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Vincenzo Di Pilato [a], Federica Ceccherini [b], Samanta Sennati [b], Federico D'Agostino [b], Fabio Arena [b,#], Noemi D'Atanasio [c], Francesco Paolo Di Giorgio [c], Serena Tongiani [c], Lucia Pallecchi [b,*] and Gian Maria Rossolini [a,d,*]

[a] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

[b] Department of Medical Biotechnologies, University of Siena, Siena, Italy

[c] Angelini RR&D (Research, Regulatory & Development) – Angelini S.p.A., S. Palomba-Pomezia, Rome, Italy

[d] Microbiology and Virology Unit, Florence Careggi University Hospital, Florence, Italy

[#] Current affiliation: Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

*** Corresponding authors**

Lucia Pallecchi

Department of Medical Biotechnologies, S. M. alle Scotte University Hospital, Viale Mario Bracci 1, 53100, Siena, Italy; tel.: +39-0577-233328; fax: +39-0577-233870. E-mail address: lucia.pallecchi@unisi.it

Gian Maria Rossolini

Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla 3, 50134, Florence, Italy; tel.: +39-055-7949239. E-mail address: gianmaria.rossolini@unifi.it

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Abstract

Staphylococcus aureus and *Staphylococcus epidermidis* are leading pathogens of biofilm-related infections and represent the most common cause of osteomyelitis and biomedical implants infections. Biofilm-related infections usually require long-term antibiotic treatment, often associated to surgical interventions. Dalbavancin is a newer lipoglycopeptide approved for the treatment of acute skin and skin-structure infections caused by Gram-positive pathogens. In addition, dalbavancin has recently been considered as a potential option for the treatment of staphylococcal osteomyelitis and orthopedic implant infections.

In this study, time-kill kinetics of dalbavancin against *S. aureus* and *S. epidermidis* biofilms were determined over prolonged exposure times (up to 7 days), using both a standardized biofilm susceptibility model and biofilms grown onto relevant orthopedic biomaterials (i.e. titanium and cobalt-chrome disks). Dalbavancin (at concentrations achievable in bone and articular tissue) showed a potent activity against established staphylococcal biofilms in both tested models, and was overall superior to the comparator vancomycin.

1. Introduction

Bacterial biofilms play a crucial role in the pathogenesis of relevant human infections, such as endocarditis, osteomyelitis and infections related to the use of biomedical devices (e.g. central vascular catheters, urinary catheters, orthopedic implants, joint prostheses, prosthetic cardiac valves, vascular grafts and pacemakers) (Arciola et al. 2018; Beck-Broichsitter et al., 2015; Lebeaux et al., 2014).

Staphylococcus aureus and *Staphylococcus epidermidis* are leading pathogens of biofilm-related infections, and represent the most common causative species of osteomyelitis and biomedical implants infections (Arciola et al., 2018; Kavanagh et al., 2018; Suresh et al., 2018). Treatment of staphylococcal biofilm-related infections is complicated by the recalcitrance to antibiotic therapy, typical of bacterial biofilms, and by the frequent multidrug resistance phenotype expressed by *S. aureus* and coagulase-negative staphylococci (CoNS) (Arciola et al., 2018; Kavanagh et al., 2018; Suresh et al., 2018).

Concerning orthopedic biofilm-related infections, interdisciplinary approaches comprising surgical interventions (e.g. debridement, resection of infected implant) and long-term antibiotic treatment are usually required (Arciola et al., 2018; Kavanagh et al., 2018; Rodríguez-Pardo et al., 2015; Suresh et al., 2018). Dalbavancin is a lipoglycopeptide antibiotic approved in 2014 by the US Food and Drug administration (FDA), and in 2015 by the European Medicines Agency (EMA), for the treatment of acute bacterial skin and skin-structure infections (ABSSIs). By virtue of its long half-life, good distribution in bone and articular tissues, and potent activity against both methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant CoNS, dalbavancin has recently been considered as a potential option for the treatment of staphylococcal osteomyelitis and orthopedic implant infections (Almagour et al., 2018; Barnea et al., 2016; Dunne et al., 2015; Kavanagh et al., 2018; Kussmann et al., 2018; Morata et al., 2019; Pfaller et al., 2018; Sader et al., 2018; Xhemali et al., 2019).

