Role of Extracellular DNA in Dalbavancin Activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA) Biofilms in Patients with Skin and Soft Tissue Infections

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ABSTRACT Methicillin-resistant *Staphylococcus aureus* (MRSA) has become the leading cause of skin and soft tissue infections (SSTIs). Biofilm production further complicates patient treatment, contributing to increased bacterial persistence and antibiotic tolerance. The study aimed to explore the efficacy of different antibiotics on biofilm-producing MRSA isolated from patients with SSTI. A total of 32 MRSA strains were collected from patients with SSTI. The MIC and minimal biofilm eradication concentration (MBEC) were measured in planktonic and biofilm growth. The study showed that dalbavancin, linezolid, and vancomycin all inhibited MRSA growth at their EUCAST susceptible breakpoint. Of the MRSA strains, 87.5% ($n = 28$) were strong biofilm producers (SBPs), while only 12.5% ($n = 4$) were weak biofilm producers (WBPs). The $MBEC_{90}$ values for dalbavancin were significantly lower than those of linezolid and vancomycin in all tested strains. We also found that extracellular DNA (eDNA) contributes to the initial microbial attachment and biofilm formation. The amount of eDNA differed among MRSA strains and was significantly higher in those isolates with high dalbavancin and vancomycin tolerance. Exogenously added DNA increased the $MBEC_{90}$ and protection of biofilm cells from dalbavancin activity. Of note, the relative abundance of eDNA was higher in MRSA biofilms exposed to $MBEC_{90}$ dalbavancin than in untreated MRSA biofilms and those exposed to sub-MIC_{90}. Overall, dalbavancin was the most active antibiotic against MRSA biofilms at concentrations achievable in the human serum. Moreover, the evidence of a drug-related increase of eDNA and its contribution to antimicrobial drug tolerance reveals novel potential targets for antibiofilm strategies against MRSA.

IMPORTANCE *Staphylococcus aureus* is the most common cause of skin and soft tissue infections (SSTIs) worldwide. In addition, methicillin-resistant *S. aureus* (MRSA) is increasingly frequent in postoperative infections and responsible for a large number of hospital readmissions and deaths. Biofilm formation by *S. aureus* is a primary risk factor in SSTIs, due to a higher antibiotic tolerance. Our study showed that the biofilm-forming capacity varied among MRSA strains, although strong biofilm producers were significantly more abundant than weak biofilm producer strains. Notably, dalbavancin demonstrated a potent antibiofilm activity at concentrations achievable in human serum. Nevertheless, dalbavancin activity was affected by an increased concentration of

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extracellular DNA in the biofilm matrix. This study provides novel insight for designing more targeted therapeutic strategies against MRSA and to prevent or eradicate harmful biofilms.

KEYWORDS biofilm, dalbavancin, *Staphylococcus aureus*, MRSA, extracellular DNA

Skin and soft tissue infections (SSTIs) represent severe forms of infectious diseases that involve deeper soft tissues, responsible for significant risk of relapse, prolonged hospitalization, and death (1, 2). Although many hospitals have adopted specific measures to reduce the emergence of adverse events and increase the effectiveness of surgical procedures, SSTIs remain a challenging and costly problem (1, 2). *Staphylococcus aureus* is the most common cause of SSTIs worldwide. In addition, methicillin-resistant *S. aureus* (MRSA) is increasingly frequent in postoperative infection and responsible for a significant increase in the risk of death and hospital readmission compared to uninfected surgical patients (1, 3–8). Biofilm represents an additional risk factor in SSTIs, primarily due to a higher antibiotic tolerance (9–11). Indeed, biofilm structure hinders antibiotic penetration, depending on the matrix composition and structure.

In particular, *S. aureus* biofilm matrix is mainly composed of polysaccharides, extracellular DNA (eDNA), and proteins (12). *S. aureus* biofilm production is achieved by two major pathways, alternatively leading to the assembly of a polysaccharide-based or an eDNA/protein biofilm. Although these distinctive forms of biofilms are not mutually exclusive, the polysaccharide-based biofilm is predominantly observed in methicillin-sensitive *S. aureus* (MSSA) strains, while the eDNA/protein biofilm is more represented in MRSA strains (13–16).

Biofilm eradication is critical for the effective treatment of SSTIs; however, no specific drugs are available as yet. Dalbavancin is a novel lipoglycopeptide with a half-life of 14.4 days and is approved for the single-dose treatment of acute bacterial SSTIs in adults (17). Like other glycopeptides, dalbavancin inhibits cell wall peptidoglycan cross-linking, showing activity against certain vancomycin-resistant enterococci (VanB and VanC phenotype) (17). Previous data indicate that dalbavancin has a remarkable efficacy against MRSA, thus representing a promising antimicrobial agent against staphylococcal biofilms (18–21). Currently, no data are available on the efficacy of dalbavancin in the treatment of biofilm-growing MRSA from SSTIs. The present study explores the activity of dalbavancin against planktonic and biofilm-growing MRSA isolated from patients with SSTIs. Furthermore, we investigated the potential impact of eDNA on microbial drug tolerance and dalbavancin efficacy for future, more targeted eradication strategies.

RESULTS

From January 2018 to November 2020, 32 MRSA strains were isolated from patients presenting with SSTIs (Table 1). The sites of isolation were surgical wound ($n = 13$; 40.6%), ulcer ($n = 9$; 28.2%), abscess ($n = 5$; 15.6%), cellulitis ($n = 4$; 12.5%) and necrotizing fasciitis ($n = 1$; 3.1%).

