Influence of biofilm growth age, media and antibiotics exposure time on Staphylococcus aureus and Pseudomonas aeruginosa biofilm removal in vitro

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SUBJECT AREAS

General Microbiology
Abstract
Biofilm is known to be tolerant towards antibiotics and difficult to eradicate. Numerous studies have reported Minimum Biofilm Eradication Concentration (MBEC) values of antibiotics for many known biofilm pathogens. However, the experimental parameters applied in these studies differ considerably, and often the rationale behind the experimental design are not well described. This makes it difficult to compare the findings. To demonstrate the importance of experimental parameters, we investigated the influence of biofilm growth age, antibiotic treatment duration and growth media on biofilm eradication in this study. The commonly used biofilm model Calgary biofilm device was used to grow 24 h and 72 h biofilms of Staphylococcus aureus and Pseudomonas aeruginosa, which were treated with time-dependent vancomycin and concentration-dependent tobramycin, respectively. Two common bacteriological growth media Tryptic Soy Broth (TSB) and Cation-adjusted Mueller Hinton Broth (CaMHB) were tested. We found for both species that biofilms were more difficult to kill in TSB than in CaMHB. Furthermore, young biofilms (24 h) were easier to eradicate than old biofilms (72 h). In agreement with vancomycin being time-dependent, extension of the vancomycin exposure increased killing of S. aureus biofilms. Tobramycin treatment of 24 h P. aeruginosa biofilms was found concentration-dependent and time-independent, however, increasing killing was indicated for 72 h P. aeruginosa biofilms. This study demonstrated biofilm removal efficacy was influenced by media, biofilm age and antibiotics treatment duration. It is therefore necessary to taking these parameters into consideration when designing experiments.

Introduction
To improve diagnosis, treatment and prevention of infections, it is necessary to differentiate between acute infections with primarily planktonic microorganisms and biofilm infections with overweight of clusters of microbial cells (1–4). Most microorganisms in a biofilm grow slowly with down-regulated virulence and are heterogeneously distributed. They are less susceptible to antibiotics compared with their planktonic counterpart and can often not be cleared by the immune system (5–7). Biofilm related infections can be device-related biofilm infections, such as prosthetic joint infections, or native tissue infections e.g. chronic osteomyelitis and cystic fibrosis. The most effective treatment of biofilm
related infections is to remove the infected medical device and to debride the infected tissue in combination with antibiotic therapy (8).

An early and correct diagnosis is necessary for proper antibiotic administration. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antibiotics preventing visible bacterial growth, while minimum bactericidal concentration (MBC) is the lowest concentration required to kill the bacteria. MICs are used by diagnostic laboratories mainly to confirm resistance. Determination of MIC and MBC is based on planktonic cells, whereas the minimum biofilm eradication concentration (MBEC) is defined as the lowest concentration of antibiotic required to eradicate the biofilm. MBEC has not been implemented in the clinical setting yet, and the published MBEC data are often incomparable because of different experimental conditions. Tables 1 - 2 illustrate examples of two important biofilm pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and their MIC and MBEC values determined in several studies. As shown in Tables 1 - 2, tryptic soy broth (TSB) and cation-adjusted Mueller Hinton Broth (CaMHB) media are often used in these studies. TSB is a complex nutrient-rich general-purpose medium, while CaMHB is recommended for MIC testing of non-fastidious organisms according to ISO standard 20776-1 (2006) and is the standard medium in clinical laboratories in the US and European committee on antimicrobial susceptibility testing. High throughput methods for MBEC determination are most frequently used including 96-well microtiter plate combined with crystal violet staining, the Calgary biofilm device (CBD), or its commercial version the MBEC™ Assay (Innovotech, Canada) (9). As shown in Table 1-2, MIC values were similar for most of the studies. However, MBEC of vancomycin towards *S. aureus* varies from 1 to more than 8000 mg/L. Similarly, the MBEC of tobramycin towards *P. aeruginosa* varies from 2 to 2560 mg/L. This large discrepancy in MBEC values is surprising, especially in light of some studies using the same strain. We hypothesize that the different test parameters and lack of standardization contributed to the large disparity.

