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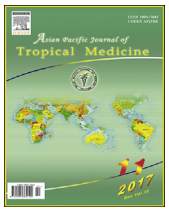

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Antimicrobial resistance and underlying mechanisms in *Staphylococcus aureus* isolates

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ABSTRACT

Objective: To investigate the antimicrobial susceptibility of 97 clinical *Staphylococcus aureus (S. aureus)* strains against 14 antimicrobials and corresponding resistance mechanisms.

Methods: The antimicrobial susceptibility of the isolates was determined using a disk diffusion method and antimicrobial resistance genes were screened by polymerase chain reaction. Mutations responsible for ciprofloxacin and rifampicin resistance were investigated by polymerase chain reaction and DNA sequencing.

Results: All isolates were found to be susceptible to vancomycin. Various rates of resistance to penicillin (83.5%), ampicillin (77.3%), erythromycin (63.9%), tetracycline (16.5%), amoxicillin/clavulanic acid (16.5%), ciprofloxacin (15.5%), trimethoprim/sulfamethoxazole (15.5%), oxacillin (13.4%), fusidic acid (12.4%), rifampin (6.2%), clindamycin (6.2%), gentamicin (6.2%) and mupirocin (5.2%) were determined. In addition, different combinations of resistance genes were identified among resistant isolates. Ciprofloxacin resistant isolates had mutations in codon 84 (Ser84Leu) and 106 (Gly106Asp) in the *gyr*A gene. Mutations in *grl*A were mostly related to Ser80Phe substitution. Leu466Ser mutation in the *rpo*B gene was detected in all rifampin resistant isolates. All methicillin resistant *S. aureus* isolates were SCC*mec* type V.

Conclusions: In conclusion, it was determined that the isolates were resistant to different classes of antimicrobials at varying rates and resistance was mediated by different genetic mechanisms. Therefore, continuous monitoring of resistance in *S. aureus* strains is necessary to control their resistance for clinically important antimicrobials.

1. Introduction

Staphylococcus aureus (S. aureus) is one of the most common human pathogens causing different sequelae of infections in both genders and all age groups [1]. The emergence and spread of antimicrobial resistant *S. aureus* isolates, particularly methicillin resistant *S. aureus* (MRSA), constitutes a global challenge for the treatment of infections caused by these bacteria [2]. Infections caused by resistant bacteria extend the duration of stay at the hospital, increase the cost of health care services, and most importantly, lead to a significant increase in both morbidity and mortality rates [3].

S. aureus develops resistance to antimicrobials by different mechanisms. These mechanisms include limiting uptake of the drug, modification of the drug target, enzymatic inactivation of the drug, and active efflux of the drug. Depending on the antimicrobial involved, the bacteria may use one or several of these resistance mechanisms. In particular, the localization of resistance genes on transferable genetic elements such as plasmids and transposons facilitates horizontal transfer of resistance between bacteria [4].

Rapid and accurate determination of the antimicrobial resistance phenotype and resistance mechanisms has great importance, not only for treatment options but also public health risks [5]. In the present study, the susceptibilities of 97 *S. aureus* strains from various clinical specimens in Hatay, Turkey were tested by the disk diffusion method and the underlying molecular mechanisms were investigated.