The results of the few *in vitro* studies that have so far investigated the activity of dalbavancin against staphylococcal biofilms point to a potential efficacy of the drug in both the prevention and treatment of *S. aureus* and *S. epidermidis* biofilms (Baldoni et al., 2013; Darouiche et al.,2005; Fernandez et al., 2016; Knafl et al., 2017; Meeker et al., 2016; Neudorfer et al., 2018). However, information on antibiofilm activity of dalbavancin using models with long exposure times is still limited.

The purpose of this study was to evaluate the time-kill kinetics of dalbavancin against *S. aureus* and CoNS biofilms over prolonged exposure times (i.e. in order to better mimic the long-term treatment regimens required in case of biofilm-related orthopedic infections), using both a standardized biofilm susceptibility model and biofilms grown onto relevant orthopedic biomaterials (i.e. titanium and cobalt-chrome disks).

2. Materials and Methods

2.1 Bacterial strains, identification and susceptibility testing

A total of 12 *Staphylococcus* spp. strains were investigated, including both reference strains (n=3) and clinical isolates (n=9) from biofilm-associated infections, of which some exhibiting relevant resistance phenotypes (Table 1). In particular, the studied strains included one daptomycin-resistant and heterogeneous vancomycin-intermediate *S. aureus* (hVISA), and three linezolid-resistant *S. epidermidis* (Table 1). *S. aureus* IT-1 had been previously categorized as hVISA based on the population analysis profile-area under the curve (PAP-AUC) method (Landini et al., 2015). Species identification was performed by the MALDI-TOF MS. Antimicrobial susceptibility was determined using the reference broth microdilution method (CLSI, 2018a, 2018b).

2.2 Biofilm time-kill assays on the Nunc-TSP lid system

Biofilm time kill assays were performed using the Nunc-TSP lid system (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (Harrison et al., 2010). Briefly, biofilms were grown in cation adjusted Mueller Hinton Broth (CAMHB) containing 0.5% glucose, for 7 days (orbital shaker, 150 rpm, 35°C), with a daily replacement. Seven-day-old biofilms were then challenged with three dalbavancin concentrations (i.e.1, 4 and 16 µg/mL), based on data available on dalbavancin distribution in bone and articular tissue (i.e. 4.1, 15.9 and 6.2 µg/mL in cortical bone, synovial tissue and synovial fluid, respectively, 14 days after a single 1000-mg infusion) (Dunne et al., 2015). Vancomycin was used as a comparator at the same concentrations as dalbavancin (Bue et al., 2018). According to the Clinical and Laboratory Standards Institute (CLSI) recommendations, starting stock solutions of dalbavancin and vancomycin were prepared from pure powders using the appropriate solvents and diluents; moreover, in experiments evaluating the antibiofilm activity of dalbavancin, the CAMHB medium was supplemented with polysorbate-80 (P-80, 0.002%) to prevent adsorption of dalbavancin to plastic surfaces (CLSI, 2018b). In order to ensure comparable experimental conditions in time-kill assays, potential changes in the vancomycin antibiofilm activity likely due to the presence\absence of P-80 were evaluated following preliminary experiments performed on a representative strain (i.e. *S. aureus* ATCC 6538). No differences were observed when vancomycin was tested alone or in combination with P-80 (data not shown); therefore, the following time-kill kinetics to evaluate the antibiofilm activity of vancomycin were performed using a standard CAMHB not supplemented with P-80.

The antibiofilm effect of dalbavancin and vancomycin was evaluated at T_0 (i.e., before antibiotic exposure) and after 2, 4 and 7 days of antibiotic exposure (35°C, statically, with antibiotic-containing medium being refreshed every day). After each exposure time, biofilms were washed twice with phosphate-buffered saline (PBS) (Sigma Aldrich, Milan, Italy) to remove loosely adherent bacteria, and sessile cells were removed from pegs by sonication for 30 min (Elma Transsonic T 460, Singen, Germany) in tryptic soy broth (TSB) (Oxoid, Milan, Italy) supplemented with 0.1% Tween 20 (Sigma Aldrich) (i.e., the recovery medium). Mean cultivable cell counts per peg (CFU/peg) were then determined by plating 10 µL of appropriate dilutions of the recovery medium onto tryptic soy agar (TSA) (Oxoid) and incubating for 48 h at 35°C (detection limit, 20 CFU/peg). Data were obtained from two independent experiments, with six replicates per condition per experiment (with the exception of ATCC 6538 for which data were obtained in three independent experiments, with three replicates per condition per experiment).