The purpose of this study was to demonstrate the influences of biofilm age, growth media, and antibiotics exposure time on *S. aureus* and *P. aeruginosa* biofilm removal using vancomycin and tobramycin, respectively. These two antibiotics were chosen because they are recommended for
serious and life-threatening infections caused by Gram-positive bacteria and Gram-negative bacteria. In addition, we investigated the possibility of biofilm eradication by local antibiotics treatment by testing OSTEOmycin, an allograft bone product loaded with either vancomycin or tobramycin (10, 11).

Results
Four biofilm formation strains were chosen for this study. S. aureus strains SAU060112 (12) was isolated from prosthetic knee infection while S. aureus ATCC 49230 was originally from chronic osteomyelitis. Both infections are known to be associated with biofilms. P. aeruginosa strain PA14 is a well-known biofilm former (13) and P. aeruginosa ATCC 15442 is also known to form biofilms (14, 15). All four tested strains in this study were found susceptible to the tested antibiotics. The vancomycin MIC for both S. aureus strains was determined to be 1.25 mg L⁻¹, which is lower than breakpoint (2 mg L⁻¹) for S. aureus. Likewise, the tobramycin MIC for both P. aeruginosa strains was 0.63 mg L⁻¹, which is lower than breakpoint (4 mg L⁻¹) for P. aeruginosa according to Clinical breakpoints – bacteria (v 9.0) in European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf).

Influence of biofilm age
Biofilm growth is dynamic and mature biofilms are thought to be more antibiotic tolerant. In this study biofilms grew for 24 h or 72 h first and then were subjected to antibiotics challenge for different duration. It was found that the number of colony forming units (CFUs) were higher for 72 h biofilms than for 24 h biofilms by up to 1-log difference (P<0.01, Figure S2 and S3). Additionally, 72 h biofilms were more difficult to eradicate than 24 h (P<0.001) as shown in Figures 1-2. It is important to stress that each data point in Figures 1 - 4 represents results for a minimum of 20 replicates from two independent experiments. Instead of MBEC value which defines complete killing of biofilms, biofilm survival ratio was chosen to present the percentage of replicates survived after treatment. The reason is because biofilm eradication was found varied among the replicates and a single MBEC value cannot provide the information.

For complete killing of 24 h SAU060112 biofilms in TSB medium, i.e. MBEC, exposure of the biofilms
with a minimum 1000 mg L\(^{-1}\) of vancomycin for four days or 100 mg L\(^{-1}\) for seven days was required (Figure 1a), whereas some 72 h biofilms still survived even with 3000 mg L\(^{-1}\) of vancomycin after seven days (Figure 1c). In CaMHB medium it required 10 mg L\(^{-1}\) of vancomycin for seven days to remove 24 h SAU060112 biofilms (Figure 1b) and 10-fold more for 72 h biofilms (Figure 1d).

In the case of strain PA14, a minimum of 10 mg L\(^{-1}\) of tobramycin killed almost all 24 h biofilms in TSB media regardless of exposure duration (Figure 2a) while for 72 h biofilms 80 mg L\(^{-1}\) of tobramycin for at least 2 days was needed (Figure 2c). In CaMHB medium, complete killing of 24 h biofilms was achieved with 5 mg L\(^{-1}\) of tobramycin regardless of exposure duration (Figure 2b), while it required more than 10 mg L\(^{-1}\) for 72 h biofilms when the treatment was shorter than 7 days (Figure 2d).

**Media**

Biofilm formation depends on many factors including nutrient availability. The main nutrients in both TSB and CaMHB media are amino acids. In addition, TSB contains glucose (2.5 g L\(^{-1}\)) while CaMHB has starch (1.5 g L\(^{-1}\)). The number of CFUs in the biofilms growing in these two media were different (P < 0.05, Figure S2 and S3). On average, slightly more CFUs were found in biofilms growing in CaMHB than TSB, except 72 h PA14 biofilms.

