RESEARCH ARTICLE / ARAŞTIRMA

DOI: 10.4274/mjima.galenos.2022.2021.14
Mediterr J Infect Microb Antimicrob 2022;11:14
Erişim: http://dx.doi.org/10.4274/mjima.galenos.2022.2021.14

The Relationship between Biofilm Production and Antimicrobial Resistance in Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* Isolates: *In vitro* Evaluation

Metisilin Duyarlı ve Metisilin Dirençli *Staphylococcus aureus* İzolatlarında Antimikrobiyal Direnç ve Biofilm Üretimi Arasındaki İlişkinin *In vitro* Değerlendirilmesi

Bahise Çağla TAŞKIN DALGIÇ[1], Gülgün YENİŞEHİRLİ[2], Barış OTLU[3], Elif Seren TANRIVERDİ[3], Aydan YENİŞEHİRLİ[4], Yunus BULUT[2]

[1]*Turhal State Hospital, Medical Microbiology Laboratory, Tokat, Turkey*
[2]*Tokat Gaziosmanpaşa University Faculty of Medicine, Department of Medical Microbiology, Tokat, Turkey*
[3]*İnönü University Faculty of Medicine, Department of Medical Microbiology, Malatya, Turkey*
[4]*Tokat Gaziosmanpaşa University Faculty of Medicine, Department of Medical Pharmacology, Tokat, Turkey*

Abstract

Introduction: The biofilm formation ability plays an important role in the pathogenesis of *Staphylococcus aureus* infections. This study aimed to investigate the biofilm production ability of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolates and evaluate the relationship between their antimicrobial resistance profile and biofilm formation ability.
Materials and Methods: A total of 50 MRSA and 50 MSSA isolates were examined. The antimicrobial susceptibility testing of isolates was performed using the disk diffusion method. The broth microdilution method was used to determine the minimum inhibitor concentrations (MICs) of vancomycin and teicoplanin. The biofilm formation ability of isolates was tested on Congo Red Agar. The presence of *icaA*, *icaD*, *IS256*, and *eno* genes was investigated by polymerase chain reaction.
Results: Both MRSA and MSSA isolates were found susceptible to vancomycin, teicoplanin, chloramphenicol, and linezolid. Two MRSA and 2 MSSA isolates were determined as heterogeneous vancomycin-intermediate *S. aureus*. No significant difference was observed between the biofilm formation ability of MRSA and MSSA isolates. The *eno* and *icaD* genes were detected in 100% of both MSSA and MRSA isolates. The *icaA* gen was detected in all MRSA and 49 MSSA isolates. The *IS256* was detected in 35 of the 50 MRSA isolates. None of the MSSA isolates were positive for the *IS256*. The amikacin, gentamicin, ciprofloxacin, levofloxacin, rifampin, clindamycin, and tetracycline resistance rates in *IS256*- positive MRSA isolates were significantly higher than those *IS256*-negative MRSA isolates. The mean MIC values of vancomycin and teicoplanin in *IS256*-positive MRSA isolates were significantly higher than those in *IS256*-negative MRSA isolates.
Conclusion: This study revealed that the presence of the *IS256* sequence was correlated with antimicrobial resistance, especially MRSA isolates.
Keywords: Methicillin-resistant *S. aureus*, methicillin-sensitive *S. aureus*, antimicrobial susceptibility, biofilm formation

Öz

Giriş: *Staphylococcus aureus* infeksiyonlarının patogenezinde biofilm oluşturma yeteneklerinin önemli rolü vardır. Bu çalışmanın amacı metisilin duyarlı *Staphylococcus aureus* (MSSA) ve metisilin dirençli *Staphylococcus aureus* (MRSA) izolatlarının biofilm oluşturma kapasitelerinin araştırılması ve antimicrobial direnç profilleri ile ilişkilerinin değerlendirilmesidir.
Gereç ve Yöntem: Çalışmaya 50 MRSA ve 50 MSSA izolatı dahil edildi. İzolatların antimikrobiyal duyarlılıkları disk difüzyon yöntemi ile test edildi. İzolatların vankomisin ve teikoplanin için minimum inhibitör konsantrasyonları (MİK) sıvı mikrodilüsyon yöntemiyle biofilm oluşturma kapasiteleri Kongo Red Agar besiyeri kullanılarak değerlendirildi. *icaA*, *icaD*, *IS256* and *eno* gen varlığı polimeraz zincir reaksiyonu yöntemi ile araştırıldı.

Cite this article as: Taşkın Dalgıç BÇ, Yenişehirli G, Otlu B, Tanrıverdi ES, Yenişehirli A, Bulut Y. The Relationship between Biofilm Production and Antimicrobial Resistance in Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* Isolates: *In vitro* Evaluation. Mediterr J Infect Microb Antimicrob. 2022;11:14.

