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Phenotypic and Genotypic Analysis of Gentamicin, Penicillin, Methicillin, Vancomycin, Linezolid and Tetracycline Resistance in Clinical Isolates of *Staphylococcus aureus*

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ABSTRACT

In this study, it was aimed to investigate the resistance rates of gentamicin, penicillin, methicillin, vancomycin, linezolid and tetracycline by phenotypic and genotypic methods in *Staphylococcus aureus* isolates and to determine plasmid content. Between the months of January and September in 2015, 100 clinical isolates of *S. aureus* were obtained from different samples such as wound, blood, urine. The automated bacteria identification and antibiotic susceptibility system (BD Phoenix™, Sparks, MD, USA) was used to determine of antibiotic sensitivities. The resistance to methicillin was also investigated by Kirby-Bauer disc diffusion method using a 30 µg cefoxitin disc. The presence of *aac(6')/aph(2")*, *blaZ, mecA, femA, vanA, vanB, cfr, tetK and tetM* genes related to antibiotic resistance was investigated by PCR amplification in all isolates. Plasmid DNAs were isolated by using a Thermo Scientific GeneJET Plasmid Miniprep Kit. The cefoxitin resistance of *S.aureus* isolates, identified according to the results of disk diffusion and automated system, was calculated as 19%. Vancomycin and linezolid resistance were not observed in isolates while gentamicin 2%, penicillin 100%, methicillin 19%, tetracycline 18% resistance were identified using the automated system. According to the results of molecular analysis *aac(6')/aph(2")*, *blaZ, mecA, femA, tetK and tetM* genes frequencies were determined as 2%, 100%, 19%, 100%, 17% and 3% respectively, but *vanA, vanB and cfr* genes were not amplified by PCR. In order to determine the relationship between antibiotic resistance and plasmid presence, plasmids were isolated from identified bacterial isolates. It is found that most of bacterial isolates (79%) contain different numbers plasmids. Rapid and reliable method for antibiotic susceptibility is important to determine the appropriate therapy decision. PCR can be used for confirmation of the results obtained by automated system or could be used as an alternative diagnostic method in the routine diagnosis for rapid, sensitive, and specific detection of MRSA associated antibiotic resistance genes.

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Research Article

Staphylococcus aureus Klinik İzolatlarında Gentamisin, Penisilin, Metisilin, Vankomisin, Linezolid ve Tetrasiklin Direncinin Fenotipik ve Genotipik Olarak Araştırılması

ÖZET

Bu çalışmada, *Staphylococcus aureus* izolatlarında gentamisin, penisilin, metisilin, vankomisin, linezolid ve tetrasiklin direnç oranlarının araştırılması ve plazmid içeriğinin belirlenmesi amaçlanmıştır. 2015 yılı Ocak-Eylül ayları arasında yara, kan, idrar gibi farklı örneklerden 100 tane *S. aureus* izolatı elde edilmiştir. Antibiyotik duyarlılıklarını belirlemek için otomatik bakteri identifikasyon ve antibiyotik duyarlılık sistemi (BD PhoenixTM, Sparks, MD, ABD) kullanıldı. Ayrıca, metisilin direnci 30 µg sefoksitin disk kullanılarak Kirby-Bauer disk difüzyon yöntemi ile de araştırıldı. Antibiyotik direncine bağlı *aac (6 ')/aph (2' ')*, *blaZ, mecA, femA, vanA, vanB, cfr, tetK* ve *tetM* genlerinin varlığı tüm izolatlarda

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Anahtar Kelimeler
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PCR,
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plazmit içeriği,
Staphylococcus aureus