When challenged with antibiotics, biofilms were more difficult to kill in TSB than in CaMHB. For 24 h SAU060112 biofilms (Figure 1a and 1b), seven-day treatment with 100 mg L\(^{-1}\) and 10 mg L\(^{-1}\) of vancomycin were required to kill all biofilms in TSB and CaMHB media, respectively. For 72 h SAU060112 biofilms, none of the vancomycin treated achieved complete killing in TSB medium, while 100 mg L\(^{-1}\) of vancomycin removed all biofilms after seven days exposure in CaMHB (Figure 1c and 1d). For near complete killing of 24 h PA14 biofilms, four-fold more tobramycin was needed in TSB than in CaMHB (10 and 2.5 mg L\(^{-1}\), respectively) (Figure 2a and 2b), while for 72 h biofilms, two-fold more tobramycin was required (80 mg L\(^{-1}\) in TSB and 40 mg L\(^{-1}\) in CaMHB) (Figure 2c and 2d).

**Antibiotics exposure time**

Vancomycin is known to be a time-dependent antibiotic (16). Extending vancomycin exposure time
from one to four days reduced survival ratio of SAU060112 biofilm in TSB (Figure 1a and 1c, Table 4) and CaMHB media (Figure 1b and 1d, Table 4). Prolonging treatment from four to seven days showed no further killing except 24 h biofilms in TSB (Table 4). Increased killing by prolonging vancomycin exposure was also found for S. aureus ATCC 49230 biofilms (Figure 3a).

In contrast to vancomycin, tobramycin is known to exhibit concentration-dependent bactericidal activity (17). Removal efficacy of 24 h P. aeruginosa PA14 biofilm was not enhanced when duration was extended (Figure 2a and 2b, Table 5). However, increasing killing was indicated for 72 h PA14 biofilms (Figure 2c and 2d, Table 5) as well as for 72 h P. aeruginosa ATCC 15442 biofilms (Figure 3b).

**Strains**

The two S. aureus strains have the same vancomycin MIC value. Although the necessary concentration of vancomycin for biofilm eradication differed slightly, the same tendency is indicated for both strains that prolonged vancomycin treatment eradicated more biofilms. Similarly, the two P. aeruginosa strains have the same tobramycin MIC value and extended tobramycin treatment lowered MBEC values for 72 h biofilms for both strains.

**OSTEOmycin**

OSTEOmycin V™ removed all S. aureus ATCC 49230 biofilm except three replicates of 72 h biofilms survived after two days treatment. None of the PA14 biofilms survived exposure to OSTEOmycin T™ (Figure 4).

**Discussion**

**Biofilm age**

Several biofilm models have been developed, each with many experimental parameters that can be adjusted. This flexibility inevitably makes it difficult to compare results obtained with varying conditions chosen in different studies. In the current study we confirmed previous findings that mature biofilm have reduced antibiotics susceptibility compared with young biofilms (18–21). However, no definition of young and mature biofilm has been universally adopted. In the case of P. aeruginosa, some considered 4 h biofilm as young and 24 h as mature (22), while others considered 24 h as young and 12 days biofilm as mature (20). Similarly, 6 h S. aureus biofilm was considered as
young and 24 h as mature (23), whereas some considered 7 days old biofilm as mature (24). The inconsistency in the different biofilm studies underlines the need for a form of consensus definition and a simple way to measure maturity. The textbook version of biofilm formation involves bacterial initial attachment to a solid surface, the formation of microcolonies on the surface, and finally differentiation of microcolonies into exopolysaccharide-encased, mature biofilms. However, studies often assume the maturity of the biofilm without looking into the structure of the biofilms or even CFUs of biofilm. In the case of MIC testing, a crucial parameter is inoculum size which is set to be $5 \times 10^5$ CFU mL$^{-1}$. It is because MIC values can increase concurrently with increasing number of CFUs (25).

