Turk J Pharm Sci 2025;22(2):131-139
DOI: 10.4274/tjps.galenos.2025.26723

ORIGINAL ARTICLE

The Effect of Sub-Minimal Inhibitory Concentrations of Daptomycin and Linezolid on Biofilm Formation of Methicillin Resistant *Staphylococcus aureus* Isolated from Clinical Samples

Halil BAL[1]*, Nurten ALTANLAR[2], Sulhiye YILDIZ[3]

[1]Sivas Cumhuriyet University Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Sivas, Türkiye
[2]Ankara University Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara, Türkiye
[3]Lokman Hekim University Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara, Türkiye

ABSTRACT

Objectives: The aim of this study was to determine the development of *in vitro* resistance and changes in biofilm forming abilities in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates exposed to sub-minimal inhibitory concentrations (sub-MICs) of daptomycin and linezolid; and to investigate the presence of the methicillin resistance gene (*mecA*) and the biofilm-associated genes (*icaA*, *icaD*) by polymerase chain reaction.

Materials and Methods: This study was carried out with thirty-two MRSA isolates. The susceptibility of the isolates to daptomycin and linezolid was investigated by the broth microdilution method, and MIC values were determined (1st MIC). After serial passages, the 2nd MIC and the 3rd MIC values were similarly detected. Before and after serial passages, the biofilm-forming abilities of MRSA isolates were examined using the microtiter plate (MTP) method.

Results: When the daptomycin and linezolid 1st MIC and 3rd MIC values of the isolates were compared, there was a 2-8 fold increase in linezolid *($p<0.05$)* and a 4-32 fold increase in daptomycin *($p<0.05$).* According to the MTP method, 20 (62.5%) of the 32 isolates formed biofilm at various levels, while 12 (37.5%) did not form biofilm. After the second series of passages, biofilm formation was observed in 19 (59.4%) isolates with daptomycin ($p>0.05$) and in 16 (50%) isolates with linezolid ($p>0.05$). The *mecA* gene was found in all isolates. Also, *icaA* and *icaD* genes were detected in 31 (96.9%) of 32 MRSA isolates.

Conclusion: MRSA isolates exposed to sub-MICs of the antibiotics daptomycin and linezolid were observed to form biofilms at varying levels or to lose their ability to form biofilms. The induction, reduction or eradication of biofilm depended on the type of antibiotic and the MRSA isolate.

Keywords: Biofilm, daptomycin, linezolid, MRSA, sub-minimal inhibitory concentration

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogenic bacterium. These bacteria are found in the body parts of healthy individuals, such as the nose, intestines, skin, skin glands and mucous membranes.[1] *S. aureus* is resistant to many antibiotics, including various mechanisms. In the early 1940s, shortly after the clinical use of penicillin, penicillin-resistant isolates appeared that produced beta-lactamase. In 1959, the

***Correspondence:** halilbal@cumhuriyet.edu.tr, **ORCID-ID:** orcid.org/0000-0002-0017-3425
Received: 31.10.2023 **Accepted:** 28.03.2025 **Publication Date:** 14.05.2025

Cite this article as: BAL H, ALTANLAR N, YILDIZ S. The effect of sub-minimal inhibitory concentrations of daptomycin and linezolid on biofilm formation of methicillin-resistant *Staphylococcus aureus* isolated from clinical samples. Turk J Pharm Sci. 2025;22(2):131-139

problem was solved with beta-lactamase-resistant methicillin, but in 1961, methicillin-resistant *Staphylococcus aureus* (MRSA) isolates were identified.[2]

MRSA is resistant not only to beta-lactam group antibiotics but also to several other antibiotics, including macrolides and tetracyclines. MRSA isolates have become the leading cause of nosocomial infections worldwide due to limited treatment options.[3] Vancomycin is used for serious MRSA infections. In treatment with vancomycin, the occurrence of less sensitive isolates, poor clinical results, and increased nephrotoxicity with high-dose therapy has been observed. In the early 2000s, daptomycin and linezolid began to be used in the treatment of MRSA infections. Linezolid is used orally or intravenously to treat skin and soft tissue infections and pneumonia. Daptomycin is recommended for the secondary treatment of bacteremia, the treatment of right valve endocarditis, and complicated skin infections.[4-7] Minimal inhibitory concentration (MIC) was defined through sensitivity and antibiotic dosing studies on bacteria. The MIC is the lowest concentration of antibiotic that inhibits the visible growth of bacteria under *in vitro* conditions. During treatment, the antibiotic concentration between two consecutive doses should be higher than the MIC. After a certain period, the concentration applied in the various tissues becomes lower than the MIC value. These antibiotic concentrations below MIC are defined as sub-MIC. Bacteria can be exposed to sub-MIC levels of non-lethal antibiotics in humans, animals, and the environment. Although sub-MICs do not kill bacteria, they can affect various virulence factors such as morphology, surface properties, pathogenicity, biofilm formation, and toxin production.[8] Sub-MICs can affect the emergence of resistant isolates, mutation, recombination, and horizontal gene transfer. In addition, as a signal molecule, it can affect bacterial virulence, altering biofilm formation, quorum sensing, and gene expression.[9-11] This study aimed to investigate the effects of sub-MICs of daptomycin and linezolid on biofilm formation of MRSA isolates and the presence of methicillin resistance gene (*mecA*) and biofilm-associated genes *(icaA and icaD)* of MRSA isolates by polymerase chain reaction.