Araştırma Makalesi

PCR amplifikasyonu ile araştırıldı. Plazmid DNA'ları Thermo Scientific GeneJET Plazmid Miniprep Kiti kullanılarak izole edildi. Disk difüzyon ve otomatik sistem sonuçlarına göre belirlenen S.*aureus* izolatlarının sefoksitin direnci %19 olarak belirlendi. Vankomisin ve linezolid direnci izolatlarda görülmezken, otomotize sistem ile %2 gentamisin, %100 penisilin, %19 metisilin, %8 tetrasiklin direnci belirlendi. Moleküler analiz sonuçlarına göre *aac (6')/aph (2'')*, *blaZ, mecA, femA, tetK* ve *tetM* genleri sırasıyla % 2, % 100, % 19, % 100, % 17 ve %3 olarak belirlendi. Fakat *vanA, vanB* ve *cfr* genleri PCR ile amplifiye edilmedi. Antibiyotik direnci ve plazmid varlığı arasındaki ilişkiyi belirlemek için plazmidler, tanımlanmış bakteriyel izolatlardan izole edildi. Bakteriyel izolatların çoğunun (% 79) farklı sayılarda plazmid içerdiği bulundu.
Antibiyotik duyarlılığı için hızlı ve güvenilir bir yöntem, uygun tedavi kararını belirlemek için önemlidir. Otomatik sistem ile elde edilen sonuçların doğrulanması için PCR kullanılabilir veya MRSA ile ilişkili antibiyotik direnç genlerinin hızlı, hassas ve spesifik saptanması için rutin tanıda alternatif bir tanı yöntemi olarak kullanılabilir.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important microbial cause of serious hospital- or society-borne infections that are associated with high morbidity and mortality as well as a rapid resistance development (Salem-Bekhit, 2014). Recent reports have revealed that *S. aureus* has developed resistance against the many classes of antibiotics (Aires de Sousa et al., 2005; Appelbaum, 2007; Matsuo and Komatsuzawa, 2012). The emergence of antibiotic-resistant *S. aureus* isolates evokes difficulty with treatments as well as an increase in the burden on health systems and the need for new antibiotics (Dudhagara et al., 2011). PCR-based molecular techniques are generally preferred for the determination of antibiotic resistance in genes. Therefore, in order to determine the antibiotic resistance of many antibiotic-resistant pathogens, the availability of sensitive and specific methods has become an important tool in clinical diagnosis (Pillai et al., 2012).

The gentamicin resistance of *S. aureus* may be explained by the fact that they have any one of many modification enzymes (Freitas et al., 1999). The most commonly found modification enzyme in Staphylococcus isolates, aminoglycoside modifying enzyme (AME), is a two-functional enzyme AAC(6')/APH(2'') and is encoded by the *aac(6')/aph(2'')* gene (Hauschild et al., 2008).

Methicillin-resistant *S. aureus* (MRSA) is resistant against all penicillin including semi-synthetic penicillinase-resistant penems, carbapenems and cephalosporins. The most crucial mechanism of penicillin is driven by an exogene *mecA*, and these codes are also known as B-lactam-resistant penicillin-binding protein (PBP) codes: PBP-2 (or PBP-2a). Another gene Staphylococcal resistance to penicillin is mediated by *blaZ,* the gene that encodes ß-lactamase (Malachowaet al., 2010; Foster, 2017; Nasution, 2018).

Glycopeptide antibiotics, such as vancomycin and teicoplanin, can inhibit synthesis of the cell wall, which consists of glycosylated non-ribomosomal peptides. In VRSA isolates, an alternative cell wall structure with D-ala-D-lac is found instead of the normal structure of D-ala-D-ala (Gardete et al., 2014). Linezolid resistance mainly occurs through the structural modification of the oxazolidinone binding site of the 50S peptidyl transferase centre. It has been determined that linezolid-resistant isolates possess both *cfr* gene and chromosomal coded mutations (Locke et al., 2014; Boswihi and Udo, 2018).

Tetracycline group antibiotics block protein synthesis by binding to the 30S ribosome, which in turn restricts the connection between aminoacyl-tRNA and the receptive region. The efflux protein code, *tetK*, is an energy-dependent membrane-bound protein that prevents tetracycline from being accumulated in the cells. *tetM*, another gene, encodes a ribosomal protection protein that decreases the affinity of tetracycline (Ullah et al., 2012; Khoramrooz et al., 2017). According to previous studies, tetracycline-resistant MRSA isolates generally possess either *tetA(M)* or *tetA(K)* alone, or both together (Trzcinski et al., 2000; Schmitz et al., 2001; Michalova et al., 2004).