2.3 Biofilm time-kill assays on titanium and cobalt-chrome disks

Titanium and cobalt-chrome are among the most commonly used materials in orthopedic implants (Gallo et al., 2014). In this perspective, the antibiofilm activity of dalbavancin and vancomycin was also tested using *in vitro* biofilm models grown onto titanium and cobalt-chrome disks (Innovotech, Edmonton, AB, Canada). Three reference strains were selected for these experiments: MSSA ATTC 6538, MRSA ATCC 43300, and MSSE ATCC 14990. Starting from an initial inoculum of 0.5 McFarland, biofilms were grown for 48 hours in daily refreshed CAMHB containing 0.5% glucose (35°C, statically). The antibiofilm effect of dalbavancin and vancomycin was evaluated at T_0 (i.e., before antibiotic exposure) and after 2, 4 and 7 days of antibiotic exposure (35°C, statically, with antibiotic-containing medium being refreshed every day). After each exposure time, two disks per conditions were washed twice with PBS to remove loosely adherent bacteria, and sessile cells were removed from disks by sonication for 30 min in the recovery medium. Mean cultivable cell counts per disk (CFU/disk) were then determined by plating 10 µL of appropriate dilutions of the recovery medium onto TSA and incubating for 48 h at 35°C (detection limit, 25 CFU/disk). Data were obtained from two independent experiments, with two replicates per condition per experiment.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (San Diego, CA, USA). D'Agostino-Pearson and Shapiro-Wilk normality tests were applied. For each time point, multiple comparison tests (one-

way Anova with Holm-Sidak's correction, or Kruskal-Wallis test with Dunn's correction) were used to assess differences of biofilms exposed to diverse dalbavancin and vancomycin concentrations compared to the respective controls.

3. Results and Discussion

3.1 Antimicrobial activity of dalbavancin and vancomycin against planktonic cells of Staphylococcus spp.

All the studied strains were susceptible to both dalbavancin and vancomycin (Table 1). The MICs of dalbavancin ranged 0.06-0.12 µg/mL and 0.03-0.25 µg/mL, for *S. aureus* and *S. epidermidis* strains, respectively (Table 1). Consistently with previous studies (Pfaller et al., 2018), vancomycin MICs were overall higher, ranging from 1 to 2 µg/mL (Table 1).

3.2 Activity of dalbavancin and vancomycin against 7-day old biofilms of Staphylococcus spp. grown in Nunc-TSP lid system

Biofilms were grown in the Nunc-TSP lid system for 7 days before antibiotic exposure. Such a prolonged time of growth was required in order to achieve an acceptable biofilm mass in CAMHB. Indeed, although this medium is not ideal as other media (e.g. TSB) for staphylococcal *in vitro* biofilm growth, it was chosen because it represents the reference medium for *in vitro* susceptibility testing (CLSI, 2018a). Preliminary experiments performed with biofilms grown in TSB and then switched to CAMHB showed a significant decrease of CFU/peg for control biofilms, indicating that medium change could represent a stress condition potentially able to affect biofilm time-kill kinetics (data not shown).

Seven-day old biofilms on the Nunc-TSP lid system ranged from 4.2 ± 0.5 to 7.3 ± 0.3 log CFU/peg for *S. aureus* strains, and from 5.2 ± 0.4 to 6.8 ± 0.4 log CFU/peg for S*. epidermidis* strains (Supplementary Fig. S1 to S6). Overall, control biofilms remained quite stable during the following 7 days of antibiotic challenge (i.e. variations within 1 log CFU/peg), with the exception of three *S. aureus* strains (i.e. MSSA DB-12, MSSA DB-13 and MRSA IT-23), for which an increase of >1 log CFU/peg was observed (Supplementary Fig. S1 to S2). The presence of 0.002% P-80 did not affect control biofilms, except for two *S. aureus* strains (i.e. MSSA DB-12 and MRSA IT-23), for which non-homogeneous biofilms were observed in the medium without P-80 (i.e. in vancomycin control biofilms) (Supplementary Fig. S1 to S2). This behavior could be likely related to a higher propensity of these strains to form aggregates, which were possibly in part prevented or reduced in their size by the presence of P-80 (i.e. in dalbavancin control biofilms).