MATERIALS AND METHODS

This study was conducted with 32 MRSA (abscess, bronchoalveolar lavage, catheter tip, joint fluid, prosthetic-tissue, tracheal aspirate, tissue, wound) isolates. MRSA isolates were obtained from Ankara University Faculty of Medicine Hospitals (Ankara University İbni Sina Hospital and Ankara University Cebeci Central Laboratory). Ethics Committee approval was obtained from Ankara University Faculty of Medicine Clinical Research Ethics Committee (approval number: 08-499-18, dated: 07.05.2018).

Gram staining, catalase, tube coagulase, DNase, and mannitol fermentation tests were performed on MRSA isolates. Then, following the cefoxitin disk diffusion test, the *mecA* gene was investigated by polymerase chain reaction (PCR), and all MRSA isolates were confirmed.[12-16] The susceptibilities of MRSA isolates to daptomycin (DEVA Holding, Türkiye) and linezolid (Haver Farma, Türkiye) were determined by the broth microdilution method.[16] Antibiotic susceptibility experiments were performed in 96-well U-bottom microtiter plates (Eppendorf, Germany) using cation-adjusted Mueller-Hinton broth (CAMHB) medium (Becton Dickenson BBL, Sparks, MD, USA). For the daptomycin MIC test, the calcium concentration in CAMHB medium was adjusted to 50 mg/L.

Linezolid was dissolved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a final concentration of 640 µg/mL, and daptomycin was dissolved in sterile distilled water at a final concentration of 160 µg/mL. Two-fold serial dilutions of linezolid (32-0.0625 µg/mL) and daptomycin (8-0.0156 µg/mL) were prepared in CAMHB medium. A 0.5 McFarland culture was prepared in CAMHB and added to the wells. The final bacterial concentration in each well was $5x10^5$ colony-forming units (CFU)/mL. The wells with bacterial suspension without antibiotics were used as positive controls, and the wells with only CAMHB medium were used as negative controls. Microtiter plates were incubated at 35 °C for 24 hours. After incubation, MIC values were determined considering the susceptibility breakpoints approved by the European Committee on Antimicrobial Susceptibility Testing for *S. aureus* (S≤1 µg/mL for daptomycin, S≤4 µg/mL for linezolid).[17] Each experiment was repeated three times. *S. aureus* ATCC 29213 was used as a quality control strain.

Sub-MIC exposure and serial passage

Serial passage experiments were performed for each strain with ½ MIC values (sub-MICs), which were determined against daptomycin and linezolid. Each MRSA strain was passaged through its respective sub-MICs on the first day and incubated at 35 °C for 24 hours. On the second day, each strain was transferred to CAMHB (Becton Dickenson BBL, Sparks, MD, USA) medium and incubated at 35 °C for 24 hours. After sixteen consecutive days, the first series of passages was completed. After the first series of passages, MICs and sub-MICs values were determined again. Similarly, second serial passages were performed to determine the third MIC values.[16-18] *S. aureus* ATCC 29213 used as quality control strain.

Microtiter plate biofilm assay

Biofilm-forming abilities of MRSA isolates were determined by the microtiter plate method (MTP).[19] Biofilm formation levels of all isolates before and after serial passages were examined. Bacteria suspension was prepared in 1% glucose supplemented Tryptic Soy Broth (TSB, Merck, Germany) medium and 0.5 McFarland ($1x10^8$ CFU/mL) turbidity was adjusted. The bacterial suspension was diluted to 1/20 and 20 µL was added to the wells in the flat-bottom microtiter plate with 96 wells (BD Falcon 96 Flat Bottom Transparent, Corning, USA) 180 µL of TSB (Merck, Germany) medium supplemented with 1% glucose was added to the reach a concentration of $5x10^5$ CFU/mL. Microtiter plates were incubated (35 °C, 24 hours). After incubation, it was discharged, washed three times with sterile phosphate buffer solution (Sigma-Aldrich S.R.L., Milan, Italy) using a micropipette, inverted, and left to dry. After the microtiter plates dried, 150 µL of methanol (Merck, Darmstadt,

Germany) was added for fixation, left for 20 minutes, the methanol was removed, and the microtiter plates were left to dry. After the drying process, 0.1% safranin (Sigma-Aldrich, St. Louis, MO) dye was added and left for 15 minutes; the microtiter plates were washed and turned over and left to dry. Three wells were used for each bacterium. TSB (Merck, Germany) medium supplemented with 1% glucose was used as a negative control. *S. aureus* ATCC 6538, *S. epidermidis* ATCC 35984 were used as positive controls. The optical density cut-off value was determined and the results interpreted.[19]