The MIC of conventional antibiotics against MRSA isolates are summarized in Fig. 1. Levofloxacin showed the highest level of antimicrobial resistance (83.9%; MIC = 8 μg/mL, range < 0.12 to 8). Conversely, MRSA strains were totally susceptible to dalbavancin (MIC = 0.06 μg/mL, range = 0.015 to 0.125), linezolid (MIC = 2 μg/mL, range = 0.5 to 8 μg/mL), and vancomycin (MIC = 1μg/mL, range = 0.25 to 2).

The assessment of biofilm formation showed that strong biofilm-producers (SBPs) ($n = 28$, 87.5%) were significantly ($P < 0.001$) more abundant than weak biofilm-producers (WBPs) ($n = 4$, 12.5%) among MRSA isolates (Fig. 2A). The resazurin conversion into resorufin revealed that the strains classified as WBPs showed a significantly ($P < 0.001$) lower level of resazurin reduction (absorbance at 570 nm = 1.62 $\pm$ 0.15) than that of SBPs (Fig. 2B). Apotome microscopy analysis of the biofilms examined after 20 h of incubation showed that all the SBPs gave a full coverage throughout the

TABLE 1 Demographic and clinical features of patients at enrollment ($n = 32$)[a]

Characteristic or site	Value	Percent
Demographic characteristics		
Sex (no. female)	13	40.6
Age, yrs (range)	58.9 (41.5–79.1)	
Site of isolation (no.)		
Surgical wound	13	40.6
Ulcer	9	28.2
Abscess	5	15.6
Cellulitis	4	12.5
Necrotizing fasciitis	1	3.1

[a]Age is expressed as the mean, with the range in parentheses.

extension of the substrate with the development of uniform and thick biofilms of 25 to 60 μm in height, while WBPs achieved a partial coverage of the substrate with the development of uneven biofilms of 10 to 30 μm in height (Fig. 2C).

Most of the MRSA strains were classified as SBP. Thus, we evaluated whether dalbavancin, linezolid, and vancomycin, the most effective antibiotics against planktonic MRSA, were active against all MRSA strains in the biofilm phase. To this end, we compared the differences in MIC_{90} and minimal biofilm eradication concentration at which 90% of the tested isolates are inhibited ($MBEC_{90}$) (Fig. 3). The MIC_{90} was 0.06 μg/mL (range, 0.015 to 0.125 μg/mL) for dalbavancin, 2 μg/mL (range, 0.5 to 4 μg/mL) for linezolid, and 1 μg/mL (range, 0.25 to 2 μg/mL) for vancomycin. The MIC_{90} for dalbavancin was significantly lower than those for linezolid ($P < 0.001$) and vancomycin ($P < 0.001$). In additions, the MIC_{90} for linezolid was found to be significantly ($P < 0.001$) higher than that for vancomycin. Next, dalbavancin, linezolid, and vancomycin activity was assessed against MRSA biofilms. The $MBEC_{90}$ was 0.5 μg/mL (range, 0.12 to 0.5 μg/mL) for dalbavancin, 8 μg/mL (range, 2 to 8 μg/mL) for linezolid, and 4 μg/mL (range, 2 to 8 μg/mL) for vancomycin. Notably, dalbavancin showed *in vitro* activity against MRSA biofilms with $MBEC_{90}$ values significantly lower than those of linezolid ($P < 0.001$) and vancomycin ($P < 0.001$) (Fig. 3). No significant difference in the $MBEC_{90}$ values was observed between linezolid and vancomycin.

The attachment and initiation of biofilm formation were investigated by measuring the relative difference (RD) (equation 1 in Materials and Methods) of bead immobilizations in
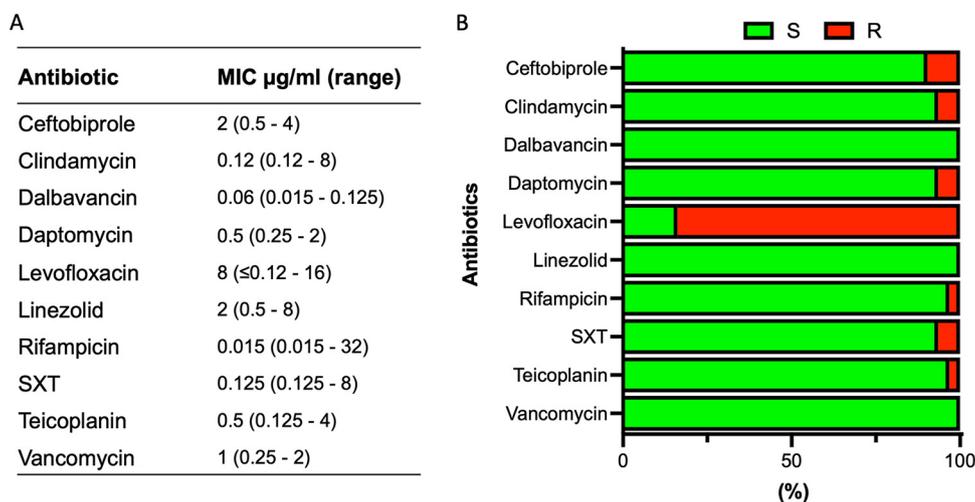
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Antibiotic	MIC µg/ml (range)
Ceftobiprole	2 (0.5 - 4)
Clindamycin	0.12 (0.12 - 8)
Dalbavancin	0.06 (0.015 - 0.125)
Daptomycin	0.5 (0.25 - 2)
Levofloxacin	8 (≤0.12 - 16)
Linezolid	2 (0.5 - 8)
Rifampicin	0.015 (0.015 - 32)
SXT	0.125 (0.125 - 8)
Teicoplanin	0.5 (0.125 - 4)
Vancomycin	1 (0.25 - 2)