Overall, both dalbavancin and vancomycin exhibited a time-dependent and concentration-dependent antibiofilm activity (Fig. 1 and Fig. 2). In the tested conditions, dalbavancin worked as a more effective antibiofilm agent compared to vancomycin, showing a significant reduction of cultivable biofilm cells being observed earlier or at lower concentrations (Fig. 1 and Fig. 2). This trend was particularly evident in experiments involving *S. epidermidis* strains (Fig. 2).

It should be noted that in this model only cultivable cells were assessed to evaluate the biofilm mass, thus not excluding that some differences in the dalbavancin and vancomycin activity might be observed in the viable but non-culturable subpopulation of biofilm cells. Therefore, further experiments will be required to verify this hypothesis.

Of note, dalbavancin remained active also against MRSA IT-1, a hVISA and daptomycin resistant strain. Indeed, a decrease of cultivable biofilm cells below the detection limit was observed, in the majority of replicates, at day7 of exposure to dalbavancin 4 µg/mL and 16 µg/mL (i.e. 9 out of 12, and 11 out of 12 replicates, respectively) (Fig. 1).

Overall, the higher antibiofilm activity of dalbavancin could possibly be related to its significantly lower MICs compared to vancomycin (Table 1). Further experiments would be required to support this hypothesis, by performing biofilm time-kill kinetics using both compounds at equal multiple MIC concentrations. However, the antibiofilm activity of both dalbavancin and vancomycin was not strictly related only to the respective MIC. Indeed, as an example, a similar effect of dalbavancin was observed against MRSE DB-5 and MRSE DB-8, which showed similar biofilm mass at T_0 (i.e. 6.2 ± 0.5 CFU/peg and. 6.3 ± 0.8 CFU/peg, respectively), but very different dalbavancin MICs (i.e. 0.03 µg/mL and 0.25 µg/mL, respectively) (Fig 2 and Supplementary Fig. S4). On the other hand, different time-kill curves were observed for both dalbavancin and vancomycin against MRSA ATCC 43300 and MRSA IT-23, which showed identical dalbavancin and vancomycin MICs, but differed for the biofilm mass at T_0 (i.e. 5.7 ± 0.1 CFU/peg and. 4.2 ± 0.5 CFU/peg, respectively) (Fig. 1 and Supplementary Fig. S2).

Additional analyses were performed with MSSA ATCC 6538, in order to explain cases of clearly divergent data among replicates (e.g. all replicates below the detection limit after antibiotic exposure, except one or two replicates which were similar to controls). For this purpose, dalbavancin MIC was determined for three randomly selected colonies from a biofilm which had not be affected after 4 days of exposure to 16 µg/mL dalbavancin (while all the other 8 replicates were below the detection limit) (Fig.1). Results showed no MIC variation and suggested a biofilm-related tolerance. However, this heterogeneous biofilm response to

antibiotic exposure was observed also with other tested strains, with both dalbavancin and vancomycin, and should be further investigated to unravel potential mechanisms of biofilm adaptation after long-term exposure to such antibiotics. Indeed, Kussmann et al. recently described the emergence of a dalbavancin induced glycopeptide/lipoglycopeptide non-susceptible *S. aureus* during a long-term treatment of a cardiac device-related endocarditis (Kussmann et al., 2018).

3.3 Activity of dalbavancin and vancomycin against 2-day old biofilms of Staphylococcus spp. grown on titanium and cobalt-chrome disks

Biofilms on titanium and cobalt-chrome disks were formed by a mean of 7.3 ± 0.6 and 7.9 ± 0.8 log CFU/peg, 7.5 ± 0.3 and 7.2 ± 0.6 log CFU/peg, and 7.4 ± 0.2 and 7.5 ± 0.1 log CFU/peg for MSSA ATCC 6538, MRSA ATCC 43300 and MSSE ATCC 14990, respectively (Supplementary Fig. S5 and S6). Control biofilms on both supports remained stable over the seven days of antibiotic exposure (Supplementary Fig. S5 and S6).

Time-kill curves obtained with biofilm grown in titanium and cobalt chrome disks overall fitted data obtained with the Nunc-TSP lid system (Fig. 3). Even in these two biofilm models, dalbavancin exhibited a stronger antibiofilm activity compared to vancomycin, both in terms of effective antibiotic concentrations and time required to achieve a relevant decrease of cultivable biofilm cells.