DNA isolation of MRSA isolates

DNA isolation from MRSA samples was performed with a DNA isolation kit (Thermo Scientific GeneJET, Van Allen Way, Carlsbad, California) according to the manufacturer's recommendations.

mecA, icaA, icaD genes amplifications

Amplifications of the methicillin resistance gene *(mecA)* and biofilm-associated genes (*icaA* and *icaD*) were determined by conventional PCR.[20,21] The PCR reaction (50 µL): 35.35 µL (ddH_2O), 2 µL (10 µM each of the primers), 0.4 µL (25 mM dNTPs, GeneDireX Inc. USA), 0.25 µL (5U/µL Taq DNA polymerase, GeneDireX Inc. USA), 5 µL (10X PCR buffer), 5 µL (template DNA). PCR amplification conditions for the *mecA* gene: initial denaturation, 94 °C/5 min; denaturation, 94 °C/30 s; annealing, 55 °C/30 s; extension, 72 °C/1 min; 40 cycles; final extension, 72 °C/5 min. PCR amplification conditions for the *icaA* and *icaD* genes: (initial denaturation: 92 °C/5 min), (denaturation: 92 °C/1 min), (annealing: 49 °C/50 s), (extension: 72 °C/1 min), (30 cycles), (final extension: 72 °C/7 min). Positive controls (*S. aureus* ATCC 6538 and *S. aureus* ATCC 43300), negative control (sterile distilled water).

Statistical analysis

The data were analyzed using SPSS version 24 software (SPSS Inc., IBM, Chicago, IL, USA). Wilcoxon Signed-rank test was used for changes in MIC values and biofilm formation of isolates; Mann-Whitney U test was used for comparison of MIC values; McNemar's test was used for comparison of biofilm formation experiments. *P* value of <0.05 was considered statistically significant.

RESULTS

The *mecA* gene was detected by conventional PCR, and a 533 bp amplicon was obtained. The *mecA* gene was detected in all 32 MRSA. This result was confirmed genotypically for methicillin resistance of all isolates (Figure 1). According to the initial MIC values, all MRSA isolates were found to be susceptible to daptomycin and linezolid. After serial passages with the antibiotics daptomycin and linezolid, there was an increase in the second and third MIC values in all MRSA strains. When we evaluated the first MIC and third MIC results of daptomycin, we observed that there was a 4-32-fold increase in MIC values. A significant difference was found between the first and third MIC values of daptomycin (z=-4.945; $p<0.05$). When linezolid's initial MIC and third MIC values were compared, there was a 2- to 8-fold increase. There was a statistically significant difference between the first MIC and third MIC values of linezolid (z=-5.018; $p<0.05$). In the third MIC value, it was observed that 10 (31.25%) of the samples (Samples IDs: 4, 17, 18, 19, 20, 22, 25, 26, 28, 32) were resistant to daptomycin, whereas all were susceptible to linezolid (Tables 1 and 2). According to the MTP method, 20 (62.5%) of 32 MRSA produced various levels of biofilm (Table 3). Twelve (37.5%) did not produce biofilm. Amplification of the *icaA* and *icaD* genes associated with biofilm formation was performed by conventional PCR.

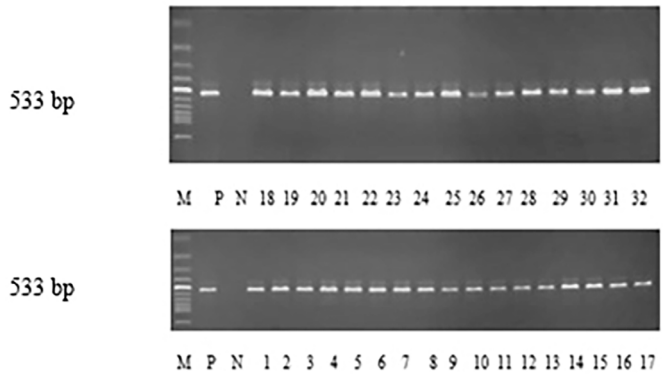

Figure 1. Agarose gel electrophoresis (*mecA* gene, 533 bp) in MRSA isolates. 1-32; [positive samples (533 bp)]. M: 100 bp DNA ladder (Thermo Fisher Scientific, St. LeonRot, Germany) P: (*Staphylococcus aureus* ATCC 43300) positive control, N: Negatif control (sterile distilled water)
MRSA: Methicillin-resistant *Staphylococcus aureus*

Amplicons of 1315 bp for the *icaA* gene and 381 bp for the *icaD* gene were obtained.[22] (96.9%) of 32 MRSA were positive for both *icaA* and *icaD* genes, and 1 (3.1%) strain was negative for both *icaA* and *icaD* genes (Figures 2 and 3). After the second series of passages performed with daptomycin, it was found that 19 (59.4%) of the 32 MRSA were biofilm producers, 13 (40.6%) were not biofilm producers (Table 3). There was no statistically significant difference between the biofilm formation levels of MRSA before serial passages and the biofilm formation levels after the second serial passages performed with daptomycin (z=-0.171; $p>0.05$).