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2. Materials and methods

2.1. Bacterial isolates

A total of 97 *S. aureus* isolates obtained from various clinical specimens such as wound swabs (63, 64.90%), urine (17, 17.50%), blood cultures (3, 3.10%), sputum (4, 4.12%) and other samples (1, 1.03%) between January and July 2011 at the Microbiology Laboratory of Antakya Public Hospital (Hatay) were used in the study. Antimicrobial susceptibility and molecular analysis of the isolates were performed at Department of Microbiology, Faculty of Veterinary Medicine, Mustafa Kemal University. Isolation and identification of the isolates involved standard biochemical tests such as colony morphology, Gram staining, catalase reaction and tube coagulase test. All isolates were confirmed using species-specific polymerase chain reaction [6].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility of the isolates was determined by the disk diffusion method according to Clinical Laboratory Standards Institute guidelines (CLSI) [7]. The following antimicrobial disks were used: vancomycin (VA, 30 μg), penicillin (P, 10 U), ampicillin (AM, 10 μg), erythromycin (E, 15 μg), tetracycline (TE, 30 μg), amoxycillin-clavulanic acid (AMC, 20 μg/10 μg), ciprofloxacin (CIP, 5 μg), trimethoprim-sulfamethoxazole (SXT, 1.25 μg/23.75 μg), oxacillin (OXA, 1 μg), fusidic acid (FA, 10 μg), rifampicin (RA, 5 μg), clindamycin (DA, 2 μg), gentamicin (CN, 10 μg), and mupirocin (MUP, 5 μg). Since there are no standardized CLSI breakpoints for mupirocin and fusidic acid, the results of these antibiotics were interpreted as described previously [8,9]. *S. aureus* ATCC 29213 was also used as a quality control. The isolates resistant to at least three different antimicrobial classes were accepted as multidrug resistant.

2.3. Oxacillin disk diffusion test

The oxacillin susceptibility test was performed according to CLSI [7] recommendations using a 1 μg oxacillin disk. *S. aureus* ATCC 25923 (susceptible) and *S. aureus* ATCC 43300 (resistant) were used as control strains.

2.4. Determination of antimicrobial resistance genes and mutations

Bacterial DNA samples were prepared according to the method as previously described [10]. Antimicrobial resistance genes for macrolide (*erm*A, *erm*B, *erm*C, *msr*A, *mph*C) [11–13], lincosamide (*lnu*A) [14], aminoglycoside (*aac(6')-aph(2'')*, *aph(3')-IIIa*, *ant(4)-Ia*) [15], tetracycline (*tet*K, *tet*M) [16], mupirocin (*ileS*-2) [17] and fusidic acid (*fus*B, *fus*C) [18] were researched as previously reported.

In order to determine the mutations, *grl*A (469 bp), *gyr*A (398 bp) and *rpo*B (1052 bp) genes were amplified, and the nucleotide sequences of the amplified products were subsequently determined commercially (Macrogen, Netherlands). Mutations were determined by comparison with the published sequences (for *grlA* gene of *S. aureus* D67074 and D67075, for *gyr*A gene of *S. aureus* D10489, for *rpo*B gene of *S. aureus* CAA45512) [19–22].

2.5. SCCmec typing

SCC*mec* types of *mec*A positive isolates were determined using the method and primers described by Kondo *et al.* [23]. SCC*mec* type assignment of the isolates was carried out according to *ccr* and *mec* gene complexes.

3. Results

3.1. Antimicrobial susceptibility testing

Of 97 *S. aureus* isolates, 9 (9.3%) were susceptible to all the antimicrobials tested. None of the isolates were resistant to vancomycin. Various rates of resistance were observed to penicillin (83.5%), ampicillin (77.3%), erythromycin (63.9%), amoxicillin/clavulanic acid (16.5%), tetracycline (16.5%), ciprofloxacin (15.5%) and trimethoprim/sulfamethoxazole (15.5%), followed by oxacillin (13.4%), fusidic acid (12.4%), rifampin (6.2%), clindamycin (6.2%), gentamicin (6.2%) and mupirocin (5.2%). Multidrug resistant was detected among 28 (28.7%) isolates and multidrug resistant to 8, 7, 6, 5, 4 and 3 antimicrobials was detected in 3 (10.7%), 1 (3.6%), 3 (10.7%), 6 (21.4%), 7 (25.0%) and 8 (28.6%) isolates, respectively. Resistance phenotypes determined among *S. aureus* isolates are given in Table 1.

Table 1
Resistance phenotypes determined among *S. aureus* isolates.