4. Conclusions

Biofilm-related orthopedic infections represent a relevant clinical challenge, requiring long-term antibiotic treatment. In this study, the time-kill kinetics of dalbavancin against *S. aureus* and *S. epidermidis* biofilms were determined over prolonged exposure times, using both a standardized biofilm susceptibility model and biofilms grown onto relevant orthopedic biomaterials (i.e. titanium and cobalt-chrome disks).

Dalbavancin (at concentrations achievable in bone and articular tissue) showed a potent activity against established staphylococcal biofilms, in both tested models, and was overall superior to the comparator vancomycin. The antibiofilm activity of tested molecules was here studied by the conventional colony-forming units method, an evaluation assessing only the cultivable cells of the biofilm system. As a consequence, in future studies it will be worth implementing microscopy techniques (e.g. confocal laser scanning microscopy), in order to gather a more comprehensive understanding of antimicrobial effects also on biofilm spatial structures and associated functions.

In conclusion, the present results suggest that dalbavancin might represent a valid therapeutic option for the treatment of biofilm-associated orthopedic infections and encourage further *in vitro* and *in vivo* studies on this topic.

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Declaration of interest

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Table

Features of the *Staphylococcus* spp. strains included in the study. Evaluation of antimicrobial susceptibility profiles was performed on planktonic cells.

Strain	Origin	MS/MR	MIC (µg/mL)										
			DAL	**VAN**	**TEI**	**ERY**	**CLI**	**LEVO**	**GEN**	**SXT**	**DAP**	**LZD**	**TGC**
S. aureus ATCC 6538	ATCC	MSSA	0.12	1	025	0.25	≤0.12	0.25	≤0.5	0.12	0.5	1	≤0.12
S. aureus DB-12	blood	MSSA	0.12	1	0.5	2	≤0.12	0.25	≤0.5	0.06	0.5	4	≤0.12
S. aureus DB-13	blood	MSSA	0.06	1	0.5	1	≤0.12	≤0.25	≤0.5	0.06	0.5	4	≤0.12
S. aureus ATCC 43300	ATCC	MRSA	0.06	1	0.5	>16	>4	0.25	>2	0.06	0.5	4	≤0.12
S. aureus IT-1	blood	MRSA	0.12	2	4	0.5	0.25	8	2	0.06	4	2	0.25
S. aureus IT-23	CSF	MRSA	0.06	1	2	>16	>4	>8	>2	1	1	2	0.5
S. epidermidis ATCC 14990	ATCC	MSSE	0.06	2	1	0.25	≤0.12	≤0.25	≤0.5	0.12	0.5	1	≤0.12
S. epidermidis MR996	blood	MSSE	0.03	1	2	>16	0.25	8	>2	0.25	0.5	1	0.25
S. epidermidis DB-5	blood	MRSE	0.03	1	8	>16	1	>8	>2	4	0.5	>8	≤0.12
S. epidermidis DB-6	blood	MRSE	0.06	2	8	>16	0.12	>8	>2	0.12	0.5	1	≤0.12
S. epidermidis DB-7	blood	MRSE	0.12	2	8	>16	1	>8	>2	4	1	>8	≤0.12
S. epidermidis DB-8	CVC	MRSE	0.25	2	8	>16	1	>8	>2	4	0.5	>8	≤0.12

CSF, cerebrospinal fluid; CVC, central venous catheter. DAL, dalbavancin; VAN, vancomycin; TEI, teicoplanin; ERY, erythromycin; CLI, clindamycin; LEVO, levofloxacin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole (MICs are referred to the component trimethoprim considering a trimethoprim-sulfamethoxazole 1:19 ratio); DAP, daptomycin; LZD, linezolid; TGC, tigecycline. Red color indicates resistance phenotypes. *S. aureus* IT-1 was categorized as heterogeneous vancomycin-intermediate *S. aureus* (hVISA) based on the population analysis profile–area under the curve (PAP-AUC) method (Landini et al., 2015).