FIG 1 Antibiotic susceptibility profile of 32 methicillin-resistant *Staphylococcus aureus* (MRSA) strains to the indicated antimicrobials as determined by broth microdilution test. (A) Median MIC. (B) Percentage of susceptible (green) and resistant (red) strains to the indicated antimicrobials. Classification was performed according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoint tables (EUCAST clinical breakpoint). SXT, trimethoprim/sulfamethoxazole.
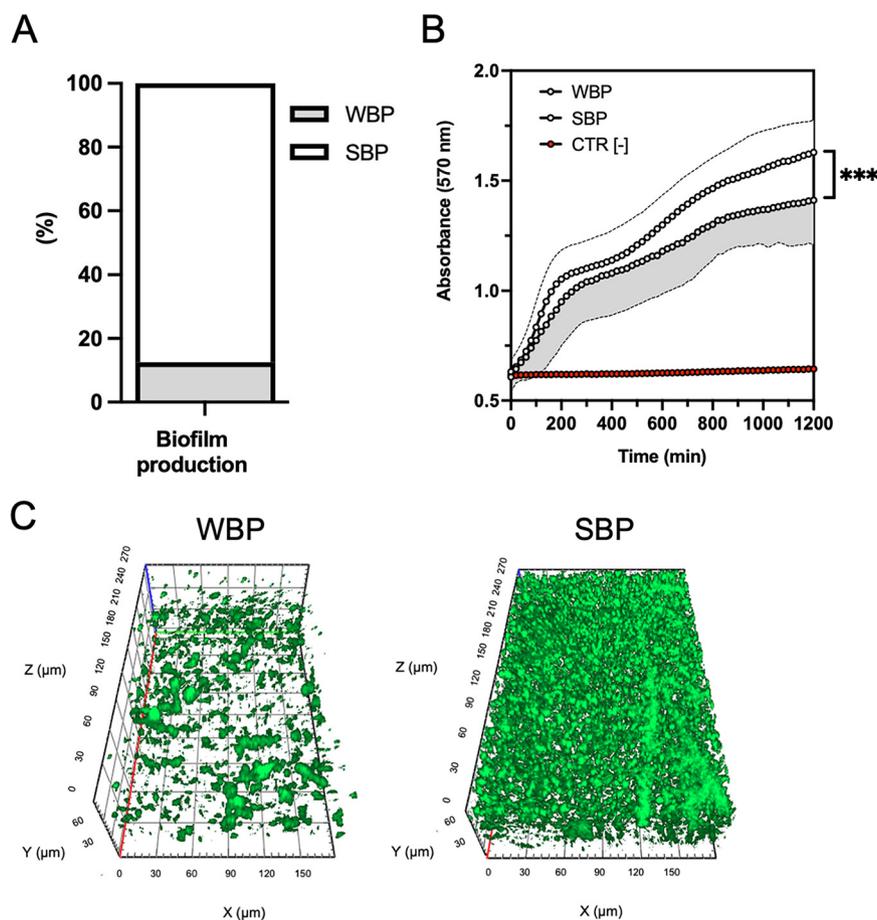
FIG 2 (A) Biofilm formation measured by the clinical BioFilm Ring Test (cBRT) for MRSA strains. Clinical isolates were classified as weak biofilm producers (WBP) and strong biofilm producers (SBP). All results were expressed as a percentage of strains with the specific biofilm-forming ability. (B) Resazurin conversion into resorufin measured after 20 h of incubation, for WBP and SBP. ***, $P < 0.001$, using the Mann-Whitney test for the absorbance values after 1,200 min. Data represent means and the standard errors of two independent experiments analyzed in duplicate. (C) Representative Apotome microscopy images of WBP (left panel) and SBP (right panel) MRSA biofilms, stained with the Live/Dead BacLight bacterial viability kit and analyzed with ZEN 3.1 software, after 20 h of incubation at 37°C. Orthogonal sections displaying horizontal (z) and side views (x and y) of reconstructed 3-dimensional (3D) biofilm images are shown.

the presence and absence of DNase I. The results showed that the treatment with DNase I leads to a significant ($P < 0.001$) reduction of $26.0 \pm 20.4\%$ in the initial attachment of MRSA strains compared to untreated isolates (Fig. 4A). Notably, eDNA was detected in all biofilm samples at a median concentration of 0.95 ng/μL (95% confidence interval [CI], 0.82 to 1.08) (Fig. 4B). However, the eDNA concentration in the biofilm did not correlate with the level of biofilm production measured by the clinical BioFilm Ring Test (cBRT).

It has been reported that eDNA can contribute to antibiotic tolerance in MRSA (22). Thus, the ratio between $MBEC_{90}$ and MIC_{90} was used to quantify the biofilm tolerance (BT) score to dalbavancin, linezolid, and vancomycin. We calculated the correlation coefficient between the eDNA levels and BT to dalbavancin, linezolid, and vancomycin (Fig. 5). The correlation matrix revealed a significant correlation of eDNA with the BT for dalbavancin ($\rho = 0.68$; $P < 0.001$) and a positive correlation with vancomycin ($\rho = 0.36$; $P = 0.04$). Thus, MRSA strains were more likely to present an increase in BT for dalbavancin and vancomycin when the level of eDNA was high. In particular, the glycopeptide antibiotics dalbavancin and vancomycin also revealed a positive correlation ($\rho = 0.48$; $P = 0.005$), suggesting the presence of a common mechanism for BT likely based on the level of eDNA in the biofilm matrix. Conversely, no correlation was observed between BT to linezolid and the levels of eDNA.