Phenotype	No of isolates
OXA, P, AMP, AMC, MUP, CIP, FA, SXT, TE, CN, E	1
OXA, P, AMP, AMC, RA, CIP, FA, SXT, DA, TE, E	1
OXA, P, AMP, AMC, MUP, FA, SXT, DA, CN, E	1
OXA, P, AMP, AMC, CIP, SXT, TE, CN, E	1
OXA, P, AMP, AMC, RA, CIP, FA, SXT, E	1
P, AMP, AMC, RA, CIP, DA, TE, E	1
P, AMP, AMC, MUP, CIP, DA, TE	1
P, MUP, CIP, SXT, DA, TE, CN, E	1
P, AMP, AMC, RA, CIP, TE, E	1
P, AMP, AMC, CIP, DA, TE, E	1
OXA, P, AMP, FA, TE, E	1
OXA, P, AMC, FA, SXT, E	1
P, AMP, AMC, CIP, TE, E	1
P, AMP, AMC, CIP, TE, E	1
P, AMP, MUP, FA, SXT, E	1
OXA, P, AMP, SXT, CN, E	1
P, AMP, AMC, CIP, E	2
FA, SXT, TE, CN, E	1
P, AMP, CIP, TE, E	1
OXA, P, RA, SXT, E	1
OXA, P, AMP, E	1
P, AMP, AMC, E	1
P, AMP, AMC, E	1
P, AMP, E, TE	2
P, AMP, FA, E	2
P, AMP, FA	1
P, AMP, CIP	1
P, AMP,TE	1
OXA, P, E	1
P, SXT, E	1
P, AMP, E	28
P, AMP	20
FA, TE	1
SXT, E	2
E	3
Pan-susceptible	9

3.2. Distribution of resistance genes

Among erythromycin-resistant isolates ($n = 62$), *erm*C, *msr*A, *mph*C, *erm*A and *erm*B were detected in 57 (91.9%), 20 (32.3%), 17 (27.4%), 12 (19.4%) and 4 (6.5%) isolates, respectively. Of the tetracycline resistant isolates ($n = 16$), 9 (56.3%) harbored *tet*M and 7 had *tet*K (43.7%). Out of 6 gentamicin resistant isolates, 3 (50.0%) carried both *aac(6')/aph(2")* and *aph(3')-IIIa,* 2 (33.3%) *aac(6')/aph(2")* and 1 (16.7%)*aph(3')-IIIa.* The *bla*Z and *mec*A gene were detected in 91.4% (74/81) and 84.6% (11/13) of *S. aureus* isolates, respectively. The *lnu*A gene was present in five (83.6%) of six of the isolates. Out of 12 fusidic acid resistant *S. aureus* isolates, 6 contained *fus*B, 2 had *fus*C and 2 had both *fus*B and *fus*C genes. The *ileS-2* gene, which is related to mupirocin resistance, was detected in one of five mupirocin resistant isolates.

3.3. Quinolone and rifampicin gene mutations

Among 15 ciprofloxacin-resistant *S. aureus* isolates, 6 different combination of mutations were observed in *grl*A and *gyr*A genes. Only one isolate had single mutation of *gyr*A (Ser84Leu). The most common combination was *grl*A mutation of Ser80Phe and *gyr*A mutation of Ser84Leu, which was detected in 7 isolates. The *grl*A mutation of Ser80Phe and *gyr*A mutations of Ser84Leu and Gly106Asp were observed in 3 isolates. Two isolates had Ser80-Phe in *grl*A and Ser84Leu - Gly106Asp in *gyr*A gene. One isolate had mutations in *grl*A (Ser80Phe-Glu84Asp) and in *gyr*A (Ser84Leu) and *grl*A mutation of Ser80Phe in combination with *gyr*A mutation of Glu88Lys-Gly106Asp was detected in one isolate. Leu466Ser point mutation in the *rpo*B gene was the only substitution detected among rifampicin isolates.

3.4. SCCmec typing

All MRSA isolates presented only SCC*mec* type V.