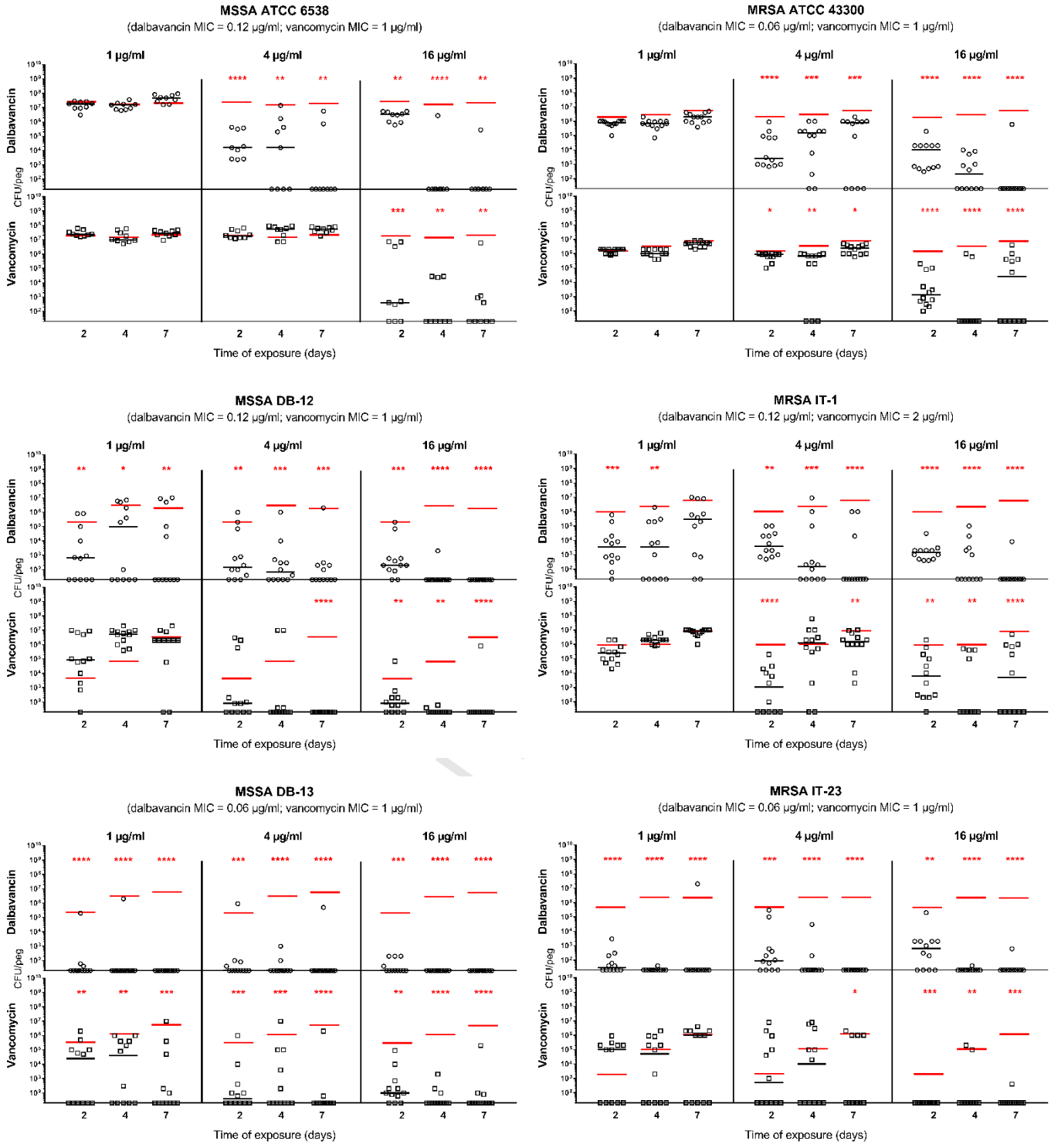

Figure 1. Biofilm time-kill curves of dalbavancin and vancomycin against MSSA and MRSA strains grown in the Nunc-TSP lid system. Red lines indicate the median of controls for each time point. For each time point, multiple comparison tests (Kruskal-Wallis test with Dunn's correction) were applied to assess differences of biofilms exposed to diverse dalbavancin and vancomycin concentrations compared to the respective controls. The x-axis is set at the limit of detection (20 CFU/peg).* p <0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