After the second series of passages with linezolid, it was observed that 16 (50%) of 32 MRSA were biofilm producers, 16 (50%) were non-biofilm producers (Table 3). There was no statistically significant difference between the biofilm formation levels of MRSA isolates before serial passages and those levels after the second serial passages performed with linezolid (z=-0.531, $p>0.05$). When we compared the effect of linezolid and daptomycin on the biofilm formation levels of the isolates after the second series of passages, there was no statistically significant difference between the two antibiotics ($p>0.05$).

DISCUSSION

During treatment, the concentration of the antibiotic varies in different body parts. Due to contamination from human activities, sub-MICs of antibiotics are found in sewage, soil, and many aquatic environments.[23] Bacteria in these environments

may be exposed to sub-MICs of antibiotics. sub-MICs can have various effects on bacteria (selection of resistant isolates, genotypic and phenotypic variability, and bacterial signaling).[9] "By Müller et al.[24] *S. aureus* HG001, *S. aureus* SG511 strains were exposed to the sub-MICs of daptomycin by serial passage for 4 months, they found that MIC values increased 100-fold in *S. aureus* HG001 and 800-fold in *S. aureus* SG511." As a result of genetic, proteomic, and transcriptomic analyses, they determined that the cell wall structure thickens and the autolysis rate decreases in both isolates. Similarly, in another study involving daptomycin, a 16-fold increase in MIC values and a significant increase in carotenoid pigment synthesis

Table 1. Linezolid MIC values of MRSA isolates

MRSA sample no.	LNZ 1st MIC (ug/mL) Susceptible (S) Resistant (R)		LNZ 2nd MIC (ug/mL) Susceptible (S) Resistant (R)		LNZ 3rd MIC (ug/mL) Susceptible (S) Resistant (R)		Total increase in fold LNZ	*p* value
1	0.5	S	1	S	1	S	2	$p<0.05$
2	0.25	S	0.5	S	2	S	8	$p<0.05$
3	0.5	S	1	S	2	S	4	$p<0.05$
4	0.5	S	1	S	2	S	4	$p<0.05$
5	0.5	S	1	S	2	S	4	$p<0.05$
6	1	S	2	S	2	S	2	$p<0.05$
7	1	S	2	S	2	S	2	$p<0.05$
8	0.25	S	1	S	2	S	8	$p<0.05$
9	0.5	S	1	S	2	S	4	$p<0.05$
10	0.25	S	0.5	S	2	S	8	$p<0.05$
11	0.25	S	1	S	2	S	8	$p<0.05$
12	0.25	S	1	S	2	S	8	$p<0.05$
13	0.25	S	1	S	2	S	8	$p<0.05$
14	0.25	S	1	S	2	S	8	$p<0.05$
15	1	S	2	S	2	S	2	$p<0.05$
16	0.5	S	2	S	2	S	4	$p<0.05$
17	1	S	2	S	2	S	2	$p<0.05$
18	0.5	S	1	S	2	S	4	$p<0.05$
19	0,25	S	1	S	2	S	8	$p<0.05$
20	0.5	S	1	S	2	S	4	$p<0.05$
21	0.25	S	1	S	2	S	8	$p<0.05$
22	0.5	S	1	S	2	S	4	$p<0.05$
23	0.5	S	1	S	1	S	2	$p<0.05$
24	0.5	S	1	S	2	S	4	$p<0.05$
25	0.25	S	1	S	2	S	8	$p<0.05$
26	0.5	S	1	S	2	S	4	$p<0.05$
27	0.5	S	1	S	2	S	4	$p<0.05$
28	0.5	S	1	S	2	S	4	$p<0.05$
29	0.5	S	1	S	2	S	4	$p<0.05$
30	0.5	S	1	S	2	S	4	$p<0.05$
31	0.5	S	1	S	1	S	2	$p<0.05$
32	0.5	S	1	S	2	S	4	$p<0.05$
S. aureus ATCC 29213	1	S	2	S	2	S	2	$p<0.05$

LNZ: Linezolid, MRSA: Methicillin-resistant *S. aureus*, *S. aureus: Staphylococcus aureus* No.: Number, MIC: Minimal inhibitory concentration

were determined in MRSA strains.[25] "In Lahiri and Alm[26] in their experiments with *S. aureus* ATCC 29213 strain, found a 16-fold increase in the MIC value of ceftaroline, while the MIC values of non-β-lactam antibiotics (vancomycin, levofloxacin, linezolid) remained in a 2-fold dilution." They stated that this situation is not a general resistance mechanism, but may be associated with a mechanism specific to β-lactam. In our study, after serial passages with daptomycin and linezolid antibiotics, an increase in the 2nd and 3rd MIC values was observed in all MRSA isolates. Staphylococcal biofilms can appear on medical devices such as catheters, prosthetic joints, and implants.[27] Staphylococcal biofilm expression can be influenced by different physical