The current study treated 24 h biofilms as young and 72 h as mature. The CFU per biofilm shown in Figure S2 and S3 indicated continuous growth in cell number after 24 h for up to 1-log. In batch culture, bacterial growth curve defines the different stages of planktonic culture growth. Similarly, the biofilm formation curves can be established for each strain and growth condition. It was shown previously (26) that using CBD the number of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 continuously increased over 24 h while the growth of *S. aureus* stagnated after 7 h under the same condition. As the growth phase of the biofilm influences antimicrobial susceptibility, it is therefore important to construct the biofilm growth curve for each strain under the chosen conditions.

**Growth media**

Biofilm eradication was found different with the two media (Figure 1 and Figure 2). Different composition of media is reported to change the activity of antibiotics (27–29). The $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ ions in CaMHB media are required for a correct antimicrobial susceptibility testing because those ions reflect the divalent cation concentration in human blood (30–33). However, *in vivo* conditions are far more complex, and the biofilms formed *in vivo* often incorporate human factors such as blood components. Hence, the antibiotics concentration needed for biofilm eradication will mostly likely be different from *in vitro* results. For comparison between different studies, a simple and widely available culture media is suitable, but for estimation of *in vivo* biofilm killing host factors in form of, for
example, serum, plasma, or blood should be included in testing medium.

**Antibiotics exposure time**

Vancomycin displayed a time-dependent eradication of *S. aureus* biofilms (Table 2) which has been demonstrated in other studies (24, 34, 35). Post et al. have shown continuous reduction of viable *S. aureus* biofilm cells over 28 days (24). This indicates that further killing could be possible by prolonging the antibiotic exposure time in the current study and complete eradication could be achieved at lower vancomycin concentration.

In contrast to vancomycin, tobramycin exhibits concentration-dependent activity (36–39). The current study indicated that tobramycin displayed concentration-dependent activity for 24 h PA14 biofilms. However, increased killing of 72 h biofilms were observed with prolonged exposure. Castaneda et al. found increased biofilm antimicrobial susceptibility with increasing antimicrobial exposure time including tobramycin against *P. aeruginosa* biofilms (40), whereas Walters et al. only found little reduction in *P. aeruginosa* biofilm cell count with longer tobramycin treatment (41). Futures studies are needed to investigate the time-dependency of tobramycin antibiofilm effect.

Regardless of the antimicrobials being time-dependent or concentration-dependent on planktonic bacteria, it may be different on biofilm cells because of the presence of biofilm matrix. Exposure time may play an important role in determination of killing effect, because the biofilm matrix may slow down antimicrobial penetration (42). Therefore, a killing curve is much more informative than a definitive MBEC value determined at a fixed time point.

**OSTEOmycin**

Since the antibiotic concentration needed for biofilm eradication is far above the parenterally administrated levels, local delivery of antibiotics may achieve concentrations high enough for biofilm killing. In this study, OSTEOmycin showed a strong biofilm eradication efficacy and completely removed biofilm in all tested conditions except three 72 h *S. aureus* biofilms. OSTEOmycin is a product developed based on Winkler et al. 2000 (43). According to the study, 1 g human cancellous bone impregnated with vancomycin released around 20000 mg L⁻¹ vancomycin in 3 mL of 5% human
albumin solution after one day and decreased to around 100 mg L\(^{-1}\) after seven days. When impregnated with tobramycin, it released more than 10000 mg L\(^{-1}\) tobramycin after one day and decreased to around 30 mg L\(^{-1}\) after seven days (43). These concentrations are much higher than the MBEC values found in Figure 1 and 2, which likely explains the high efficacy. Osteomycin was also shown to be a promising product for local treatment of osteomyelitis in the clinic although recurrence may still occur in complex cases within an unknown period of time (11).

**Conclusion**

This study showed biofilm removal efficacy was influenced by media, biofilm age and antibiotics treatment duration. It is therefore necessary to take these parameters into consideration when designing experiments. We recommend choosing the conditions most similar to the in vivo situation and explaining the rationale when reporting. This study also showed that in vitro biofilms were possible to be eradicated when treated with long-term high concentrations of antibiotics. This finding needs to be confirmed by in vivo studies.