In the treatment of staphylococcal infections, the accurate and rapid diagnosis of antibiotic resistance genes is crucial in preventing the spread of infections. PCR-based molecular methods for the detection of antibiotic resistance genes are frequently preferred. This study aimed to investigate the resistance properties of *S. aureus* isolates gentamicin, penicillin, methicillin,

vancomycin, linezolid and tetracycline by phenotypic and genotypic methods and determine the plasmid content.

MATERIALS and METHODS

Bacterial strains

One hundred *S. aureus* strains were isolated from clinical specimens regardless of their methicillin resistance or sensitivity status. The study was carried out in Kahramanmaras Sutcu Imam University, Medical Microbiology Laboratory of Medicine Faculty and Microbiological Genetics Laboratory in University-Industry-Public Cooperation Development Application and Research Center between January and September of 2015.

The samples were cultured on agar supplemented with sheep blood in a microbiology laboratory and incubated at 37 ° C for 24 hours. After the incubation, the isolates were used in certain morphological and chemical tests: colony morphology, gram staining and catalase reaction. Tube coagulase and weak coagulase isolates were performed by DNase tests and BD Phoenix by using a fully automated system. All isolates identified as *S. aureus* were stored in 5% glycerin containing Tryptone Soya Broth (Thermo Scientific™ CM0129B).

Biochemical analyses

Methicillin resistance was investigated by using 30 µg of cefoxitin disc (BD BBL™ Sensi-Disc™ 231590) on agar at 37 °C for 24 hours incubations according to Mueller-Hinton Agar (BD, ABD). The isolates that produced less than (or equal to) 21 mm of cefoxitin inhibition zone in diameter were considered resistant, while those produced zones that were higher than (or equal to) 22 mm in diameter were labelled as sensitive (CLSI, 2015).

S. aureus ATCC 25923 were used as control strain. For the identification and antibiotic sensitivity tests of the isolates, the BD Phoenix automated microbiology system was also used, according to the manufacturer's instructions.

Molecular analyses

Reaction Conditions: 5 µl Standard *Taq* reaction buffer, 1 µl of forward and reverse primers, 1µl dNTPs, 0.5 µl of *Taq* DNA polymerase (5 U/mL) and 1 µl of DNA template in a total reaction volume of 30 µl. One µl of colonies dissolved in 20 µl of distilled water was used as DNA template. The sequences of the oligonucleotide primers used in the study are presented in Table 1.

PCR reaction was initiated at 94 °C for 5 min for pre-denaturation followed by 30 cycles at 94 °C for 30 seconds, with an annealing temperature for *aac(6')/aph(2")* of 49 °C, for *blaZ* of 50 °C, for *mecA* of 50 °C, for *femA* of 49 °C, for *vanA* of 55 °C, for *vanB* of 58 °C, for *cfr* of 57 °C, for *tetK* of 50 °C and for *tetM* of 54 °C). The elongation step was at 72 °C for 45 seconds, and the final elongation was at 72 °C for 10 min. PCR products were visualized and photographed under UV light after having been run on 1% agarose gel through electrophoresis. Positive and negative controls were used throughout the study.

Calculation of plasmid presence

Plasmid DNAs were isolated by using a Thermo Scientific GeneJET Plasmid Miniprep Kit with the modification of adding 0.50% of lysozyme into 1000 ml.

RESULTS

Clinical characteristics

Staphylococcus isolates were obtained from wounds (53%), blood (20%), the nose (9%), mucus (7%), urine (7%), the throat (3%) and cerebrospinal fluid (1%). Methicillin resistance of the isolates was determined as 19% in total and the total was sourced from 10% wounds, 5% blood, 1% the nose, 2% urine and 1% the throat.

Antibiotic resistance

The both methods showed that isolates have 2% gentamicin resistance (Figure 1), 100% penicillin and *blaZ* resistance (Figure 2).

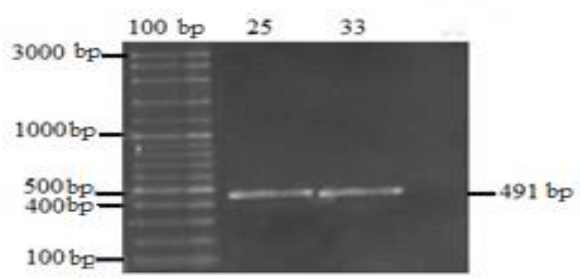

Figure 1. Agarose gel electrophoresis picture of *aac(6')/aph(2")* genes amplified through PCR from the isolates 25 and 33 M: molecular size marker (vivantis VC 100bp Plus DNA ladder NL1405).