Address for Correspondence/Yazışma Adresi: Gülgün Yenişehirli MD, Tokat Gaziosmanpaşa University Faculty of Medicine, Department of Medical Microbiology, Tokat, Turkey
Phone: +90 356 212 95 00/7209 **E-mail:** gulgun.yenisehirli@gop.edu.tr **ORCID ID:** orcid.org/0000-0001-7030-0752
Received/Geliş Tarihi: 22.10.2021 **Accepted/Kabul Tarihi:** 01.01.2022

Published: 17 January 2022

Öz

Bulgular: Çalışmaya dahil edilen tüm MRSA ve MSSA izolatları vankomisin, teikoplanin, kloramfenikol ve linezolide duyarlı bulundu. İki MRSA ve 2 MSSA izolatının heterojen vankomisin orta dirençli *S. aureus* (hVISA) olduğu belirlendi. Biofilm oluşturma kapasiteleri bakımından MRSA ve MSSA izolatları arasında istatistiksel olarak anlamlı fark bulunmadı. MSSA ve MRSA izolatlarının tamamında, *eno* ve *icaD* genlerinin varlığı saptandı. *icaA* geni ise tüm MRSA ve 49 MSSA izolatlarında gözlendi. *IS256* geni 50 MRSA izolatının 35'inde saptanırken, MSSA izolatlarının hiçbirinde *IS256* geni varlığı tespit edilmedi. *IS256* pozitif MRSA izolatlarında *IS256* negatif MRSA izolatlarına göre; amikasin, gentamisin, siprofloksasin, levofloksasin, rifampin, klindamisin ve tetrasiklin direnç oranları anlamlı olarak yüksek bulundu. Benzer şekilde vankomisin ve teikoplanin ortalama MİK değerleri *IS256* pozitif MRSA izolatlarında *IS256* negatif MRSA izolatlarına göre anlamlı olarak yüksek olduğu gözlendi.
Sonuç: Bu çalışma *IS256* gen varlığının antimikrobiyal dirençle özellikle de metisilin direnci ile ilişkili olduğunu göstermiştir.
Anahtar Kelimeler: Metisilin dirençli *Staphylococcus aureus*, metisilin duyarlı *Staphylococcus aureus*, antimikrobiyal duyarlılık, biofilm oluşumu

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is accepted as the most common resistant pathogen that leads to both community and hospital-acquired infections worldwide. Most MRSA isolates are not only resistant to beta-lactams, but also other antimicrobial agents, such as macrolides, tetracyclines, fluoroquinolones, aminoglycosides, etc[1]. Limited options are available for the treatment of infections caused by these resistant isolates. The glycopeptide antibiotics, especially vancomycin, were used for the treatment of serious infections caused by MRSA isolates[2].

S. aureus strains with vancomycin minimum inhibitory concentration (MIC) of >2 µg/ml is accepted as non-susceptible according to the European Committee on Antimicrobial Sensitivity Testing (EUCAST) criteria[3]. The Clinical Laboratory Standards Institute (CLSI) guidelines define vancomycin breakpoints for *S. aureus* strains as follows: susceptible at a vancomycin MIC of 2 µg/ml, intermediate susceptible at 4-8 µg/ml, and resistant at 16 µg/ml[4]. A recent global report has documented the prevalence of vancomycin-resistant *S. aureus* (VRSA), vancomycin-intermediate *S. aureus* (VISA), and heterogeneous VISA (hVISA) has been significantly increasing worldwide[5]. The hVISA phenotype contains VISA subpopulations with high levels of vancomycin MICs. An infection caused by hVISA is usually correlated with vancomycin treatment failure[2].

Moreover, another reason for the treatment failure in MRSA infections is the biofilm formation ability of MRSA isolates. Biofilm plays an important role in the pathogenesis of MRSA infections. The biofilm formation ability of *S. aureus* isolates is correlated with many serious chronic infections, such as osteomyelitis, urinary tract infections, catheter-related infections, and endocarditis. Methicillin-resistant *Staphylococcus aureus* isolates can colonize and produce biofilm matrix on implanted medical devices. This biofilm is mainly composed of a specific polysaccharide antigen called polysaccharide intercellular antigen (PIA). The *icaADBC* (*intercellular adhesionADBC*) operon in *S. aureus* isolates is responsible for PIA synthesis[6]. The *icaADBC* operon contains *icaA*, *icaD*, *icaB*, and *icaC* genes. Thus, the presence of these genes in *S. aureus* isolates is directly linked with the biofilm formation ability. The *eno* gene encodes the α-enolase in *S. aureus* isolates. This enzyme is responsible for laminin-binding and biofilm formation activation[7]. The presence of insertion sequence element IS256 has been also associated with biofilm formation in staphylococci[8]. The insertion or excision of *IS256* into the intercellular adhesion (*ica*) gene locus changes the biofilm phases of *S. aureus*[9]. The biofilm matrix supplies a physical barrier against the entrance of the antimicrobial agents and the host immune system[10]. Infection associated with a biofilm that is formed by MRSA is an important medical problem because of its limited therapeutic strategies[6].

This study aimed to investigate the biofilm production ability of MRSA and MSSA isolates and evaluate the relationship between their antimicrobial resistance profile and biofilm formation ability.