Next, MRSA biofilms were exposed to the $MBEC_{90}$ of dalbavancin in the presence or in the absence of salmon sperm DNA to verify whether the addition of exogenous DNA
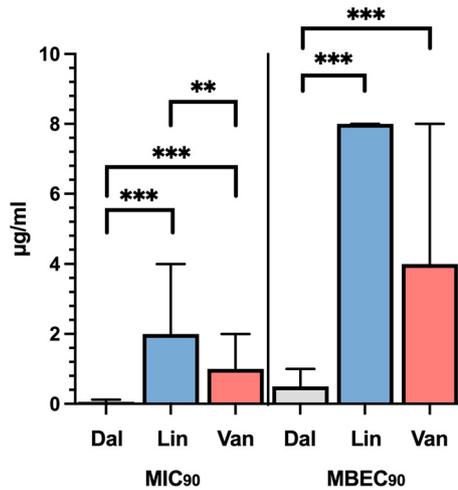
FIG 3 Dalbavancin showed *in vitro* activity against MRSA biofilms. Median (range) MIC_{90} and $MBEC_{90}$ (minimum biofilm eradication concentration) for dalbavancin (Dal), linezolid (Lin), and vancomycin (Van) determined for MRSA strains. Statistical differences were determined using the Kruskal-Wallis test followed by Dunn's *post hoc* test for multiple comparisons; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

could protect biofilm cells. Data showed that exogenous DNA alone did not affect the growth of biofilm cells. As expected, dalbavancin at the $MBEC_{90}$ concentration caused a significant ($P < 0.001$) reduction in the number of biofilm cells compared to untreated biofilm cells (Fig. 6). Notably, preincubation of dalbavancin at a concentration of $MBEC_{90}$ with salmon sperm DNA significantly ($P = 0.011$) increases the antimicrobial tolerance of biofilm cells compared to dalbavancin alone.

Previous work suggested that staphylococcal biofilms treated with antibiotics at sub-MIC levels contained more eDNA than untreated controls (22, 23). To this end, eDNA was analyzed by Apotome microscopy in 20-h-old MRSA biofilms exposed to sub-MIC and $MBEC_{90}$ levels of dalbavancin (Fig. 7A). Distinct differences in the biofilm structure and thickness were observed between untreated biofilms and those treated with the sub-MIC_{90} and $MBEC_{90}$. The presence of eDNA was determined by staining with TOTO-1, which is impermeable to live bacterial cells and normalized to the living cells in the biofilms as measured by the Live/Dead assay. The relative amount of eDNA in MRSA biofilms treated with dalbavancin at sub-MIC_{90} levels was comparable to that in untreated biofilms (Fig. 7B). Conversely, MRSA biofilms treated with dalbavancin at
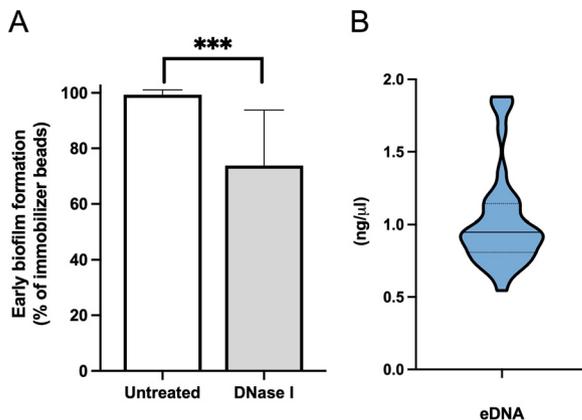

FIG 4 DNase I reduces MRSA biofilm. (A) Role of extracellular DNA (eDNA) on early biofilm formation for MRSA strains. Results are expressed as relative differences (equation 1) in the amounts of biofilm as measured by the BioFilm Ring Test after 6 h of incubation in the presence of DNase compared with untreated control strains. Data represent means and the corresponding standard errors of two independent experiments analyzed in duplicate. ***, $P < 0.001$, using the Mann-Whitney test. (B) Quantification of eDNA in MRSA biofilm cultures. Black bars and dots represent medians and quartiles.
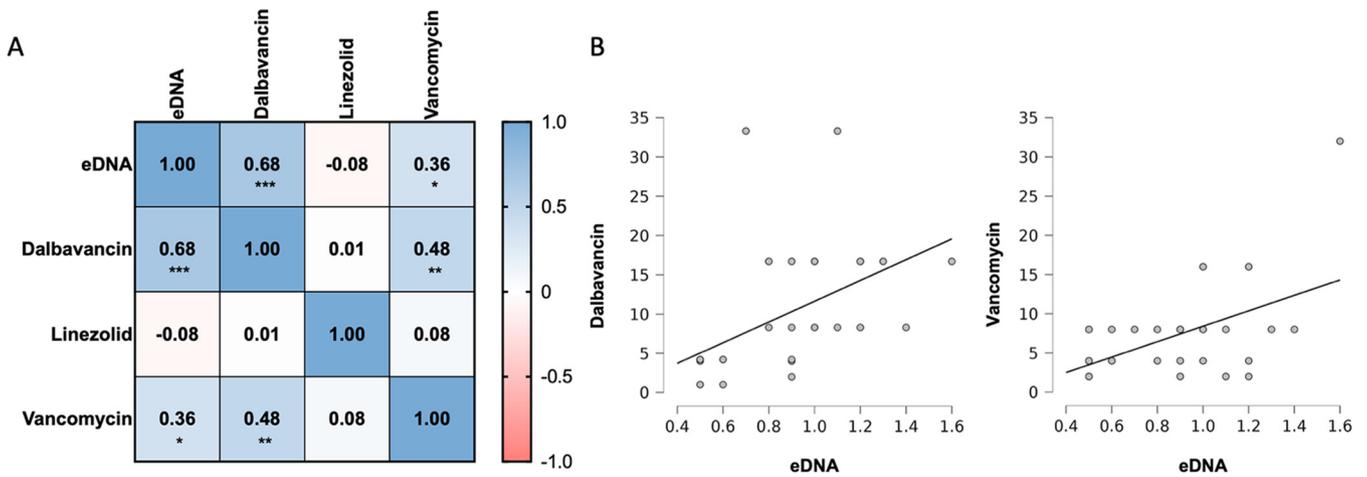
FIG 5 (A) Correlation matrix based on Spearman's rank correlation coefficient between the level of eDNA and the biofilm tolerance (BT) for dalbavancin, linezolid, and vancomycin. The BT is the ratio between the $MBEC_{90}$ (minimum biofilm eradication concentration) and MIC_{90} values calculated for each antimicrobial agent and strain. (B) Scatter graphs with detailed illustration of the relation between eDNA level and BT for dalbavancin and vancomycin.