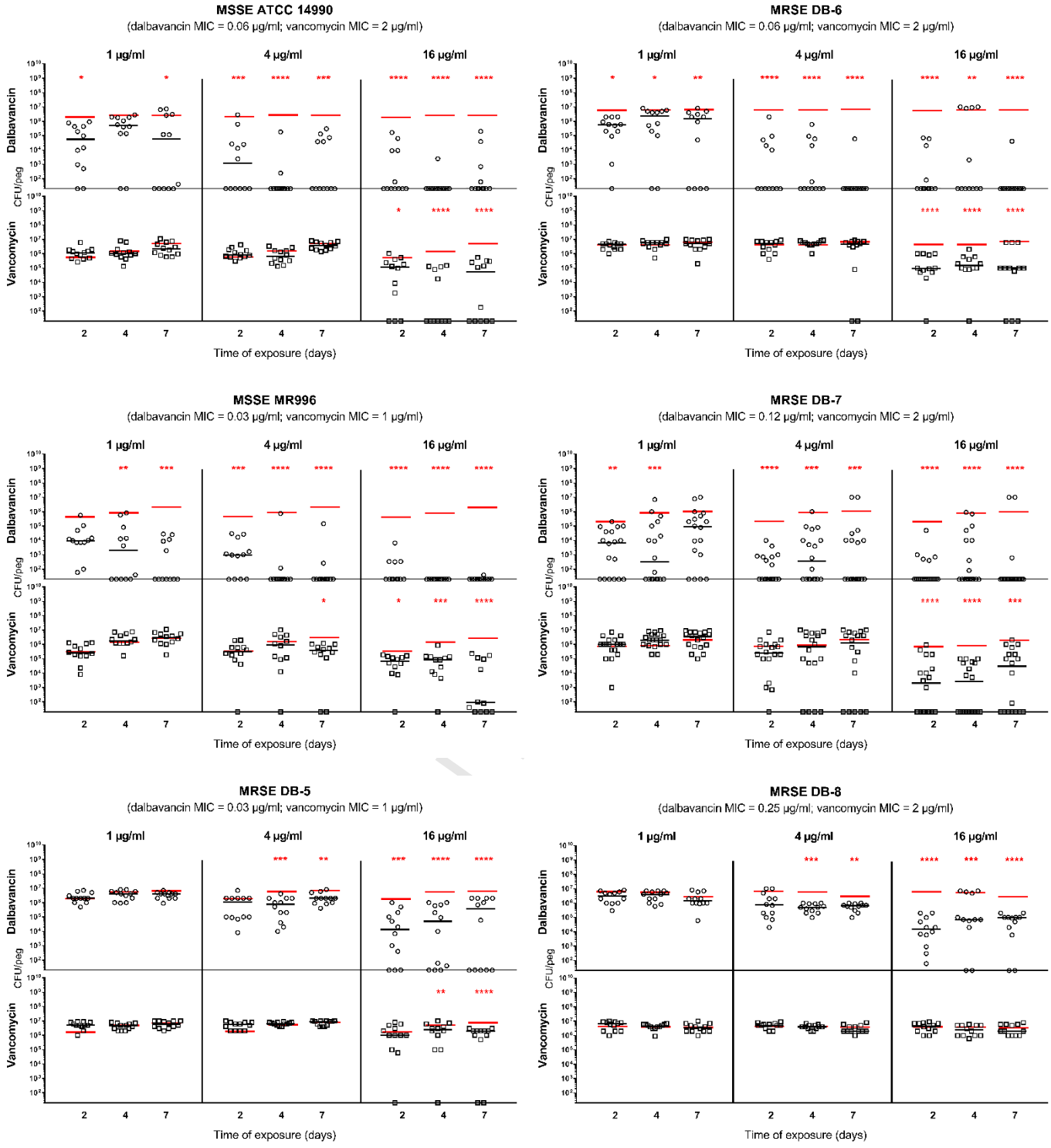

Figure 2. Biofilm time-kill curves of dalbavancin and vancomycin against MSSE and MRSE strains grown in the Nunc-TSP lid system. Red lines indicate the median of controls for each time point. For each time point, multiple comparison tests (Kruskal-Wallis test with Dunn's correction) were applied to assess differences of biofilms exposed to diverse dalbavancin and vancomycin concentrations compared to the respective controls. The x-axis is set at the limit of detection (20 CFU/peg).* p <0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