4. Discussion

Antimicrobial resistance has become an important public health problem in Turkey as it has spread all over the world, limiting the use of antimicrobial drugs in the treatment of infectious diseases. In this study, antimicrobial resistance among *S. aureus* isolates from various clinical materials was investigated by both phenotypic and genotypic tests.

In our study, *S. aureus* isolates showed higher rates of resistance to penicillin (83.5%) and ampicillin (77.3%). This result is not surprising because β-lactams are widely prescribed agents for the treatment of infectious diseases in Turkey. Previous studies carried out in Turkey revealed higher prevalence rates of β-lactam resistance. In a study conducted in Hatay [24], the prevalence of penicillin resistance among *S. aureus* strains was reported as 92.8%. Rağbetli *et al.* [25] evaluated antimicrobial resistance in *S. aureus* isolates according to years from 2009 to 2014 and determined resistance to penicillin G as 100% during those years. The erythromycin-resistance rate (63.9%) was consistent with the findings of a resistance rate of 60.4% in Hatay [24], but inconsistent with previous studies conducted by Çalık *et al.* [26] in Kars and Rağbetli *et al.* [25] in Van. Çalık *et al.* [26] reported resistance rates of 38.7% in methicillin sensitive *S. aureus* (MSSA) and 84.3% in MRSA. Rağbetli *et al.* [25] reported a lower prevalence rate (17.7%) among *S. aureus* isolates. The resistance rate to tetracycline (16.5%) was lower than that from the findings (41.0%) of Duran *et al.* [24], but higher than that from the findings (11.0%) of Rağbetli *et al.* [25]. Amoxicillin/clavulanic acid and ciprofloxacin are important antimicrobials for human medicine [27]. Duran *et al.* [24] found that 23.0% of *S. aureus* strains showed resistance to amoxicillin/clavulanic acid and trimethoprim-sulfamethoxazole, 41.0% to ciprofloxacin, and 22.3% to trimethoprim-sulfamethoxazole Çalık *et al.* [26] reported a resistance rate of 35.5% in MSSA and 71.9% in MRSA to ciprofloxacin, and a resistance rate of 32.2% in MSSA and 65.6% in MRSA to trimethoprim-sulfamethoxazole. In contrast to these studies, low level resistance rates for trimethoprim-sulfamethoxazole (6.1%) and for norfloxacin (10.3%) were reported [25]. In our study, resistance rates for amoxicillin/clavulanic acid, ciprofloxacin, and trimethoprim/sulfamethoxazole were determined as 16.5%, 15.5% and 15.5%, respectively. Resistance rate to gentamicin (6.2%) was similar to the findings (6.4%) of Çalık *et al.* [26], but lower than that from the findings (38.1% and 13.0%) of Duran *et al.* [24] and Rağbetli *et al.* [25].

Clindamycin is an alternative drug for the treatment of skin and soft-tissue infections caused by both MSSA and MRSA. Also, this antibiotic is an alternative drug to be used in penicillin-allergic patients. On the other hand, resistance to this antibiotic reduces the efficacy of the drug [28]. In this study, the clindamycin resistance rate was determined as 6.2%. In other studies, the resistance rate was reported as 11.1% in Van [25], 38.1% in Hatay [24], and 25.8% in MSSA and 62.5% in MRSA in Kars [26].

Fusidic acid and mupirocin are topical drugs which are used for the treatment of staphylococcal skin infections. Increased use of these antibiotics has led to the emergence and dissemination of resistant staphylococci [29,30]. In this study, resistance rates of fusidic acid and mupirocin were found to be 12.4% and 5.2%, respectively. Deveci *et al.* [31] investigated fusidic acid resistance phenotypically in 37 *S. aureus*strains from various clinical materials and such resistance was detected in 13.5% of the isolates. Nergiz *et al.* [32] compared fusidic acid resistance rates in MSSA and MRSA strains isolated at an interval of ten years, between 2001 and 2011. Fusidic acid resistance rates for MSSA strains in the years 2001 and 2011 were found to be 4.2% and 5.7%, respectively. However, the rates for MRSA strains were found to be 18.9% and 22.2%, respectively. Yiğit *et al.* [33] reported a resistance rate of mupirocin of 14.2% in MRSA and 4.7% in MRSA isolates, and a resistance rate of fusidic acid of 14.2% in MRSA and 14.3% in MSSA. Oğuzkaya-Artan *et al.* [34] determined fusidic acid resistance in 2 (5.6%) of 36 *S. aureus* strains isolated from the nasal cavities of healthy preschool children, but no resistance against mupirocin. In contrast to these studies, Sareyyupoğlu *et al.* [35] reported a higher prevalence of mupirocin resistance (47%) among clinical *S. aureus* isolates.