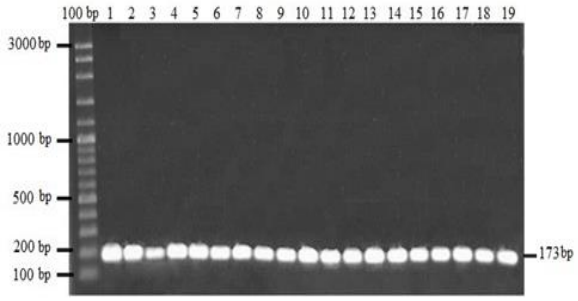

Figure 2. Agarose gel electrophoresis picture of *blaZ* gene amplified by PCR from the isolates 1 to 19. M: molecular size marker (vivantis VC 100bp Plus DNA ladder NL1405).

Table 1. Sequence of oligonucleotide primers to detect antibiotic resistance

Gene	Primers	Antibiotic	Oligonucleotide (5' →3')	Amplicon size (bp)	Reference
aac(6')/ aph(2")	*aac*-F *aac*-R	gentamicin	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491	(Choi et al., 2003)
blaZ	*blaZ*-F *blaZ*-R	penicillin	ACTTCAACACCTGCTGCTTTC' TGACCACTTTTATCAGCAACC	173	(Martineau et al., 2000)
mecA	*mecA*-F *mecA*-R	methicillin	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	532	(Strommenger et al., 2003)
femA	*femA*-F *femA*-R	factor essential for resistance to methicillin resistance	AAAAAAGCACATAACAAGCG GATAAAGAAGAAACCAGCAG	132	(Mehrotra et al., 2000)
vanA	*vanA*-F *vanA*-R	vancomycin	CATGAATAGAATAAAAGTTGCTGCAATA	1032	(Farhadian et al., 2014)
vanB	*vanB*-F *vanB*-R		CCCCTTTAACGCTAATACGATCAA GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	628	
Cfr	*cfr*-F *cfr*-R	linezolid	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	746	(Kehrenberg et al., 2006)
tet(K)	*tetK*-F *tetK*-R	tetracycline	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360	(Strommenger et al., 2003)
tet(M)	*tetM*-F *tetM*-R		AGTGGAGCGATTACAGAA CATATGTCCTGGCGTGTCTA	158	

All *femA* molecular isolates were also found to be resistant (Figure 3). In all three methods (cefoxitin disc diffusion, Phoenix cefoxitin and PCR (*mecA*)) showed that methicillin resistance was the same value (19%) (Figure 4).

No resistance was found for vancomycin (*vanA*, *vanB*) and linezolid (*cfr*) in either of the methods.

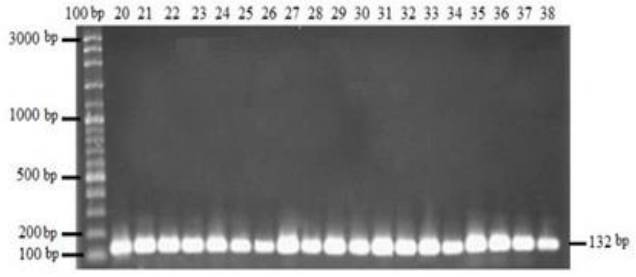

Figure 3. Agarose gel electrophoresis picture of *femA* gene from the isolates 20–38 amplified through PCR. M: molecular size marker (vivantis VC 100bp Plus DNA ladder NL1405).

