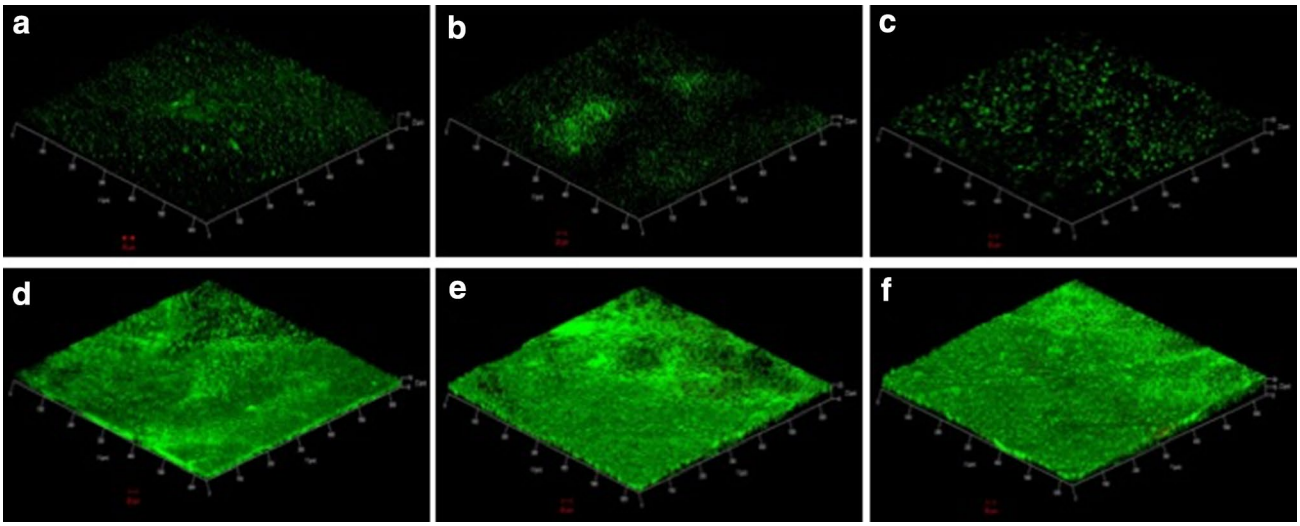

Fig. 4 3D reconstruction of the biofilm of *Pseudomonas aeruginosa*. The pictures (**a**–**c**) refer to bacterial biofilm formation on colistin ALBC at 48-h (**a**), 72-h (**b**), and 96-h observation (**c**). The panels (**d**–**f**) represent the *Pseudomonas aeruginosa* biofilm on plain PMMA bone cement specimens (controls), at 48-h (**d**), 72-h (**e**), and at 96-h (**f**) observation. The green fluorescence is associated with live cells. Scale bar 20 µm

formation on colistin-loaded cement specimens (Fig. 4) was significantly lower at all time intervals compared with controls. The volume occupied by the cells into the matrix on colistin ALBC discs was steadily less than 1.5% of the overall biofilm volume, with live and dead cells occurring with equal frequency. The CLSM data were consistent with the viable cell count of surface-attached bacteria (supplemental data: Table S3). The susceptibility of the tested bacterial strains to antibiotics at 96 h post-inoculation was identical to the baseline pattern.

Discussion

The most important finding of the present study was that ALBC discs loaded with any antibiotic (except for colistin) were able to inhibit biofilm formation within the first 48 h of bacterial inoculation compared to plain PMMA. The subsequent formation of biofilms over time on cement discs containing antibiotics was differentially inhibited depending on the microbial strain and the antibiotic. At the 72-h analysis, MRSA adhesion and colonization was detected only on ALBC samples containing clindamycin or tobramycin, even in association with vancomycin. Simultaneously, interval, biofilm growth by *S. epidermidis* was present on aminoglycoside (gentamycin or tobramycin) discs. However, biofilm formation by both of these microorganisms was significantly reduced on ALBC discs in comparison with plain cements. When exposed to bacteria for 96 h, none of the tested ALBCs completely prevented the MRSA biofilm growth. In MRSA cultures, the volume of biofilm on ALBC discs containing vancomycin alone or in combination was comparable to the controls. Conversely, linezolid-loaded samples showed significantly less biofilm formation. At 96 h, aminoglycoside-loaded ALBC no longer showed activity in reducing the biofilm formation by *S. epidermidis* in comparison with control samples. In *P. aeruginosa* cultures, discs containing tobramycin and gentamicin showed complete absence of biofilms over the entire period of observation. Colistin-loaded PMMA samples did not display the same effectiveness,