Rifampicin has been the focus of attention due to the spread of MRSA infections. However, it cannot be used as a single agent to treat such infections due to the emergence of rapid resistance, even during therapy [36–38]. The use of rifampicin in combination with other agents in treatment seems useful as long as the bacteria are sensitive to combined antibiotics [37,38]. In previous studies, the rifampicin resistance rate was reported as 14.0% in Van [25], 6.5% in MSSA and 80.8% in MRSA in Gaziantep [39], and 5.0% in MSSA and 80.0% in MRSA in Malatya [40]. Similarly, low resistance (6.2%) was found against this agent in the present study.

In previous studies conducted in Turkey, the prevalence of methicillin resistance has been reported ranging from 10.9% to 61.1% among *S. aureus* isolates [24,25,41–44]. In this study, the resistance rate of MRSA was determined as 13.4%.

In the current study, all penicillin-resistant isolates carried the *blaZ* gene. Similarly, Duran *et al.* [24] also reported that all penicillin-resistant *S. aureus* isolates were positive for *blaZ*. Another resistant mechanism is methicillin resistance, which has gained increasing importance in human medicine throughout the world in the last two decades. MRSA showed resistance not only to β-lactams but also to other classes of antimicrobials [45,46]. In the current study, 11 of 13 oxacillin-resistant isolates were positive for the *mec*A (84.6%) gene. In a previous study, Duran *et al.* [24] detected 16.5% of the isolates by the disk diffusion method, whereas 25.9% of the isolates had *mec*A by polymerase chain reaction.

The main resistance mechanism to macrolide in staphylococci involves target-site modification following ribosome methylation via the methylase enzyme encoded by the *erm* gene (erythromycin ribosome methylase) [47]. In the present study, the *erm*C gene was the most frequent gene detected in 91.9% ($n = 57$) of erythromycin resistant isolates and was found in 35 isolates alone, in 20 isolates with *erm*A and in 2 isolates with the *erm*B gene. In contrast to our study, *erm*A was reported as the most common genotype among both erythromycin resistant MSSA and MRSA isolates in Turkey. Duran *et al.* [24] studied 84 phenotypically erythromycin resistant *S. aureus* strains and found that the most frequent genotype was the presence of *erm*A (52.4%), followed by *erm*C (28.6%) and *erm*B (9.5%). Yıldız *et al.* [45] investigated the presence of *erm*A, *erm*B, and *erm*C genes among 225 erythromycin resistant MRSA isolates and found that 48 (21.3%) carried *erm*A, 20 (8.9%) carried *erm*C and 128 (56.9%) harbored both *erm*A and *erm*C. Similarly, Aydeniz Ozansoy *et al.* [48] reported the high prevalence of the *erm*A gene alone or in combination with *erm*C in clinical *S. aureus* isolates.

The most common gentamicin resistance gene was *aac(6')/aph(2'')* (83.3%), which was detected in 2 (33.3%) isolates alone and in 3 (50.0%) isolates with *aph(3')-IIIa*. In previous studies, the *aac-aph* gene was also reported as the most common gene detected among gentamicin resistant isolates. The presence of this gene among gentamicin resistant MRSA isolates was reported as 96.0% by Yıldız *et al.* [45] and 94.1% by Ardıç *et al.* [49]. In another study, Duran *et al.* [24] reported the presence of *aac(6')-aph(2'')*,*aph(3')-IIIa* and *ant(4)-Ia* genes as 47.2%, 32.1% and 20.8% among gentamicin resistant *S. aureus* isolates, respectively.