Materials and Methods

MRSA and MSSA Isolates

A total of 50 MRSA and 50 MSSA isolates were included in this study. All isolates were randomly selected from the culture collection of the Bacteriology Laboratory of our hospital. The MRSA and MSSA isolate distribution by clinical specimens are shown in Table 1. The identification of isolates was conducted using the automated VITEK2® system (bioMérieux, France). Gram staining, catalase, coagulase, and the production of DNase were simultaneously tested to confirm the identity of isolates.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing to a penicillin (1 IU), tetracycline (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), linezolid (10 µg), erythromycin (15 µg), rifampin (5 µg), gentamicin (10 µg), amikacin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), ceftaroline (5 µg), and trimethoprim-

sulfamethoxazole (1.25/23.75 µg) (Bioanalyse, Turkey) was performed using Kirby-Bauer disk diffusion method according to the EUCAST criteria[3]. Methicillin resistance was determined with cefoxitin disk (30 µg) (Bioanalyse, Turkey) following the EUCAST (2019) guidelines[3]. Inducible and constitutive macrolide-lincosamide-streptogramin B ($iMLS_B$ and $cMLS_B$) resistance was tested using the double-disk test.

The reference broth microdilution method was used to determine the MIC of vancomycin and teicoplanin. Vancomycin hydrochloride (Sigma-Aldrich, USA) and teicoplanin (Carbosynth Ltd, UK) was dissolved in sterile water and used to prepare stock solutions. Both vancomycin and teicoplanin susceptibilities were tested at concentrations from 0.125 µg/mL to 32 µg/ml. All samples were tested in duplicate. The EUCAST criteria were used to interpret the test results[3]. *S. aureus* ATCC 29213 was used as a quality control strain.

Teicoplanin Agar Screening Method

All MSSA and MRSA isolates were screened for hVISA. Mueller Hinton agar (Conda, Spain) plates containing 5 mg/L teicoplanins (Carbosynth Ltd, UK) were prepared and used according to the EUCAST recommendations to detect hVISA[3]. After inoculating 10 µl of bacterial suspension adjusted to 2 McFarland on the agar plate surfaces, plates were incubated at 35 °C for 48 h and read. *S. aureus* ATCC 700698 (Mu3) (hVISA), *S. aureus* ATCC 700699 (Mu50) (VISA), and *S. aureus* ATCC 29213 (vancomycin susceptible *S. aureus*) were used as control strains.

Modified Population Analysis Profile-area Under the Curve Method

The population analysis profile-area under the curve (PAP-AUC) was performed as previously described[11]. The bacterial suspension was inoculated onto Brain-Heart Infusion agar plates (Conda, Spain) containing 0, 0.5, 1, 2, 4, and 8 µg/ml of vancomycin. After 24 h of incubation at 35 °C, colony growth was counted as $\log_{10}$ CFU/ml. The logarithmic count of each isolate was plotted against the vancomycin concentrations on the graph. This graph was used to calculate the AUC. The AUC ratio was calculated by dividing the AUC of the test strain by the AUC of the reference Mu3 strain. The PAP-AUC ratio was interpreted as follows: 0.90 as susceptible, 0.90-1.3 as hVISA, and 1.3 as VISA[11].

Table 1. The distribution of MRSA and MSSA isolates by clinical specimens

Sample	MRSA (n=50)	MSSA (n=50)	Total (n=100)
Nasal	1	0	1
Sputum	9	2	11
Endotracheal aspirate	7	1	8
Bronchoalveolar lavage	0	1	1
Wound	23	27	50
Urine	1	4	5
Blood	9	15	24
Total	50	50	100

MSSA: Methicillin-sensitive *S. aureus*, MRSA: Methicillin-resistant *S. aureus*

Phenotypic Analysis of Biofilm Formation

The phenotypic analysis of biofilm formation of the MSSA and MRSA isolates was tested on Congo Red Agar (CRA) medium as previously described[12]. The CRA medium was prepared by adding sucrose (Isolab, Turkey) (50 g/L), agar (10 g/L), and Congo Red stain (CDH, India) (0.8 g/L) to the brain-heart infusion broth (Conda, Spain) (37 g/L). After 48 h of incubation at 37 °C, bacterial colony morphology was evaluated. Black colonies on CRA medium were defined as biofilm producer, pink as non-producer, and burgundy as borderline[13]. *S. epidermidis* ATCC 35984 and *S. epidermidis* ATCC 12228 strains were used as positive and negative controls, respectively.

Detection of *icaA, icaD, IS256,* and *eno* Genes

Genomic DNA was extracted using the high pure polymerase chain reaction (PCR) template preparation kit (Roche, Germany) according to the manufacturer's recommendations. The primer sets described by Vancraeynest et al.[14] and Montanaro et al.[15] were used for the amplification of *eno* and *IS256* genes, respectively. The multiplex PCR method was used to detect the *eno* and *IS256* genes. The initial step (94 °C for 3 min) was followed by 35 cycles with denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 1 min, and a final step at 72 °C for 10 min. The presence of *eno* and *IS256* gene regions was confirmed in our fiftieth MRSA isolate by sequencing analysis. Then we used this strain as a positive control.

The molecular detection of *icaA* and *icaD* genes was performed as previously described by Vasudevan et al.[16] with slight modifications. PCR conditions were the following: an initial temperature of 94 °C for 3 min, followed by 35 cycles with denaturation at 94 °C for 30 sec, annealing at 57.8 °C for 30 sec, elongation at 72 °C for 1 min, and a final step at 72 °C for 10 min. The fifth MRSA isolates in our study confirmed the existence of *icaA* and *icaD* gene regions by sequencing analysis and used as a positive control. *S. epidermidis* ATCC 12228 was used as a negative control.