Table 2. Daptomycin MIC values of MRSA

MRSA sample no.	DAP 1st MIC (ug/mL) Susceptible (S) Resistant (R)		DAP 2nd MIC (ug/mL) Susceptible (S) Resistant (R)		DAP 3rd MIC (ug/mL) Susceptible (S) Resistant (R)		Total increase in fold DAP	*p* value
1	0.0625	S	0.125	S	1	S	16	$p<0.05$
2	0.0625	S	0.25	S	1	S	16	$p<0.05$
3	0.125	S	0.125	S	1	S	8	$p<0.05$
4	0.125	S	0.25	S	2	R	16	$p<0.05$
5	0.0625	S	0.125	S	0.5	S	8	$p<0.05$
6	0.125	S	0.125	S	1	S	8	$p<0.05$
7	0.125	S	0.125	S	0.5	S	4	$p<0.05$
8	0.0625	S	0.25	S	1	S	16	$p<0.05$
9	0.03125	S	0.125	S	0.25	S	8	$p<0.05$
10	0.0625	S	0.125	S	0.5	S	8	$p<0.05$
11	0.0625	S	0.125	S	0.25	S	4	$p<0.05$
12	0.0625	S	0.25	S	0.5	S	8	$p<0.05$
13	0.0625	S	0.125	S	0.25	S	4	$p<0.05$
14	0.0625	S	0.5	S	0.5	S	8	$p<0.05$
15	0.0625	S	0.125	S	0.25	S	4	$p<0.05$
16	0.0625	S	0.25	S	0.25	S	4	$p<0.05$
17	0.25	S	0.5	S	2	R	8	$p<0.05$
18	0.0625	S	0.5	S	2	R	32	$p<0.05$
19	0.0625	S	0.5	S	2	R	32	$p<0.05$
20	0.25	S	0.5	S	2	R	8	$p<0.05$
21	0.125	S	0.125	S	0.5	S	4	$p<0.05$
22	0.0625	S	0.25	S	2	R	32	$p<0.05$
23	0.125	S	0.25	S	0.5	S	4	$p<0.05$
24	0.25	S	0.5	S	1	S	4	$p<0.05$
25	0.25	S	0.5	S	2	R	8	$p<0.05$
26	0.125	S	0.5	S	2	R	16	$p<0.05$
27	0.125	S	0.5	S	1	S	8	$p<0.05$
28	0.0625	S	0.25	S	2	R	32	$p<0.05$
29	0.0625	S	0.25	S	0.5	S	8	$p<0.05$
30	0.125	S	0.25	S	1	S	8	$p<0.05$
31	0.125	S	0.5	S	1	S	8	$p<0.05$
32	0.0625	S	0.5	S	2	R	32	$p<0.05$
S. aureus ATCC 29213	0.25	S	0.5	S	0.5	S	2	$p<0.05$

DAP: Daptomycin, MRSA: Methicillin-resistant *S. aureus*, *S. aureus: Staphylococcus aureus* No.: Number, MIC: Minimal inhibitory concentration

and chemical factors and is induced in response to sub-MICs of certain antibiotics and external stress.[28-30] Numerous studies are showing that sub-MICs of some antibiotics affect bacterial biofilms *in vitro*. This is clinically important because bacteria may be exposed to sub-MICs at the beginning of the dosing regimen or between doses.[31] Adhesion factors and

Table 3. Biofilm formation levels of MRSA isolates

MRSA sample no.	Before serial passages	Daptomycin (after 2nd serial passages)	Linezolid (after 2nd serial passages)
1	I	I	I
2	III	II	0
3	I	III	II
4	II	III	II
5	0	III	0
6	III	II	III
7	III	III	0
8	I	III	I
9	0	0	0
10	I	I	III
11	0	I	II
12	0	0	0
13	0	0	0
14	I	0	0
15	I	III	I
16	I	II	III
17	II	III	II
18	0	0	0
19	0	0	0
20	III	0	0
21	0	I	0
22	0	0	0
23	I	I	II
24	III	I	III
25	0	0	0
26	II	0	0
27	II	0	II
28	0	III	III
29	I	III	II
30	III	I	0
31	III	0	III
32	0	0	0
Total biofilm*	20	19	16
S. aureus ATCC 29213	0	0	0
S. aureus ATCC 43300	III	III	III

0: No biofilm production, I: Weak biofilm production, II: Moderate biofilm production, III: Strong biofilm production, MRSA: Methicillin-resistant *Staphylococcus aureus*, *($p<0.05$)

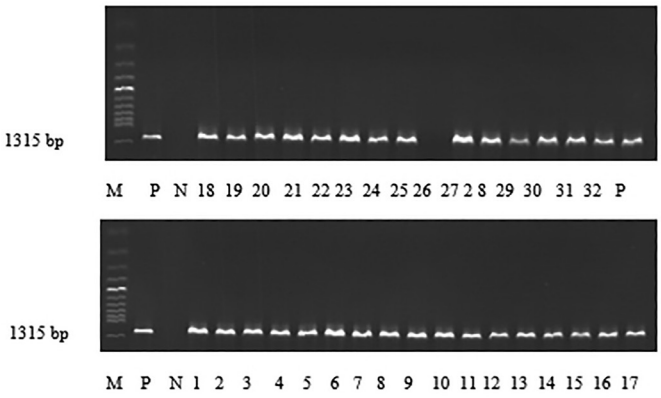

Figure 2. Agarose gel electrophoresis (*icaA* gene, 1315 bp) in MRSA isolates. M: 100 bp DNA ladder (Thermo Fisher Scientific, St. LeonRot, Germany). P: Positive control (*Staphylococcus aureus* ATCC, 43300), *icaA* positive samples (1-25, 27-32), *icaA* negative sample (26), N: Negative control (steril distilled water)