**Material And Method**

**Bacterial strains, growth media and antibiotics**

*S. aureus* strains SAU060112 (12) and ATCC 49230 were tested with vancomycin (Sigma-Aldrich). *P. aeruginosa* strains PA14 and ATCC 15442 were tested with tobramycin (Sigma-Aldrich). Both TSB (Sigma-Aldrich) and CaMHB (Sigma-Aldrich) media were employed in susceptibility testing.

**Minimum inhibitory concentration (MIC) determined by the broth microdilution method**

The broth microdilution method was used to determine the MIC of each strain according to the procedures described in Wiegand et al. (44). Briefly, each strain was inoculated on TSB agar plate for 24 h. Then five well-isolated colonies were selected and inoculated in a 50 mL tube with 20 mL CaMHB until the OD\(_{600}\) value of the culture reached around 0.6. The culture was diluted to approximately \(1 \times 10^6\) colony-forming unit (CFU) mL\(^{-1}\). Then, 100 µl of the diluted culture was added into each well of a 96-well-plate containing 100 µl of antibiotics at different concentrations. The plate was covered and inoculated at 37°C with shaking at 150 rpm for 24 h. After that, OD\(_{595}\) of each well was measured by Infinite F200 Pro (Tecan Group Ltd., Switzerland) to determine MIC.
Biofilm antibiotics susceptibility testing by Calgary Biofilm Device (CBD)

CBD (45) was used to grow biofilms. An illustration of the experimental procedure is given in Figure S1. Briefly, biofilms were formed by immersing the pegs of a microtiter lid (Nunc\textsuperscript{TM} 445497) into the biofilm growth microtiter plate (Figure S1), 150 µl of the diluted culture containing $10^4$ CFU was added into the wells of 96 well microtiter plate (Thermo Fisher Scientific) and then covered with peg lid. The biofilms were allowed to grow in TSB or CaMHB media at 37°C with shaking at 150 rpm for intended time (Table 3). After incubation, the lid with biofilms was transferred to a rinse plate containing 200 µl saline in each well and incubated for 1 minute. The rinsed lid was then transferred to a challenge plate containing 200 µl antibiotics solution in each well. The antibiotics were prepared in the media used for growing biofilms. After challenged in the antibiotics solution, the lid containing biofilms was rinsed twice with fresh saline each time and then transferred to a recovery plate containing 200 µl sterile media followed by sonication at 40KHz for five minutes. The recovery plate was inoculated for another 24 h at 37°C with shaking at 150 rpm and OD595 measured by Infinite F200 Pro to determine the biofilm removal efficacy. All tests were repeated at least on two occasions with minimum 10 replicates each time. Percentage of the surviving replicates was calculated and presented as biofilm survival ratio.

Biofilm eradication by OSTEOmycin\textsuperscript{TM}

OSTEOmycin\textsuperscript{TM} samples were obtained from European Cell and Tissue Bank (ECTB). Two clinical strains \textit{S. aureus} ATCC 49230 and \textit{P. aeruginosa} PA14 were chosen for this test. Seventy-two h (3-days) or 168 h (7-days) biofilms were challenged with OSTEOmycin\textsuperscript{TM} for 1, 2, 4 or 7 days following the method described above. \textit{S. aureus} ATCC 49230 biofilms were challenged with 186 g L\textsuperscript{-1} OSTEOmycin V\textsuperscript{TM} in CaMHB, while \textit{P. aeruginosa} PA14 biofilms were subjected to 220 g L\textsuperscript{-1} OSTEOmycin T\textsuperscript{TM}.

Statistics analysis

ANOVA was used to calculate the difference between biofilm formation on CBD pegs. Binary logistic
regression model was used to compare biofilm removal efficacy under different conditions.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information file.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The study was conceived and designed by YX, HW and TRT. XC performed laboratory experiments. XC and YX analyzed the data. YX, XC and TRT wrote the manuscript. All authors have read and approved the manuscript.