Tetracycline resistance is mediated mainly by four different mechanisms among staphylococci: active efflux pump, protection of binding site, drug modification and modification of binding site [50]. In this study, *tet*M responsible for ribosomal protection and *tet*K responsible for efflux pump were detected in 56.3% and 43.7% of the tetracycline resistant isolates, respectively. Similarly, Duran *et al.* [24] reported the prevalence of *tet*M and *tet*K as 63.2% and 36.8%, respectively. In another study conducted by Yıldız *et al.* [45], a higher prevalence rate of *tet*M gene was reported as 89.7% among 350 tetracycline resistant MRSA isolates. Wide dissemination of the *tet*M gene is explained by localization of this gene on conjugative transposon *Tn*5801 [51].

In staphylococci, variable levels of quinolone resistance are most commonly attributed to mutations in the QRDRs of *grl*A and *gyr*A caused by single-nucleotide changes [21]. In this study, we observed three types of single-point mutations in the *grl*A genes of 15 ciprofloxacin resistant *S. aureus* isolates (100%). Among these mutations, Ser80Phe mutation was the most common, detected in 14 isolates. In addition, Glu84Asp mutation was detected in 1 isolate together with Ser80Phe mutation. It was reported that Ser84Leu mutation in the *gyr*A gene was sufficient for high levels of quinolone resistance [21]. This mutation was found in nearly all ciprofloxacin resistant *S. aureus* isolates tested, except for 1 isolate. The single-point mutation of Ser84Leu was found in 9 isolates, the double-point mutation of Ser84Leu and Gly106Asp was observed in 5 isolates, and the double-point mutation of Glu88Lys and Gly106Asp was detected in 1 isolate. Although at different rates, all mutations detected in both the *grl*A and *gyr*A gene were similar to those previously reported by Coskun-Ari and Bosgelmez-Tinaz [52].

In the present study, *rpo*B sequence analysis of rifampicin resistant isolates revealed only Leu466Ser amino acid substitution. It has been reported that Leu466Ser led to low rifampicin resistance alone but high rifampicin resistance with other point mutations, especially amino acids at positions 455, 481, and 529 [53,54]. Of 12 fusidic acid-resistant isolates, 10 carried resistance genes, of which 6 had only *fus*B, 2 had both *fus*B and *fus*C, and 2 isolates had *fus*C. Castanheira *et al.* [55] studied large numbers of staphylococci from North America and Australia for fusidic acid resistance. Among 25 fusidic acid resistant isolates (MICs of ≥2 μg/mL), 4 carried *fus*B and 15 carried *fus*C gene while 6 were negative for both genes.

ileS-2 related to high level mupirocin resistance in staphylococci was found in only 1 isolate [17]. This can be attributed to the fact that the susceptibilities of the isolates to mupirocin were determined by the disk diffusion method. CLSI recommended using a mupirocin disk (200 μg) for the screening of high levels of mupirocin resistance [7].

SCC*mec* is one of the molecular techniques which is used to understand the epidemiology and the clonal relationship of MRSA strains, especially when community-acquired MRSA infections occur worldwide [56]. In addition, it has been reported that SCC*mec* type IV or V are associated with community-acquired MRSA infections strains [57]. Similarly, in this study, all MRSA isolates were found to carry SCC*mec* type V.

In conclusion, it has been found that *S. aureus* isolates are resistant to antimicrobials of various classes at different rates and underlying resistance mechanisms as well. Therefore, the results of this study indicate that continuous surveillance is needed to determine the evolution of resistance and mechanisms. Also, prudent use of antimicrobials is important to prevent the emergence and spread of resistant bacteria.

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Conflicts of interest

We declare that we have no conflict of interest.

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