MRSA: Methicillin-resistant *Staphylococcus aureus*

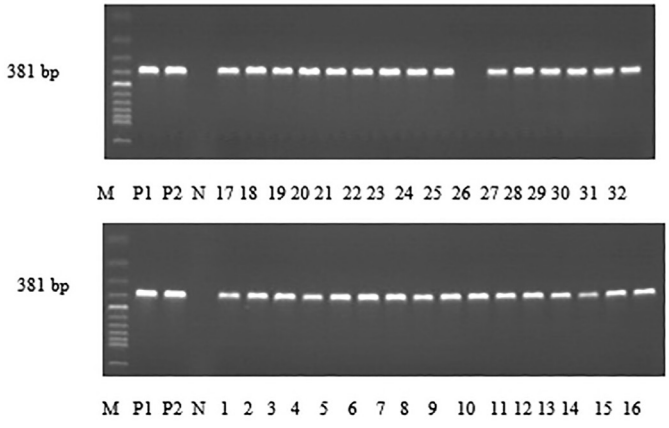

Figure 3. Agarose gel electrophoresis (*icaD* gene, 381 bp) in MRSA isolates. P1: Positive control (*Staphylococcus aureus* ATCC, 43300), P2: Positive control (*Staphylococcus aureus* ATCC 6538), *icaD* positive samples (1-25, 27-32), *icaD* negatif sample (26), N: Negative control (steril distilled water), M: 100 bp DNA ladder (Thermo Fisher Scientific, St. LeonRot, Germany), MRSA: Methicillin-resistant *Staphylococcus aureus*

polysaccharide intracellular adhesion (PIA) molecules are required for staphylococci to form biofilms. The *icaADBC* operon is located in the intercellular adhesion (ica) locus. The *icaADBC* operon contains four genes *(icaA, icaD, icaB, icaC)* encoding the proteins required for PIA production.[32,33] Cramton et al.[34] reported that *Staphylococcus* strains had *icaA* and *icaD* genes, but they could not form biofilms *in vitro*, which may be due to a point mutation in the locus. Arciola et al.[22] stated that the expression of *icaA* and *icaD* genes can be affected by various environmental factors such as medium content and anaerobic environment. Biofilm formation of *S. aureus* is explained by mechanisms independent of *ica* genes.[35] The *ArlRS* gene system in *S. aureus* is effective in autolysis, biofilm formation, capsule synthesis, and virulence.[35,36] Toledo-Arana et al.[37] reported that mutations in the ArlRS system increased biofilm formation and that the biofilm formation of the ArlRS mutant strain was not affected by the deletion of the *icaADBC* locus.34 Similarly, in our study, *icaA* and *icaD* genes were negative in 1 (3.1%) out of 32 MRSA isolates. This strain formed biofilm by the MTP. It was found to be compatible with these studies.

Some studies show that sub-MICs of antibiotics such as vancomycin, cephalexin, oxacillin, and cephalothin, which are effective on the cell wall, can induce biofilm formation of *S. aureus*.[38-40] In a similar study, Sritharadol et al.[41] observed that low mupirocin concentrations induced biofilm formation, especially in the MRSA USA 300 clone. In another study, Lázaro-Díez et al.[42] reported that sub-MICs of ceftaroline induce bacterial attachment and biofilm formation in some MRSA strains, and it is important to use effective bactericidal concentrations of ceftaroline in the treatment of biofilm MRSA-related infections. Kaplan et al.[43] stated that sub-MIC concentrations of methicillin, ampicillin, amoxicillin, and cloxacillin-induced biofilm formation were observed in.

At least one of the USA300, USA400, and USA500 MRSA strains. The induction depended on the antibiotic type and the strain, and methicillin-induced biofilm formation was observed in all three isolates. As a result of the second series of passage experiments in our study, it was determined that the induction, reduction, or destruction of biofilm formation varies according to the antibiotic and isolate, which aligns with our findings.

CONCLUSION

In this study, it was observed that the MIC values of daptomycin and linezolid in MRSA isolates increased after serial passages. After the second series of passages, it was determined that changes in the biofilm formation levels of the isolates varied by isolate and antibiotic type. Biofilm is a factor that complicates the treatment process and requires additional costs. Sub-MICs can affect biofilm formation in bacteria. In the treatment process, it is important to select the appropriate antibiotic, adjust the antibiotic dose range, and prevent random contact of antibiotics with the environment. In this way, the exposure of bacteria to sub-MICs can be reduced, preventing antibiotic resistance and biofilm formation.