After the amplification, the amplicons were run on a 1.5% agarose gel (GeneOn, Germany), containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich, USA) at 80 V for 2.5 h and visualized using a transilluminator (Sigma-Aldrich, USA).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9 demo version statistical software. The association between

the categorical variables was determined using the chi-square (χ^2) test and Student's t-test. P values of <0.05 were considered significant.

Results

Antimicrobial Drug Resistance Profiles

All isolates were found susceptible to vancomycin, teicoplanin, chloramphenicol, and linezolid. The antimicrobial resistance rates of penicillin, tetracycline, ciprofloxacin, levofloxacin, linezolid, erythromycin, rifampin, gentamicin, amikacin, chloramphenicol, clindamycin, ceftaroline, and trimethoprim-sulfamethoxazole against MRSA and MSSA isolates are shown in Table 2. The significant differences in antimicrobial-resistant rates to penicillin, tetracycline, ciprofloxacin, levofloxacin, erythromycin, rifampin, gentamicin, amikacin, clindamycin, and ceftaroline were detected between MRSA and MSSA isolates. iMLSB and cMLSB were detected in 29% and 3% of all isolates, respectively. The MIC ranges, MIC_{50}, and MIC_{90} values of vancomycin and teicoplanin for MRSA and MSSA isolates are shown in Table 3. All tested MRSA and MSSA isolates were found to be susceptible to vancomycin and teicoplanin.

Detection of hVISA

Eleven MRSA and seven MSSA isolates were found to be hVISA according to the teicoplanin agar screening method. The PAP-AUC analysis was performed to confirm the teicoplanin agar screening method results. The PAP-AUC analysis confirmed 2 of 11 MRSA and 2 of 7 MSSA isolates as hVISA. Among the 4 hVISA isolates, 3 (1 MRSA and 2 MSSA) were isolated from the wound, whereas 1 (1 MRSA) from the sputum.

Table 2. The antimicrobial resistance rates of penicillin, tetracycline, ciprofloxacin, levofloxacin, linezolid, erythromycin, rifampicin, gentamicin, amikacin, chloramphenicol, clindamycin, ceftaroline, and trimethoprim-sulfamethoxazole against MRSA and MSSA isolates

Antimicrobial agent	Resistance n (%)		p
	MSSA n=50	MRSA n=50	
Penicillin	37 (74)	50 (100)	<0.001
Tetracycline	8 (16)	43 (86)	<0.0001
Ciprofloxacin	4 (8)	40 (80)	<0.0001
Levofloxacin	2 (4)	40 (80)	<0.0001
Linezolid	0 (0)	0 (0)	-
Erythromycin	8 (16)	30 (60)	<0.0001
Rifampin	0 (0)	36 (72)	<0.0001
Gentamicin	3 (6)	32 (64)	<0.0001
Amikacin	3 (6)	32 (64)	<0.0001
Chloramphenicol	0 (0)	0 (0)	-
Clindamycin	5 (10)	27 (54)	<0.0001
Ceftaroline	0 (0)	20 (40)	<0.0001
Trimethoprim-sulfamethoxazole	1 (2)	5 (10)	>0.05

MSSA: Methicillin-sensitive *S. aureus*, MRSA: Methicillin-resistant *S. aureus*

Production of Biofilm

All MRSA and 48 MSSA isolates were determined as biofilm producers on the CRA medium. No significant difference was observed between the biofilm formation ability of MRSA and MSSA isolates (p>0.05). Non-biofilm producer 2 MSSA isolates were found to be susceptible to all antimicrobials except penicillin. Among the biofilm producer isolates, 1 of the MRSA and 4 of the MSSA isolates was defined as borderline phenotypically. Two of the 4 borderline biofilm producer MSSA isolates were susceptible to all tested antimicrobial agents, whereas the other 2 isolates were resistant only to penicillin.

Detection of *icaA, icaD, IS256*, and *eno* Genes

The *eno* and *icaD* genes were detected in 100% of both MSSA and MRSA isolates. The *icaA* gen was detected in all MRSA and 49 MSSA isolates. The agarose gel electrophoresis of *icaA* and *icaD* genes of MRSA isolates is shown in Figure 1.

The prevalence of the *eno*, *icaD*, *icaA,* and *IS256* genes among the biofilm producer *S. aureus* isolates were 100%, 100%, 99%, and 36%, respectively (Figures 1, 2).

The *eno, icaD,* and *icaA* genes were observed in 2 of the non-biofilm producer MSSA isolates. The *IS256* was detected in 35 of the 50 MRSA isolates. None of the MSSA isolates were positive for *IS256* (Figure 2). All *IS256*-positive MRSA isolates were also identified as biofilm producers. The resistance rates to penicillin, tetracycline, ciprofloxacin, levofloxacin, erythromycin, rifampin, gentamicin, amikacin, clindamycin, and ceftaroline of *IS256*-positive *S. aureus* isolates were significantly higher than those *IS256* negative *S. aureus* isolates (p<0.02, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.0001, and p<0.0002, respectively) (Table 4). Additionally, the amikacin, gentamicin, ciprofloxacin, levofloxacin, rifampin, clindamycin, and tetracycline resistance rates in *IS256*-positive MRSA isolates

Table 3. The MIC ranges, MIC_{50}, and MIC_{100} values of vancomycin and teicoplanin