the $MBEC_{90}$ showed a 2-fold increase in the relative abundance of eDNA than untreated controls and sub-MIC_{90}-treated strains. The relative amount of eDNA normalized to biofilm viability was calculated to confirm these observations. MRSA biofilms treated with dalbavancin at sub-MIC_{90} levels contained a relative abundance of eDNA comparable to that of untreated biofilms ($P > 0.05$) (Fig. 8A). Conversely, the relative abundance of eDNA in MRSA biofilms treated with dalbavancin at the $MBEC_{90}$ was significantly higher than that of untreated control strains ($P = 0.004$) and those treated with sub-MIC_{90} ($P = 0.026$) concentrations (Fig. 8B).

DISCUSSION

Data from this study are consistent with previous reports showing that dalbavancin, linezolid, and vancomycin are effective in managing SSTIs due to MRSA and further confirm that biofilm poses a significant challenge for effective treatment of SSTI (24–28). Biofilm formation by *S. aureus* is associated with a delay in reepithelialization of the infected tissues, ultimately increasing healing time (29–34). Our study showed that the biofilm-forming capacity varied among MRSA strains, although SBPs (87.5%) were significantly more abundant than WBPs (12.5%). Biofilm production analyzed by
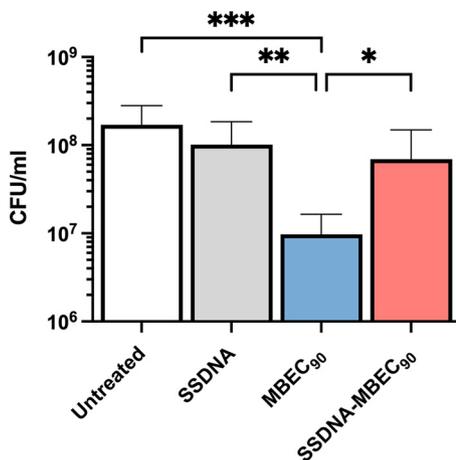

FIG 6 Exogenous DNA protects biofilm cells from dalbavancin. Number of viable cells (CFU/mL) obtained from 20-h MRSA biofilms treated with $MBEC_{90}$ of dalbavancin, 16 μg/mL of exogenous salmon sperm DNA, and a solution of salmon sperm DNA and dalbavancin. All biofilms were exposed to the treatments for 20 h. Statistical differences were determined using the Kruskal-Wallis test followed by Dunn's *post hoc* test for multiple comparisons; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
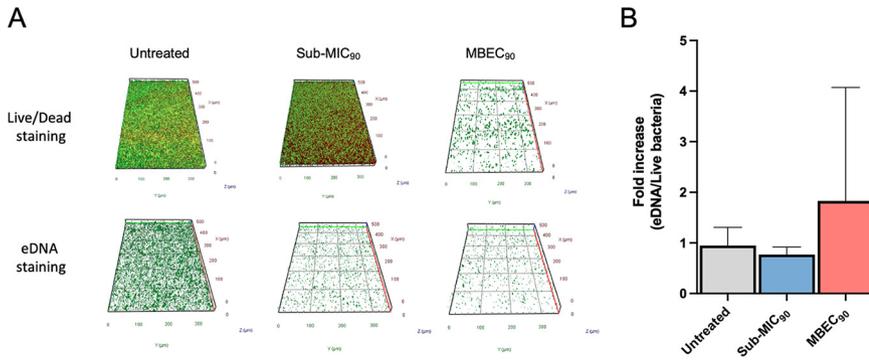
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FIG 7 (A) Representative images of biofilm cells stained with the Live/Dead BacLight bacterial viability kit (BacLight kit) and TOTO-1 for eDNA (green) exposed to dalbavancin (sub-MIC$_{90}$ and MBEC$_{90}$) compared to untreated biofilms. (B) Relative abundances of fluorescence intensity of eDNA stained by TOTO-1 and live bacteria stained with the Live/Dead BacLight bacterial viability kit, analyzed with AxioVision 4.8 software, and expressed as the fold increase compared to untreated control cells.