Ethics

Ethics Committee Approval: Ethics Committee approval was obtained from Ankara University Faculty of Medicine Clinical Research Ethics Committee (approval number: 08-499-18, dated: 07.05.2018).

Informed Consent: Not required.

Footnotes

Authorship Contributions

Concept: H.B., N.A., S.Y., Design: H.B., N.A., S.Y., Data Collection or Processing: H.B., N.A., S.Y., Analysis or Interpretation: H.B., N.A., S.Y., Literature Search: H.B., N.A., S.Y., Writing: H.B., N.A., S.Y.

Conflict of Interest: The authors declare no conflicts of interest.

Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES

1. Lakhundi S, Zhang K. Methicillin-resistant *Staphylococcus aureus*: Molecular characterization, evolution, and epidemiology. Clin Microbiol Rev. 2018;31:e00020-18.
2. Palavecino E. Community-acquired methicillin-resistant *Staphylococcus aureus* infections. Clinics in laboratory medicine. 2004;24:403-418.
3. Gonçalves E, Carvalhal R, Mesquita R, Azevedo J, Coelho MJ, Magalhães R, Ferraz MP, Manso MC, Gavinha S, Pina C, Lopes Cardoso I. Detection of *Staphylococcus aureus* (MRSA/MSSA) in surfaces of dental medicine equipment. Saudi J Biol Sci. 2020;27:1003-1008.
4. Rodvold KA, McConeghy KW. Methicillin-resistant *Staphylococcus aureus* therapy: past, present, and future. Clin Infect Dis. 2014;58 Suppl 1:S20-S27.
5. Molloy L. Methicillin-resistant *Staphylococcus aureus*: A pharmacotherapy primer. Journal of Pediatric Health Care: Official publication of National Association of Pediatric Nurse Associates & Practitioners. 2017;31:2:246-256.
6. Foster TJ. Antibiotic resistance in *Staphylococcus aureus*. Current status and prospects. FEMS Microbiol Rev. 2017;41:430-449.
7. Hashemian SMR, Farhadi T, Ganjparvar M. Linezolid: a review of its properties, function, and use in critical care. Drug Des Devel Ther. 2018;12:1759-1767.
8. Sato Y, Unno Y, Ubagai T, Ono Y. Sub-minimum inhibitory concentrations of colistin and polymyxin B promote *Acinetobacter baumannii* biofilm formation. PLoS One. 2018;13:e0194556.
9. Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol. 2014;12:465-478.
10. Liu B, Zhang X, Ding X, Wang Y, Zhu G. Regulatory mechanisms of sub-inhibitory levels antibiotics agent in bacterial virulence. Appl Microbiol Biotechnol. 2021;105:3495-3505.
11. Chen J, Zhou H, Huang J, Zhang R, Rao X. Virulence alterations in *Staphylococcus aureus* upon treatment with the sub-inhibitory concentrations of antibiotics. J Adv Res. 2021;31:165-175.
12. Procop GW, Church DL, Hall GS, Janda WM, Koneman EW, Schrecken-Berger PC. Woods. In: GL (ed). Koneman's color atlas and textbook of diagnostic microbiology, 7th ed. Wolters Kluwer, Philadelphia. 2017a; PA. Page: 693.
13. Procop GW, Church DL, Hall GS, Janda WM, Koneman EW, Schrecken-Berger PC, WOODS. In: GL (ed). Koneman's color atlas and textbook of diagnostic microbiology, 7th ed. Wolters Kluwer, Philadelphia. 2017b; PA. Page: 702.
14. Pumipuntu N, Kulpeanprasit S, Santajit S, Tunyong W, Kong-Ngoen T, Hinthong W, Indrawattana N. Screening method for *Staphylococcus aureus* identification in subclinical bovine mastitis from dairy farms. Vet World. 2017;10:721-726.
15. EUCAST Disk disk diffusion method for antimicrobial susceptibility testing. 2021a;Version 9.0.
16. EUCAST The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. 2021b; Version 11.0.
17. EUCAST Breakpoint tables for interpretation of MICs and zone diameters. Version 2021c; 11.0.
18. Woodburn KW, Jaynes JM, Clemens LE. Evaluation of the antimicrobial peptide, RP557, for the broad-spectrum treatment of wound pathogens and biofilm. Front Microbiol. 2019;10:1688.
19. Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I, Ruzicka F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *Staphylococci*. APMIS. 2007;115:891-899.
20. Radenovic M, Asanin J, Aksentijevi C K, Misic D. Investigation of presence of methicillin resistant *Staphylococci* in students of the faculty of veterinary medicine at the university of belgrade. Archives of Veterinary Medicine. 2017;9:17-28.
21. Branko S, Vlado T, Dragan V, Karabasil N, Mirjana D, Jasna D, Vera K. Detection of *icaA* and *icaD* genes of *Staphylococcus aureus* isolated in cases of bovine mastitis in the republic of serbia. Acta Veterinaria-Beograd. 2017;67:168-177.
22. Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW. Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials. 2012;33:5967-5982.
23. Sandegren L. Selection of antibiotic resistance at very low antibiotic concentrations. Ups J Med Sci. 2014;119:103-107.
24. Müller A, Grein F, Otto A, Gries K, Orlov D, Zarubaev V, Girard M, Sher X, Shamova O, Roemer T, François P, Becher D, Schneider T, Sahl HG. Differential daptomycin resistance development in *Staphylococcus aureus* strains with active and mutated gra regulatory systems. Int J Med Microbiol. 2018;308:335-348.
25. Mishra NN, Rubio A, Nast CC, Bayer AS. Differential adaptations of methicillin-resistant *Staphylococcus aureus* to serial *in vitro* passage in daptomycin: Evolution of daptomycin resistance and role of membrane carotenoid content and fluidity. Int J Microbiol. 2012;2012:683450.
26. Lahiri SD, Alm RA. Identification of non-PBP2a resistance mechanisms in *Staphylococcus aureus* after serial passage with ceftaroline: Involvement of other PBPs. J Antimicrob Chemother. 2016;71:3050-3057.
27. Kwiecinski JM, Jacobsson G, Horswill AR, Josefsson E, Jin T. Biofilm formation by *Staphylococcus aureus* clinical isolates correlates with the infection type. Infect Dis (Lond). 2019;51:446-451.
28. Götz F. *Staphylococcus* and biofilms. Mol Microbiol. 2002;43:1367-1378.
29. Rachid S, Ohlsen K, Witte W, Hacker J, Ziebuhr W. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming Staphylococcus *epidermidis*. Antimicrob Agents Chemother. 2000;44:3357-3363.
30. Rossatto FC, Pinto JB, Costa GA, Frazzon AP. *In vitro* biofilm formation ability of *Staphylococci* under different growth conditions. Int J Appl Microbiol Biotechnol Res. 2017;5:2:12-19.
31. Odenholt I. Pharmacodynamic effects of subinhibitory antibiotic concentrations. Int J Antimicrob Agents. 2001;17:1-8.
32. Silva V, Almeida L, Gaio V, Cerca N, Manageiro V, Caniça M, Capelo JL, Igrejas G, Poeta P. Biofilm formation of multidrug-resistant MRSA strains isolated from different types of human infections. Pathogens. 2021;10:970.
33. Kadkhoda H, Ghalavand Z, Nikmanesh B, Kodori M, Houri H, Taghizadeh Maleki D, Karimi Bavandpour A, Eslami G. Characterization of biofilm formation and virulence factors of *Staphylococcus aureus* isolates from paediatric patients in Tehran, Iran. Iran J Basic Med Sci. 2020;23:691-698.