Isolates	Antimicrobial agents	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC range (µg/ml)
S. aureus	Vancomycin	1	1	0.25-1
	Teicoplanin	0.5	2	0.125-2
MRSA	Vancomycin	1	1	0.5-1
	Teicoplanin	2	2	0.25-2
MSSA	Vancomycin	0.5	1	0.25-1
	Teicoplanin	0.5	0.5	0.125-1

MSSA: Methicillin-sensitive *S. aureus*, MRSA: Methicillin-resistant *S. aureus*, MIC: Minimum inhibitory concentration

were significantly higher than those *IS256*-negative MRSA isolates (p<0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.002, and p<0.0005, respectively) (Table 5). The mean MIC values of vancomycin and teicoplanin in *IS256*-positive *S. aureus* isolates were significantly higher than those in *IS256*-negative *S. aureus* isolates (p<0.0001, p<0.0001, respectively) (Table 6).

The mean MIC values of vancomycin and teicoplanin in *IS256*-positive MRSA isolates were significantly higher than those in *IS256*- negative MRSA isolates (p<0.002, p<0.0001, respectively) (Table 6).

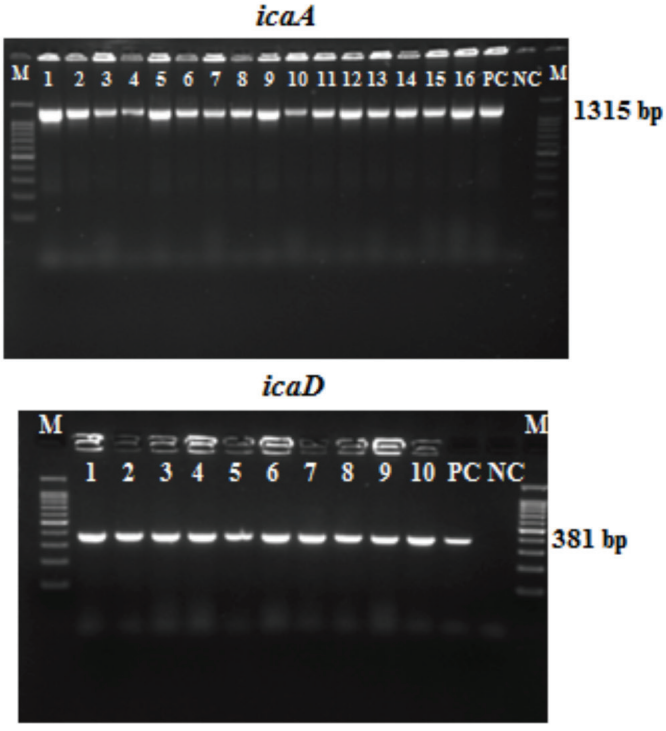

Figure 1. Agarose gel-electrophoresis of *icaA* and *icaD* genes of MRSA isolates

M: 100 bp DNA ladder, PC: Positive control, NC: Negative control

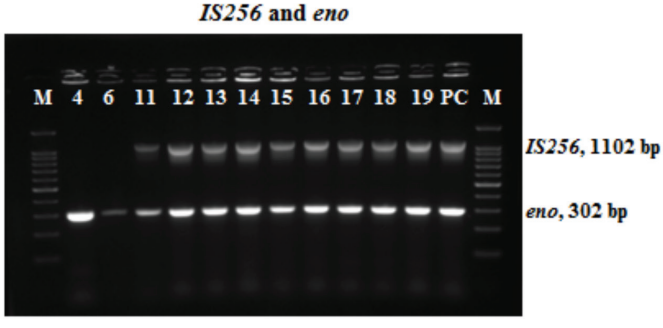

Figure 2. Agarose gel-electrophoresis of *eno* and *IS256* genes of MRSA isolates

M: 100 bp DNA ladder, PC: Positive control, NC: Negative control

Discussion

This study revealed that none of the *S. aureus* isolates were resistant to vancomycin, teicoplanin, chloramphenicol, and linezolid. All MSSA isolates were also susceptible to ceftaroline and rifampin. Methicillin-resistant *Staphylococcus aureus* isolates were more resistant to tested antimicrobial agents except for trimethoprim-sulfamethoxazole. Similar results were reported by previous researchers[1,17-19].

Table 4. The antimicrobial resistance profiles of *IS256* (+) and *IS256* (–) *S. aureus* isolates

Antimicrobial agent	*S. aureus* (n/%)		p
	IS256 (+) n=35	*IS256* (–) n=65	
Penicillin	35 (100)	52 (80)	<0.02
Tetracycline	34 (97.1)	17 (26.2)	<0.0001
Ciprofloxacin	34 (97.1)	10 (15.4)	<0.0001
Levofloxacin	34 (97.1)	10 (15.4)	<0.0001
Linezolid	0 (0)	0 (0)	-
Erythromycin	24 (68.6)	14 (21.5)	<0.0001
Rifampin	34 (97.1)	3 (4.6)	<0.0001
Gentamicin	29 (82.9)	6 (9.2)	<0.0001
Amikacin	29 (82.9)	6 (9.2)	<0.0001
Chloramphenicol	0 (0)	0 (0)	-
Clindamycin	24 (68.5)	6 (9.2)	<0.0001
Ceftaroline	14 (40)	6 (9.2)	<0.0002
Trimethoprim-sulfamethoxazole	1 (2.9)	5 (7.7)	>0.05