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Tables
Table 1 MBEC values of vancomycin for S. aureus found in a few studies. Please note ATCC 29231 were tested in several studies with different MBEC values.
| S. aureus strains | MIC (mg L⁻¹) | Challenge medium | Biofilm age (h) | Treatment duration (hours) | Biofilm model | MBEC (mg L⁻¹) |
|------------------|--------------|------------------|----------------|---------------------------|---------------|---------------|
| ATCC 49230       | 2            | TSB              | 24             | 24,72,120                 | 96-well microtiter plate | >8000, 20000 |
| ATCC BAA1556     | 2            | TSB              | 24             | 24,72,120                 | 96-well microtiter plate | >8000, 000 |
| ATCC 6538P, MRSA 16 | 0.5,1       | Not mentioned    | 24             | 24                        | Beads         | >200          |
| ATCC 29213, UOC18 | 1-2          | CaMHB            | 24             | 1-72                      | CBD           | >102          |
| ATCC 29213       | 1            | CaMHB            | 24             | overnight                 | CBD           | >102          |
| ATCC 29213       | 1            | MHB              | 48             | 24                        | CBD           | >51, 254     |
| ATCC 35556       | 1            | CaMHB            | 24             | 24                        | CBD           | >254          |
| ATCC 29213, ATCC 33591, VRS5 | 1-2 | CaMHB | 24 | 24 | CBD | >128, 102 |
| B341002, B346846 | 0.5-1        | CaMHB            | 24             | overnight                 | CBD           | 128, 102     |
| Clinical isolates | 0.5-1        | CaMHB            | 18             | 24                        | 96-well microtiter plate | 8-16         |
| Clinical isolates | 0.5-1        | CaMHB            | 18             | 24                        | 96-well microtiter plate | 4-32         |
| 40 MRSA isolates | 1            | TSB              | 24             | overnight                 | CBD           | 1-64         |

Table 2 MBEC values of tobramycin for *P. aeruginosa* in a few studies.
| Strains            | MIC (mg L⁻¹) | Challenge media | Biofilm inoculation (hours) | Treatment duration (hours) | Biofilm model       | MBEC (mg L⁻¹) |
|-------------------|--------------|-----------------|-----------------------------|----------------------------|---------------------|---------------|
| ATCC 27853        | 0.25-16      | CaMHB           | 24                          | 1,2,4                      | 96-well microtiter plate | 160-25       |
| ATCC 27853        | 0.25         | TSB             | 24                          | 24,72,120                  | 96-well microtiter plate | 2000, ≤ 250  |
| Strain K (PAK)    | Not tested   | LB              | 72                          | 18                         | 96-well microtiter plate | 200-16       |
| PAO1              | <2           | CaMHB           | 6                           | 16-20                      | CBD                 | 64(57)       |
| ATCC 27853        | 0.5          | CaMHB           | 24                          | overnight                  | CBD                 | 2(26)        |

Table 3 An overview of experimental parameters used this study.
| Strains                | Isolation source                     | Media         | Biofilm growth (hours) | Antibiotics treatment duration (days) |
|-----------------------|--------------------------------------|---------------|------------------------|---------------------------------------|
| *S. aureus* SAU060112 | Prosthetic joint infection           | TSB or CaMHB | 24, 72                 | 1, 2, 4 and 7                         |
| *S. aureus* ATCC 49230| Patient with chronic osteomyelitis   | CaMHB         | 72                     | 1, 2, 4 and 7                         |
| *P. aeruginosa* PA14  | Human burn patient                   | TSB or CaMHB | 24, 72                 | 1, 2, 4 and 7                         |
| *P. aeruginosa* ATCC 15442 | Animal room water bottle             | CaMHB         | 72                     | 1, 2, 4 and 7                         |

Table 4. P-values for difference between *S. aureus* SAU060112 biofilm survival ratio after vancomycin treatment of different durations. ** indicates P<0.001.