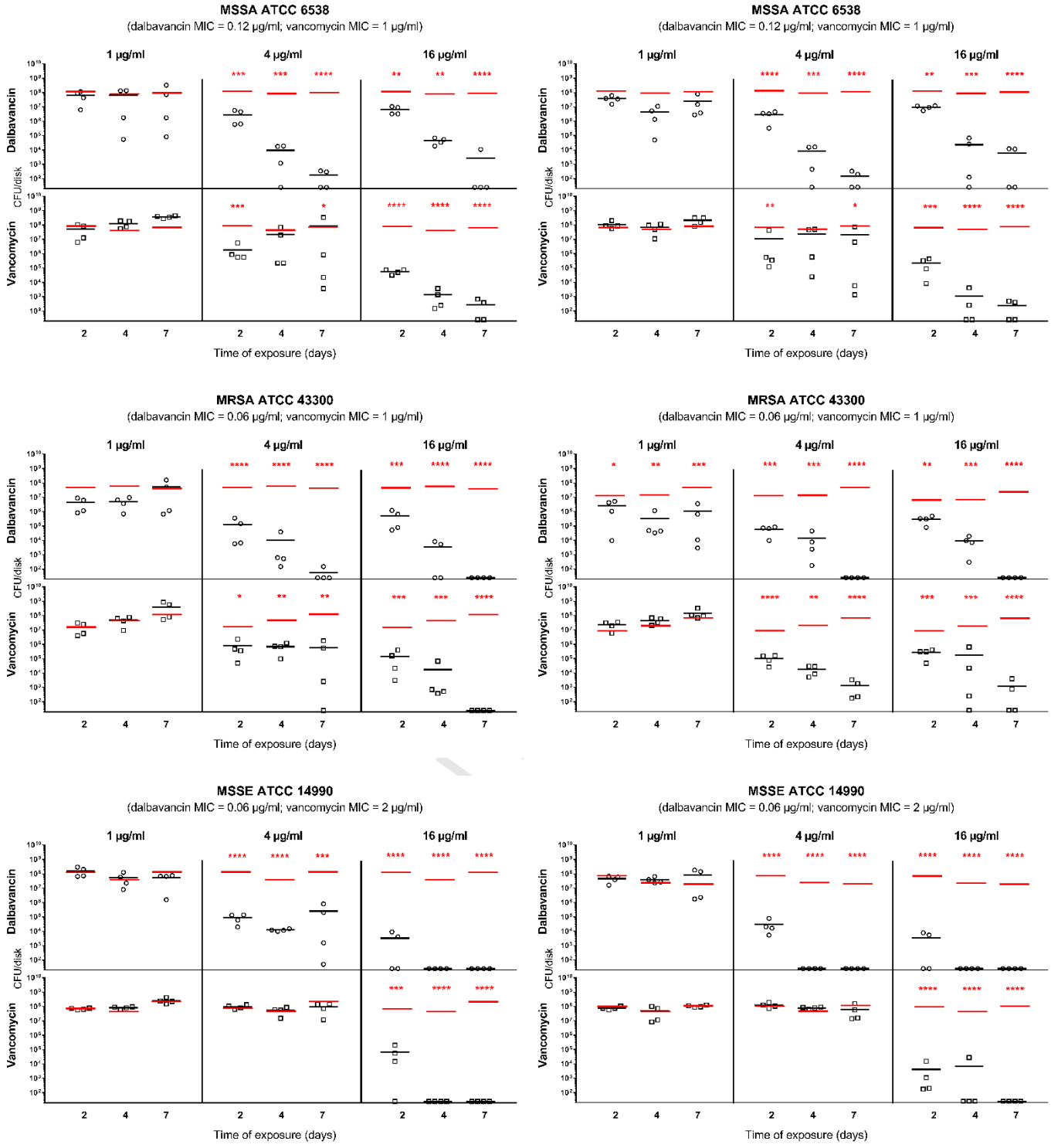

Figure 3. Biofilm time-kill curves of dalbavancin and vancomycin against MSSA, MRSA and MSSE strains grown in titanium (left part of the figure) and cobalt-chrome (right part of the figure) disks. Red lines indicate the mean of controls for each time point. For each time point, multiple comparison tests (one-way Anova with with Holm-Sidak's test, performed with log transformed data) were applied to assess differences of biofilms exposed to diverse dalbavancin and vancomycin concentrations compared to the respective controls. The x-axis is set at the limit of detection (25 CFU/disk). * p <0.05, ** p<0.01, *** p<0.001, **** p<0.0001.