Table 5. The antimicrobial resistance profiles of *IS256* (+) and *IS256* (–) MRSA isolates

Antimicrobial agent	MRSA (n/%)		p
	IS256 (+) n=35	*IS256* (–) n=15	
Penicillin	35 (100)	15 (100)	>0.05
Tetracycline	34 (97.1)	9 (60)	<0.0005
Ciprofloxacin	34 (97.1)	6 (40)	<0.0001
Levofloxacin	34 (97.1)	6 (40)	<0.0001
Linezolid	0 (0)	0 (0)	-
Erythromycin	24 (68.6)	6 (40)	>0.05
Rifampin	34 (97.1)	3 (20)	<0.0001
Gentamicin	29 (82.9)	3 (20)	<0.0001
Amikacin	29 (82.9)	3 (20)	<0.0001
Chloramphenicol	0 (0)	0 (0)	-
Clindamycin	24 (68.5)	3 (20)	<0.002
Ceftaroline	14 (40)	6 (9.2)	<0.0002
Trimethoprim-sulfamethoxazole	1 (2.9)	4 (26.7)	<0.01

MRSA: Methicillin-resistant *S. aureus*

Table 6. Vancomycin and teicoplanin mean MICs of *IS256* (+) and *IS256* (–) isolates

Antimicrobial agent	Mean MIC (µg/ml)					
	S. aureus		p	MRSA		p
	IS256 (+)	*IS256* (–)		*IS256* (+)	*IS256* (–)	
Vancomycin	0.91	0.66	<0.0001	0.91	0.7	<0.002
Teicoplanin	1.75	0.50	<0.0001	1.75	0.63	<0.0001

The results of the broth microdilution method yielded that the vancomycin MIC range was 0.5-1 µg/ml in all *S. aureus* isolates. The SENTRY Antimicrobial Surveillance Program has examined antimicrobial susceptibility of 191,460 clinical *S. aureus* isolates between 1997 and 2016 and revealed only 1 *S. aureus* isolates showing the MIC value of 8 µg/ml among all the isolates[1]. According to the teicoplanin agar screening method results, 11 of the 50 MRSA and 7 of the 50 MSSA isolates were detected as suspicious hVISA isolates; however, only 4 (2 of the 11 MRSA and 2 of the 7 MSSA) (4%) were hVISA according to modified PAP-AUC reference method. The prevalence of hVISA varies with geographical region. Results from the recent meta-analysis research have indicated that the prevalence of hVISA was 4.7% in Asia, 4.4% in Europe, and 5.2% in America. The same report also documented that the prevalence of hVISA has been increasing, especially in Asia and America[5].

PAP-AUC method is accepted as a gold standard method for detecting hVISA strains; however, it is a laborious procedure for routine application. The EUCAST has recommended a macro gradient test, glycopeptide resistance detection gradient test, and teicoplanin agar screening method for hVISA screening[3]. Alternatively, for hVISA screening, brain-heart infusion agar supplemented with 6 µg/ml of vancomycin (BHIA6V) has been recommended by the CLSI[4]. Wootton et al.[20] have revealed the teicoplanin agar screening method and macro gradient test had high sensitivity and specificity compared with BHIA6V, which compared the screening methods for hVISA. They also emphasized that the teicoplanin agar screening method was relatively low in cost compared with the macro gradient test. Therefore, we preferred the teicoplanin agar screening method for hVISA screening in our study. However, the percentage of false-positive results in our study was higher than the study by Wootton et al.[20]. Additionally, our study noticed that 2 of the 4 hVISA strain were MSSA. Previous studies on hVISA were mainly focused on MRSA strains although decreased sensitivity to glycopeptides was also observed in MSSA isolates. Hu et al.[21] revealed that 10% and 0.5% MSSA isolates were hVISA and VISA, respectively. Therefore, we have suggested that MSSA isolates must be tested for glycopeptide susceptibility to determine the true prevalence and revise the treatment strategies.

In this study, 98% of *S. aureus* isolates have been identified as biofilm producers. Similar results were reported by previous researchers[22, 23]. Among the *S. aureus* isolates, only 2 of the MSSA isolates were detected as non-biofilm producers. These 2 MSSA isolates were resistant to only penicillin among the tested antimicrobial agents. No significant difference was detected between the methicillin resistance and biofilm formation ability in *S. aureus* isolates (p>0.05). This result is in concordance with the findings reported by Smith et al.[24]. Contrary to our results, higher rates of multidrug resistance and methicillin resistance among the biofilm producer *S. aureus* isolates were reported by Belbase et al.[25].

In the present study, the genes responsible for biofilm production were analyzed using the PCR method. The *eno* and *icaD* genes were found in all MRSA and MSSA isolates. The *eno* gene encodes the laminin-binding protein responsible for the *S. aureus* adherence to the extracellular matrix[7]. This matrix is mainly composed of PIA. The PIA is produced by the *N*-acetylglucosaminyltranferase enzyme synthesized by the expression of the *icaADBC* operon, especially the *icaA* gene. Co-expression of *icaA* and *icaD* genes causes a substantially increased activity of acetylglucosaminyltranferase[26]. Our study detected *icaD* and *icaA* genes in 2 of the non-biofilm producer MSSA isolates. This may be explained by insufficient phenotypic methods to identify biofilm producer isolates or different levels of expression of the genes required for biofilm production. Beloin et al.[27] revealed that the nature of the isolates also plays role in the expression levels.