| Biofilm age (hours) | Treatment durations (days) | 1 | 2  | 4  | 7  | 1 | 2 | 4 | 7 | 1 | 2 | 4 | 7 |
|---------------------|----------------------------|---|----|----|----|---|---|---|---|---|---|---|---|
|                     | TSB                        |   |    |    |    |   |   |   |   |   |   |   |   |
|                     | CaMHB                      |   |    |    |    |   |   |   |   |   |   |   |   |
| 24                  |                            |   |    |    |    |   |   |   |   |   |   |   |   |
| 1                   |                            | - | ** | ** | ** |   | ** | ** | ** |   | ** | ** | ** |
| 2                   |                            | - |    | ** | ** |   | 0.110 | 0.251 |   |   |   |   |   |
| 4                   |                            | - |    |    | ** |   |    | 0.790 |   |   |   |   |   |
| 7                   |                            | - |    |    |    |   |    |   |   |   |   |   |   |
| 72                  |                            |   |    |    |    |   |   |   |   |   |   |   |   |
| 1                   |                            | - | ** | ** | ** |   | ** | ** | ** |   | ** | ** | ** |
| 2                   |                            | - |    | ** | ** |   | 0.220 | 0.027 |   |   |   |   |   |
| 4                   |                            | - |    |    | 0.578 |   |    | 0.391 |   |   |   |   |   |
| 7                   |                            | - |    |    |    |   |    |   |   |   |   |   |   |

Table 5. P-values for difference between *P. aeruginosa* PA14 biofilm survival ratio after tobramycin treatment of different durations. * indicates P<0.01 and ** indicates P<0.001.
| Biofilm age (hours) | Treatment durations (days) | TSB          | CaMHB       |
|--------------------|-----------------------------|--------------|-------------|
|                    | 1   | 2   | 4   | 7   | 1   | 2   | 4   | 7   |
| 24                 |     | 0.553 | 0.188 | 0.578 | -   | 0.042 | 0.535 | 0.309 |
| 2                  |     | 0.220 | 0.518 | -    | *   | 0.218 |
| 4                  |     | -    | 0.101 | -    | -   | 0.113 |
| 7                  |     | -    | -    | -    | -   | -    |
| 72                 |     | -    | *    | **  | **  | -    | 0.016 | **  | **  |
| 2                  |     | -    | **  | **  | -   | 0.128 | *    |
| 4                  |     | -    | **  | -   | -   | 0.233 |
| 7                  |     | -    | -    | -    |

Figures

a. 24 Hours S. aureus SAU060112 biofilm survival ratio in TSB after vancomycin treatment
b. 24 Hours S. aureus SAU060112 biofilm survival ratio in CaMHB after vancomycin treatment
c. 72 Hours S. aureus SAU060112 biofilm survival ratio in TSB after vancomycin treatment
d. 72 Hours S. aureus SAU060112 biofilm survival ratio in CaMHB after vancomycin treatment

Figure 1

S. aureus SAU060112 biofilm survival ratio after vancomycin treatment. Biofilms of S. aureus SAU060112 were grown for 24 h or 72 h in TSB or CaMHB medium followed by vancomycin treatment for 1, 2, 4 or 7 days. Each data point contained at least 20 replicates conducted at two occasions.
Figure 2

P. aeruginosa PA14 biofilm survival ratio after tobramycin treatment. Biofilms of P. aeruginosa PA14 were grown for 24 h or 72 h in TSB or CaMHB medium followed by tobramycin treatment for 1, 2, 4 or 7 days. Each data point contained at least 20 replicates conducted at two occasions.
Survival ratio of 72 h S. aureus ATCC 49230 biofilms after vancomycin treatment (a) and 72 h P. aeruginosa ATCC15442 biofilm after tobramycin treatment (b). Each data point contained at least 20 replicates conducted at two occasions.

Figure 3

72 h S. aureus ATCC 49230 (a) and P. aeruginosa PA14 (b) biofilm survival ratio after OSTEOmycin treatment.

Figure 4

Supplementary Files
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