34. Cramton SE, Gerke C, Schnell NF, Nichols WW, Götz F. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect Immun. 1999;67:5427-5433.

35. Arciola CR, Campoccia D, Ravaioli S, Montanaro L. Polysaccharide intercellular adhesin in biofilm: Structural and regulatory aspects. Front Cell Infect Microbiol. 2015;5:7.

36. Crosby HA, Tiwari N, Kwiecinski JM, Xu Z, Dykstra A, Jenul C, Fuentes EJ, Horswill AR. The *Staphylococcus aureus* ArlRS two-component system regulates virulence factor expression through MgrA. Mol Microbiol. 2020;113:103-122.

37. Toledo-Arana A, Merino N, Vergara-Irigaray M, Débarbouillé M, Penadés JR, Lasa I. *Staphylococcus aureus* develops an alternative, ica-independent biofilm in the absence of the arlRS two-component system. J Bacteriol. 2005;187:5318-5329.

38. Mirani ZA, Jamil N. Effect of sub-lethal doses of vancomycin and oxacillin on biofilm formation by vancomycin intermediate resistant *Staphylococcus aureus*. J Basic Microbiol. 2011;51:191-195.

39. Haddadin RN, Saleh S, Al-Adham IS, Buultjens TE, Collier PJ. The effect of subminimal inhibitory concentrations of antibiotics on virulence factors expressed by *Staphylococcus aureus* biofilms. J Appl Microbiol. 2010;108:1281-1291.

40. Subrt N, Mesak LR, Davies J. Modulation of virulence gene expression by cell wall active antibiotics in *Staphylococcus aureus*. J Antimicrob Chemother. 2011;66:979-984.

41. Sritharadol R, Hamada M, Kimura S, Ishii Y, Srichana T, Tateda K. Mupirocin at subinhibitory concentrations induces biofilm formation in *Staphylococcus aureus*. Microb Drug Resist. 2018;24:1249-1258.

42. Lázaro-Díez M, Remuzgo-Martínez S, Rodríguez-Mirones C, Acosta F, Icardo JM, Martínez-Martínez L, Ramos-Vivas J. Effects of subinhibitory concentrations of ceftaroline on methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms. PLoS One. 2016;11:e0147569.

43. Kaplan JB, Izano EA, Gopal P, Karwacki MT, Kim S, Bose JL, Bayles KW, Horswill AR. Low levels of β-lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. mBio. 2012;3:e00198-12.