although the formation of *P. aeruginosa* biofilms was inferior to controls at all time intervals. These results show that different ALBCs have variable and time-dependent windows of effectiveness at inhibiting biofilm formation. Biofilm formation on ALBC is governed by a variety of factors, including the kinetics of antibiotic release from the surface and the characteristics of bacteria [34]. Indeed, the ability of ALBCs to prevent the early bacterial adhesion and biofilm formation in comparison with plain PMMA is related to the high local concentration of antibiotics during the first hours of bacterial inoculation, as shown by the previous *in vitro* and *in vivo* studies [5, 32]. The slow release of antibiotics in sub-inhibitory doses can be responsible for bacterial colonization and biofilm formation on the surface of ALBC over time [12, 21, 24]. Some studies [11, 25, 35] have shown that the prolonged exposure of *S. aureus* cultures to antibiotics may select the phenotypic subpopulations of survivors of this microorganism, called "small colony variants" (SCVs). SCVs of *S. aureus* grow slowly and may show increased resistance to antibiotics [11, 35]. Vancomycin can preferentially select for the amplification of SCV subpopulations [18], and in vitro models have confirmed that the SCV phenotype of *S. aureus* is less susceptible to this antibiotic [33]. However, bacterial strains in this study did not change their antibiotic susceptibility at 96-h post-inoculation. Thus, the time-dependent effectiveness of vancomycin could explain the declining ability to inhibit MRSA biofilm formation on ALBC discs loaded with this antibiotic. The present study also showed that the biofilm volume by MRSA on ABLC discs containing linezolid was reduced until 96 h. Although the efficacy of oral or intravenous linezolid is well documented, a few data are available on linezolid-loaded PMMA. Our findings concur with those of the previous studies that reported favourable patterns of antibiotic elution from ALBC containing linezolid [2, 4, 29]. Anguita-Alonso et al. [4] reported that the elution of linezolid is less influenced by its concentration in the ALBC in comparison with the other antibiotics. In addition, Anagnostakos et al. [2] reported that linezolid consistently showed elution concentrations above the susceptibility breakpoint for MRSA, thereby inhibiting its growth for 8 days. This effect was increased when gentamicin was added to linezolid. In the present study, the effectiveness of colistin ALBC at inhibiting the formation of *P. aeruginosa* biofilms was less pronounced in comparison with aminoglycosides. To the best of our knowledge, a few studies have evaluated ABLCs containing colistin [13, 26, 27], and no data are available on the in vitro analysis of biofilm formation on this PMMA.

The most important limitation of the current study is that the results obtained from in vitro experiments cannot necessarily be applied to in vivo findings because of the lack of surrounding tissues, blood supply, and normal supply of nutrients. Nevertheless, this study should be seen in the wider scope of using ALBC to prevent and treat PJIs. It could be a good starting point to discuss the usefulness of preoperative bacterial strain testing using ALBC discs to choose the proper antibiotic to add to PMMA, especially in the procedures with high doses of ALBC, such as two-stage exchange for PJIs. We tried to reproduce interactions between ABLC implants and the normal 'environment' using a complex experimental protocol. Indeed, the BHI broth amount was changed daily to simulate the in vivo local perfusion. Thus, this amount may have been too high compared to the in vivo circumstances; as such, the antibiotics could have been washed off the surface in a shorter time than they would have in vivo. Should this be the case, ALBC would show more satisfactory activity in vivo. However, this methodology has been used in the previous in vitro studies [1, 7, 17, 22]. Small significant differences in biofilm volume between some ALBCs and control discs may have been missed because of the small number of cement specimens tested. Nevertheless, the clinical meaning of our study remains unchanged. Indeed, a surgeon should select only the most effective ALBCs to prevent and treat PJIs. The strengths of this study also deserve mention. First, it provided quantitative three-dimensional assessment of bacterial biomass. Second, microbial strains isolated from clinical PJIs were used for this study rather than genetically manipulated microorganisms. Third, the antibiofilm activity of ABLCs containing previously untested antibiotics was also assessed. Finally, CLSM acquisitions were obtained until 96 h from the initial bacterial inoculation, a time interval that is longer than the previous studies.

Conclusions

Bone cements containing different antibiotics have variable and time-dependent windows of activity in inhibiting surface biofilm formation within 96 h of bacterial inoculation. Our results raise concerns on the efficacy of PMMA containing vancomycin and colistin for the local control of PJIs sustained by MRSA and *Pseudomonas aeruginosa*, respectively. Preoperative testing of bacterial strains using different ALBC discs could be a promising approach to select the proper antibiotic to add to PMMA, especially in the surgical procedures with high doses of ALBC, such as two-stage exchange for PJIs.

Author contributions GB, MRC, ER, OG, GG, and MM worked on conception and study design. GB, ER, and AV performed sample acquisition and data testing. All authors played a role in data analysis and interpretation. GB, ER, MRC, TA, and MM drafted the manuscript. All authors played a role in critical review and revision of final manuscript.

Funding No funding was received for this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Research ethics committee Institutional Review Board (IRB) approval is not required due to in vitro study design.

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