Apotome microscopy showed that all the SBPs gave full coverage throughout the substrate extension, developing uniform and thick biofilms of 25 to 60 μm in height. Conversely, biofilm biomass was strongly reduced in WBPs, reaching only limited substrate coverage and the development of uneven biofilms of 10 to 30 μm in height. These results are consistent with previous studies reporting a high percentage (70 to 95%) of biofilm-forming *S. aureus* isolates from SSTI, confirming that surface adhesion is essential for skin colonization and infection (34–39). Due to the high percentage of SBP isolates, the activity of dalbavancin, linezolid, and vancomycin, which were the most effective antibiotics on planktonic cells according to the MIC values, was evaluated against MRSA biofilm. Dalbavancin showed potent activity against established MRSA biofilms, with an MBEC$_{90}$ of 0.5 μg/mL (range, 0.12 to 0.5 μg/mL). The antibiofilm activity of dalbavancin was superior to that of the other drugs, with an MBEC$_{90}$ significantly lower than that of linezolid (8 μg/mL; range, 2 to 8 μg/mL) and vancomycin (4 μg/mL; range, 2 to 8 μg/mL), which are among the most common antibiotics administered for the treatment of biofilm-related infections caused by MRSA (40). Notably, the study showed that dalbavancin was active *in vitro* against MRSA biofilms in concentrations achievable in human serum, as the mean plasma concentration
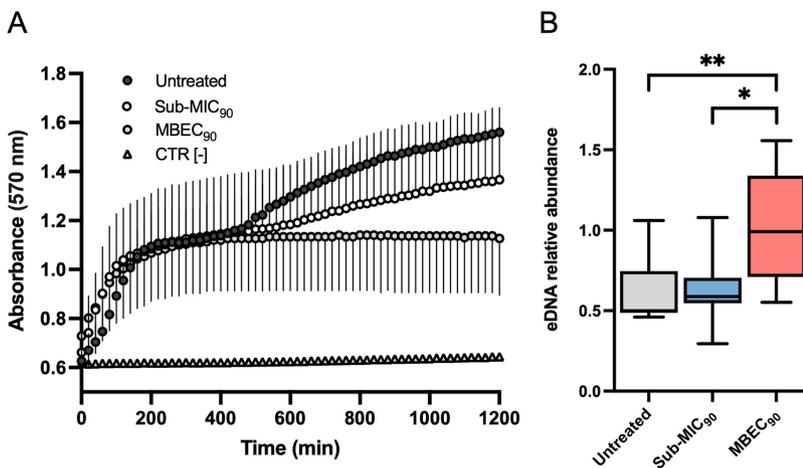
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FIG 8 Increased relative abundance of eDNA in MRSA biofilms exposed to dalbavancin at MBEC$_{90}$. (A) Biofilm viability (resazurin conversion to resorufin) at 20 h in untreated and treated at sub-MIC$_{90}$ and MBEC$_{90}$ MRSA strains. (B) eDNA relative abundances normalized to biofilm viability at absorbance at 570 nm. Statistical differences were determined using the Kruskal-Wallis test followed by Dunn's *post hoc* test for multiple comparisons; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

is >30 mg/L for 7 days after one dose of 1,000 mg (17). Previous studies have shown promising activity of dalbavancin against Gram-positive biofilms, including MRSA (20, 41–45). Moreover, an animal study model reported that dalbavancin was more effective than vancomycin in preventing *S. aureus* colonization of medical devices (44). In rat sternal osteomyelitis, dalbavancin was active in the treatment of MRSA (46). Interestingly, although dalbavancin failed to eradicate MRSA biofilms from a foreign-body infection model, it was able to eradicate biofilms when used in combination with rifampicin, achieving cure rates of 25 to 36% compared to monotherapy and preventing the rifampicin resistance (18). It has been suggested that the activity of dalbavancin can be facilitated by its mechanism of action. Indeed, this antibiotic not only inhibits bacterial cell wall synthesis like vancomycin but also can dimerize and anchor its lipophilic side chain in the bacterial membranes (47, 48). This may increase the affinity of dalbavancin for its target, improving antimicrobial potency. In addition, previous data have reported that vancomycin does not efficiently penetrate staphylococcal biofilms, suggesting that dalbavancin may better diffuse and preserve its activity against biofilm-embedded cells (19, 22, 49, 50). Unlike glycopeptides, linezolid has a different mechanism of action based on protein synthesis inhibition (51). Our findings are in line with previous studies indicating that linezolid is poorly effective in eradicating established MRSA biofilms (52, 53). Others found a decrease in bacterial counts in the presence of linezolid but could not demonstrate biofilm eradication (52–56). A previous meta-analysis showed that linezolid is more effective than vancomycin for treating people with SSTIs, including those caused by MRSA. However, the authors warned of the potential risk of bias due to several studies supported by the pharmaceutical company that produces linezolid (57, 58). Thus, the activity of linezolid against biofilm-associated infections remains a topic of controversy (52, 59–61). In staphylococcal biofilms, tolerance to different antibiotics has been linked to the presence of eDNA (22, 23, 62). Specifically, eDNA provides structural integrity limiting antibiotic diffusion and penetration through staphylococcal biofilm (22, 23, 63, 64). In this study, we used the BioFilm Ring Test to quantify the contribution of eDNA in the early stages of biofilm formation. The results showed that DNase effectively prevented biofilm formation, leading to a significant reduction of $26.0 \pm 20.4\%$ in the initial attachment of MRSA strains compared to that of untreated control strains. In addition, eDNA was detected in all biofilm samples at a median concentration of 0.95 ng/μL. Notably, the high content of eDNA within the biofilm matrix was significantly correlated with increased tolerance to dalbavancin and vancomycin but not to linezolid. These data suggested that eDNA directly affects the antimicrobial potential of glycopeptides through the biofilm but does not interfere with linezolid activity. Similar conclusions were previously observed for vancomycin, describing an eDNA-based mechanism of antibiotic tolerance (22, 65). Dalbavancin and vancomycin are positively charged under physiological conditions, suggesting that negatively charged eDNA could bind to and interact with those antibiotics (66, 67). Our data showed that exogenous DNA could increase the bacterial counts in biofilm cultures exposed for 20 h to the MBEC$_{90}$ of dalbavancin. Overall, our findings are in agreement with earlier reports showing that *S. aureus* can incorporate heterologous DNA into the biofilm matrix from an exogenous source, including salmon sperm DNA, thus providing additional evidence that eDNA in the biofilm matrix contributes to increased biofilm tolerance to dalbavancin (22, 23, 68). The exposure of *S. epidermidis* biofilms to sub-MICs of vancomycin was enriched in eDNA (22, 69). Others described that subinhibitory concentrations of clindamycin increased the ability of *S. aureus* to form biofilms, shifting the biofilm matrix's composition toward higher eDNA content (70). The relative eDNA abundance in the biofilm matrix increased after 20 h of exposure to MBEC$_{90}$ but not at sub-MIC$_{90}$ levels of dalbavancin. These results suggest that the biofilm exposure to dalbavancin at MBEC$_{90}$ increases the level of eDNA in the biofilm culture, which in turn, shields MRSA cells from the action of dalbavancin. What remains uncertain is how the relative abundance of eDNA accumulates in the biofilm after dalbavancin treatment. Lysis-independent eDNA release