Kwon et al.[28] revealed that the prevalence of *IS256* (insertion sequence) was correlated with biofilm formation. In our study, *IS256* was present in 36% of the biofilm producer *S. aureus* isolates. All *IS256*-positive *S. aureus* isolates were methicillin-resistant. The increased resistance rates were detected for penicillin, tetracycline, ciprofloxacin, levofloxacin, erythromycin, rifampin, gentamicin, amikacin, clindamycin, and ceftaroline in *IS256*-positive *S. aureus* isolates. Kwon et al.[28] documented that the prevalence of *IS256* was associated with multidrug resistance in *S. aureus*. Lyon et al.[29] revealed that *IS256* had an important role in increasing aminoglycoside resistance in *S. aureus* isolates. Our study revealed higher antimicrobial resistance rates among *IS256*-positive MRSA isolates compared with *IS256*-negative MRSA isolates, except for penicillin and erythromycin. Additionally, we observed the mean MICs of vancomycin and teicoplanin for *IS256*-positive

MRSA isolates as significantly higher than those for *IS256*-negative isolates. Maki et al.[30] demonstrated that the insertion of *IS256* into the *tcaA* gene region has caused the teicoplanin resistance in *S. aureus*.

In this study, the *IS256* sequence was not detected in any of the MSSA isolates. Our results were in concordance with Kwon et al.[28]. They have suggested that the transposition or rearrangement of *IS256* in the chromosome may contribute to the methicillin resistance of *S. aureus*[28].

The main limitation of this study was the low sampling size. Another limitation was the limited number of the tested biofilm-related genes.

Conclusion

All MRSA and MSSA isolates were found susceptible to vancomycin, teicoplanin, chloramphenicol, and linezolid. Two MRSA and two MSSA isolates were determined as hVISA. No significant difference was observed between the biofilm formation ability of MRSA and MSSA isolates. The *eno* and *icaD* genes were detected in all isolates. Our results revealed that the presence of the *IS256* sequence was correlated with antimicrobial resistance, especially methicillin resistance in *S. aureus* isolates.

Ethics

Ethics Committee Approval and Informed Consent: Our study is an in vitro study it does not require ethics committee approval and patient consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Laboratory and Medical Practices: B.Ç.T.D., G.Y., B.O., E.S.T., Concept: B.Ç.T.D., G.Y., B.O., Y.B., Design: G.Y., B.O., Y.B., Data Collection or Processing: B.Ç.T.D., G.Y., E.S.T., A.Y., Analysis or Interpretation: A.Y., Literature Search: B.Ç.T.D., G.Y., Writing: G.Y., A.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by the Tokat Gaziosmanpaşa University Research Fund, project no: 2020/51.

References

1. Diekema DJ, Pfaller MA, Shortridge D, Zervos M, Jones RN. Twenty-year trends in antimicrobial susceptibilities among Staphylococcus aureus from the SENTRY antimicrobial surveillance program. Open Forum Infect Dis. 2019;6(Suppl 1):S47-S53.
2. Tenover FC, Biddle JW, Lancaster MV. Increasing resistance to vancomycin and other glycopeptides in Staphylococcus aureus. Emerg Infect Dis. 2001;7:327-32.
3. European Committee on Antimicrobial Susceptibility Testing, Breakpoint tables for interpretation of MICs and zone diameters. European Society of Clinical Microbiology and Infectious Diseases Basel, 2019.
4. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. twenty-seventh informational supplement M100. Clinical and Laboratory Standards Institute. Perform Stand. Antimicrob. susceptibility testing. 27th ed. CLSI Suppl. M100-S27. Wayne PA: Clin Lab Stand Inst, 2017.
5. Shariati A, Dadashi M, Moghadam MT, van Belkum A, Yaslianifard S, Darban-Sarokhalil D. Global prevalence and distribution of vancomycin resistant, vancomycin intermediate and heterogeneously vancomycin intermediate Staphylococcus aureus clinical isolates: a systematic review and meta-analysis. Sci Rep. 2020;10:12689
6. Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME. Staphylococcus aureus biofilms: properties, regulation, and roles in human disease. Virulence 2011;2:445-59.
7. Carneiro CR, Postol E, Nomizo R, Reis LF, Brentani RR. Identification of enolase as a laminin-binding protein on the surface of Staphylococcus aureus. Microbes Infect. 2004;6:604-8.
8. Maki H, Murakami K. Formation of potent promoters of the mutant Ilm gene by IS256 transposition in methicillin-resistant Staphylococcus aureus. J Bacteriol. 1997;179:6944-8.
9. Kiem S, Oh WS, Peck KR, Lee NY, Lee JY, Song JH, Hwang ES, Kim EC, Cha CY, Choe KW. Phase variation of biofilm formation in Staphylococcus aureus by IS256 insertion and its impact on the capacity adhering to polyurethane surface. J Korean Med Sci. 2004;19:779-82.
10. Craft KM, Nguyen JM, Berg LJ, Townsend SD. Methicillin-resistant Staphylococcus aureus (MRSA): antibiotic-resistance and the biofilm phenotype. Medchemcomm. 2019;10:1231-41.
11. Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. J Antimicrob Chemother. 2001;47:399-403.
12. Freeman D, Falkiner F, Keane C. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989;42:872-4.
13. Różańska A, Chmielarczyk A, Romaniszyn D, Bulanda M, Walkowicz M, Osuch P, Knych T. Antibiotic resistance, ability to form biofilm and susceptibility to copper alloys of selected staphylococcal strains isolated from touch surfaces in Polish hospital wards. Antimicrob Resist Infect Control. 2017;6:80.
14. Vancraeynest D, Hermans K, Haesebrouck F. Genotypic and phenotypic screening of high and low virulence Staphylococcus aureus isolates from rabbits for biofilm formation and MSCRAMMs. Vet Microbiol. 2004;103:241-7.
15. Montanaro L, Campoccia D, Pirini V, Ravaioli S, Otto M, Arciola CR. Antibiotic multiresistance strictly associated with IS256 and ica genes in Staphylococcus epidermidis strains from implant orthopedic infections. J Biomed Mater Res Part A. 2007;83:813-8.
16. Vasudevan P, Nair MK, Annamalai T, Venkitanarayanan KS. Phenotypic and genotypic characterization of bovine mastitis isolates of Staphylococcus aureus for biofilm formation. Vet Microbiol. 2003;92:179-85.
17. Akpaka PE, Roberts R, Monecke S. Molecular characterization of antimicrobial resistance genes against Staphylococcus aureus isolates from Trinidad and Tobago. J Infect Public Health. 2017;10:316-23.
18. Horváth A, Dobay O, Sahin-Tóth J, Juhász E, Pongrácz J, Iván M, Fazakas E, Kristóf K. Characterisation of antibiotic resistance, virulence, clonality and mortality in MRSA and MSSA bloodstream infections at a tertiary-level hospital in Hungary: a 6-year retrospective study. Ann Clin Microbiol Antimicrob. 2020;19:17
19. Pignataro D, Foglia F, Della Rocca MT, Melardo C, Santella B, Folliero V, Shinde S, Pafundi PC, Sasso FC, Iovene MR, Galdiero M, Boccia G, Franci G, Finamore

E, Galdiero M. Methicillin-resistant Staphylococcus aureus: epidemiology and antimicrobial susceptibility experiences from the University Hospital 'Luigi Vanvitelli' of Naples. Pathog Glob Health. 2020;114:451-6.

20. Wootton M, MacGowan AP, Walsh TR, Howe RA. A multicenter study evaluating the current strategies for isolating Staphylococcus aureus strains with reduced susceptibility to glycopeptides. J Clin Microbiol. 2007;45:329-32.
21. Hu J, Ma XX, Tian Y, Pang L, Cui LZ, Shang H. Reduced vancomycin susceptibility found in methicillin-resistant and methicillin-sensitive Staphylococcus aureus clinical isolates in Northeast China. PLoS One. 2013;8:e73300.
22. Batista IR, Prates ACL, Santos BS, Araújo JCC, Bonfim YCO, Pimenta Rodrigues MV, Morceli G, Polettini J, Cavalleri AC, Winkelstroter LK, Pereira VC. Determination of antimicrobial susceptibility and biofilm production in Staphylococcus aureus isolated from white coats of health university students. Ann Clin Microbiol Antimicrob. 2019;18:37.
23. Piechota M, Kot B, Frankowska-Maciejewska A, Grużewska A, Woźniak-Kosek A. Biofilm formation by methicillin-resistant and methicillin-sensitive Staphylococcus aureus strains from hospitalized patients in Poland. Biomed Res Int. 2018;2018:4657396.
24. Smith K, Perez A, Ramage G, Lappin D, Gemmell CG, Lang. Biofilm formation by Scottish clinical isolates of Staphylococcus aureus. J Med Microbiol. 2008;57:1018-23.
25. Belbase A, Pant ND, Nepal K, Neupane B, Baidhya R, Baidya R, Lekhak B. Antibiotic resistance and biofilm production among the strains of Staphylococcus aureus isolated from pus/wound swab samples in a tertiary care hospital in Nepal. Ann Clin Microbiol Antimicrob. 2017;16:15.
26. Gerke C, Kraft A, Süssmuth R, Schweitzer O, Götz F. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the Staphylococcus epidermidis polysaccharide intercellular adhesin. J Biol Chem. 1998;17:273:18586-93.
27. Beloin C, Michaelis K, Lindner K, Landini P, Hacker J, Ghigo JM, Dobrindt. The transcriptional antiterminator RfaH represses biofilm formation in Escherichia coli. J Bacteriol. 2006;188:1316-31.
28. Kwon AS, Park GC, Ryu SY, Lim DH, Lim DY, Choi CH, Park Y, Lim Y. Higher biofilm formation in multidrug-resistant clinical isolates of Staphylococcus aureus. Int J of Antimicrob Agent. 2008;32:68-72.
29. Lyon BR, Gillespie MT, Skurray RA. Detection and characterization of IS256, an insertion sequence in Staphylococcus aureus. J Gen Microbiol. 1987;133:3031-8.
30. Maki H, McCallum N, Bischoff M, Wada A, Berger-Bächi B. tca A inactivation increases glycopeptide resistance in Staphylococcus aureus. Antimicrob Agent Chemother. 2004;48:1953-9.