was described for *Enterococcus faecalis*, *Bacillus subtilis*, and *Neisseria gonorrhoeae* (71–74). In *ica*-independent MSSA strains, some genes are important for the eDNA release in the biofilm matrix. A possible mechanism is correlated with the selective lysis of a subset of cells that leads to the release of genomic DNA into the matrix (62, 75, 76). From our study, it seems most reasonable to assume that eDNA accumulates due to the lysis of a subpopulation of cells instead of as a result of an active release into the biofilm matrix dependent on antibiotic exposure. Indeed, at sub-MIC$_{90}$ dalbavancin, we could not observe any significant increase in the relative abundance of eDNA compared to untreated controls.

From a clinical point of view, our study suffers from some limitations. First, our analysis was performed on MRSA strains. Previous studies have shown that *S. aureus* produces biofilms through two major pathways, alternatively leading to the assembly of a polysaccharide-based or an eDNA/protein biofilm. Although these distinctive forms of biofilms are not mutually exclusive, the polysaccharide-based biofilm is predominantly described in methicillin-sensitive *S. aureus* (MSSA) strains, while the eDNA/protein biofilm is more represented in MRSA strains (13–16). Consequently, the activity of different antibiotics observed in this study has to be related to an MRSA strain which forms biofilms where eDNA plays a relevant contribution in shaping the architecture of the exopolysaccharide (EPS) matrix (75, 77). Second, in this study, bacteria were exposed to constant concentrations of antibiotics for a prolonged period. These conditions do not mimic the pharmacokinetic profile of the drugs at the site of infection; thus, the clinical findings should be interpreted with caution.

In conclusion, our data show that dalbavancin, at concentrations achievable in human serum, has antimicrobial potential against established MRSA biofilms, representing a promising therapeutic option for treating biofilm-associated SSTI. Furthermore, the evidence that the eDNA can reduce drug-induced antimicrobial activity may offer novel insight for designing more targeted therapeutic strategies against MRSA to either prevent or eradicate harmful biofilms.

MATERIALS AND METHODS

From the Microbial Strain Repository of the laboratory of Clinical Pathology and Microbiology (San Gallicano Dermatology Institute, Rome, Italy), 32 MRSA strains, collected during the period 2018 to 2020, from 32 patients presenting with complicated SSTIs were included in the study (78). Sample collection, bacterial identification, and antimicrobial susceptibility testing were performed as previously described (79). Strains were classified as MRSA when presenting the gene for methicillin resistance (*mecA*), oxacillin resistance (MIC $\geq$ 4 mg/mL), and positive agglutination test for penicillin-binding protein (PBP2; Oxoid, Basingstoke, UK) (80).

The ethics committee I.R.C.C.S. Lazio approved the study (Protocol 4394—31.03.2020, trials registry no. 1326/20).

Biofilm production. Biofilm production was quantified with the clinical BioFilm Ring Test (cBRT) as previously described (81), using the reagents and equipment provided by the Biofilm Ring Test kit (KITC004), and analyzed with BFC Elements 3.0 software (Biofilm Control, Saint Beauzire, France). *S. aureus* strain ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 (Se12228) were included in each plate as the standard reference and internal control, respectively. Each strain was analyzed in duplicate, and experiments were repeated three times.

Susceptibility testing. MIC. MICs were determined for each strain using the broth microdilution method, and results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (http://www.eucast.org/clinical_breakpoints). After the antibiotic treatment, viable cells were determined by plate counting for the CFU/mL determination. A standard bacterial inoculum of 5×10^5 CFU/mL was used. Serial 2-fold dilutions of the antimicrobials were prepared in cation-adjusted Mueller-Hinton broth (MHB). The MIC$_{90}$ was defined as the lowest concentration of antibiotic that killed 90% of the bacteria compared to the untreated control. Experiments were conducted in triplicate.

Minimum biofilm eradication concentration (MBEC) assays. For each experiment, an overnight culture of MRSA grown on a blood agar plate was used to inoculate 2 mL of 0.45% saline solution to 0.5 ± 0.1 McFarland turbidity standard (approximately 10^8 CFU/mL). For biofilm cultures, diluted cell suspensions (approximately 10^5 CFU/mL) were used to inoculate a 96-well polystyrene flat-bottom plate with 100 μL MHB. After 5 h at 37°C, the wells were rinsed with 0.45% saline solution to remove nonadherent bacteria, and the cells were resuspended in 100 μL of MHB supplemented with serial dilutions of dalbavancin, linezolid, and vancomycin. The plate was incubated for 20 additional hours at 37°C. After 20 h of exposure, the well contents were aspirated. Each well was washed two times with sterile deionized water, and the cells were resuspended in 100 μL of MHB. Biofilms were scraped thoroughly, and the total number of viable cells was determined by serial dilution and plating on blood agar plates to

estimate the CFU number. To allow reproducibility in the cell counting procedures, the *S. aureus* strain ATCC 25923 was included in each plate as the standard reference and internal control. The $MBEC_{90}$ levels were determined as the lower concentrations of antibiotics that killed 90% of the bacteria in preformed biofilms compared to the untreated control.

Determination of metabolic activity. The metabolic activity of planktonic and biofilm MRSA isolates was determined using a resazurin-based assay as previously described (82). An overnight culture of MRSA grown on a blood agar plate was used to inoculate 2 mL of 0.45% saline solution to 0.5 ± 0.1 McFarland turbidity standard (approximately 10^8 CFU/mL). For planktonic cultures, diluted cell suspensions (approximately 10^5 CFU/mL) were used to inoculate a 96-well polystyrene flat-bottom plate with 100 μL of an MHB/resazurin solution (Promega, Madison, WI, USA). The plates were incubated for 24 h at 37°C, and absorbance (570 nm) was recorded in 20-min periods for 1,200 min using a multidetection microplate reader (Multiskan SkyHigh; Thermo Fisher Scientific, USA).

For biofilm formation, 100 μL of diluted cells suspensions (approximately 10^5 CFU/mL) in MHB was transferred to a 96-well polystyrene flat-bottom plate. After 5 h at 37°C, the wells were rinsed with 0.45% saline solution, and 100 μL of an MHB/resazurin solution (Promega, Madison, WI, USA) was added. The plate was incubated for 20 additional hours at 37°C, and absorbance (570 nm) was recorded in 20-min periods for 1,200 min using a multidetection microplate reader (Multiskan SkyHigh; Thermo Fisher Scientific, USA).

Assessment of MRSA biofilm composition. The Biofilm Ring Test method was used to quantify the attachment and initial biofilm formation in the presence of DNase (100 μg/mL) (83). Standardized bacterial suspensions containing 1 vol % magnetic beads were supplemented with DNase (100 μg/mL) and incubated at 37°C in a 96-well microplate (200 μL/well) (BD Falcon 96 flat-bottom transparent; Corning, USA). Negative controls contained 200 μL of sterile brain heart infusion (BHI) with magnetic beads and enzymes. The plate was read after 6 h of incubation, as described above. The capacity of the strains to form biofilm in the presence of DNase was expressed using the relative difference (RD):

$$RD = \left[(Pmb\ without\ enzyme - Pmb\ with\ enzyme)/Pmb\ without\ enzyme \right] \times 100 \qquad (1)$$

The analysis was performed three times in duplicate for each sample.

eDNA quantification in MRSA biofilm. eDNA was quantified as described previously (84). Briefly, a microtiter plate was inoculated with diluted starter cultures adjusted to a final concentration of approximately 1×10^5 CFU/mL in 100 μL of MHB and incubated at room temperature under static conditions for 20 h. The presence of eDNA was quantified by the addition of 100 μL Tris-EDTA (TE) buffer followed by 100 μL freshly made PicoGreen solution (1 μL PicoGreen dye in 199 μL TE buffer). Wells with PicoGreen were incubated for 5 min before measuring the fluorescence intensity (excitation 485 nm/ emission 535 nm, 0.1 s) using a fluorescence plate reader (Wallace Victor 3, 1420 Multicolor; PerkinElmer). Each analysis was performed on three biological replicates for each strain. Lambda DNA (Invitrogen Molecular Probes) was used to generate a standard curve for each run. For each time point, the biofilm was stained with PicoGreen and observed using phase-contrast and fluorescence microscopy (Zeiss Axiovert 200M).

To test the effects of exogenous DNA on the growth of MRSA biofilm, MHB was supplemented with dalbavancin, salmon sperm DNA (16 μg/mL) alone, or both dalbavancin and salmon sperm DNA. Dalbavancin and salmon sperm DNA were preincubated at 25°C for 30 min before cells were added. For biofilm formation, 100 μL of diluted cell suspensions (approximately 10^5 CFU/mL) in MHB was transferred to a 96-well polystyrene flat-bottom plate. After 5 h at 37°C, wells were rinsed with 0.45% saline solution, and 100 μL of salmon sperm DNA (16 μg/mL) and dalbavancin, separately and in combination, were added. After 20 h of incubation at 37°C, the biofilms were washed thoroughly with sterile deionized water. Biofilms were scraped, and the total number of viable cells was determined by serial dilution and plating on blood agar plates to estimate the CFU number.

Biofilm imaging. Biofilms were grown in μ-Slide slides (Ibidi, Gräfelfing, Germany) inoculated with $\sim 1 \times 10^5$ cells in 500 μL of fresh BHI medium and incubated for 48 h at 37°C. The culture medium was changed after 24 h of biofilm growth. Biofilms were stained using the Live/Dead BacLight bacterial viability kit (Life Technologies, New York, NY, USA) and/or TOTO-1 iodide staining (Thermo Fisher Scientific, catalog [cat.] no. T3600; dilution, 1:1,000) for detection of free eDNA surrounding living and dead cells (85, 86) and examined with an Apotome system (Zeiss, Oberkochen, Germany) connected to an Axio Observer inverted fluorescence microscope (Zeiss). Data were analyzed with the ZEN 3.1 (blue edition) software (Zeiss).

Statistics. All variables were summarized with descriptive statistics and tested for normality. When appropriate, comparisons between continuous variables were carried out with Student's *t* test or the Mann-Whitney U test. In contrast, when appropriate, categorical variables were tested using the χ^2 or two-tailed Fisher's exact test. Correlation analysis was performed using the Spearman rank-order correlation coefficient (ρ). A *P* value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS software version 21 (SPSS, Inc., Chicago, IL, USA).

A. Mastrofrancesco, and L.T. collected and interpreted the clinical data. All authors interpreted the data, revised the manuscript critically, and approved the submitted version.

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