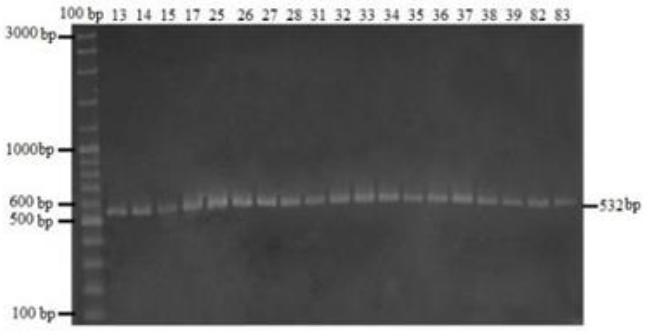

Figure 4. Agarose gel electrophoresispicture of *mecA* gene amplified through PCR from the isolates 17, 25–28, 31–39, 82, 83. M: molecular size marker(vivantis VC 100bp Plus DNA ladder NL1405).

Tetracycline resistance was determined to be phenotypically 18% of the samples. Tetracycline resistance was determined genotypically in 17% *tetK* (Figure 5) 3% *tetM* (Figure 6), 2% (both *tetK* and *tetM*). Of three isolates carrying the *tetM* gene, one did not carry the *tetK* gene. Only one of the isolates that was determined as being tetracycline-resistant phenotypically could not be found molecularly. One isolate that was not determined phenotypically was observed to have the *tetK* gene.

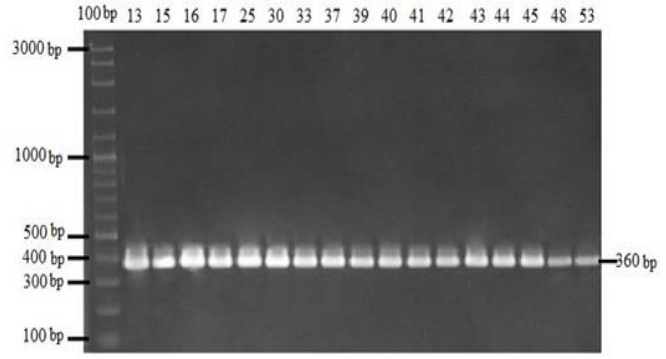

Figure 5. Agarose gel electrophoresis picture of *tetK* gene from the isolates 13, 15–17, 25, 30, 33, 37, 39, 40–45, 48, 53 amplified through PCR. M: molecular size marker (vivantis VC 100bp Plus DNA ladder NL1405).

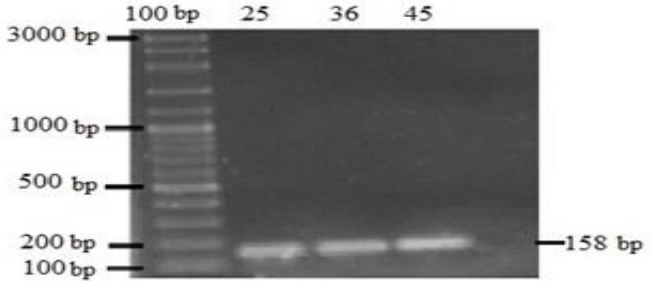

Figure 6. Agarose gel electrophoresis picture of *tetM* from the isolates 25, 36, 45 amplified through PCR. M: molecular size marker (vivantis VC 100bp Plus DNA ladder NL1405).

Presence of the Plasmids

In total, 79% isolates were determined to have plasmids, with 62% carrying one, 13% carrying two, and 4% carrying three plasmids (Figure 7).

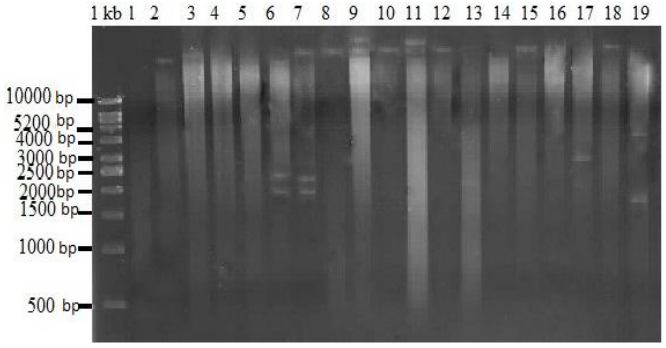

Figure 7. Plasmid contents of the isolates 1-19 on a 0.8% agarose gelelectrophoresis. M: molecular size marker (vivantisVC 1kb DNA ladder NL1409).

DISCUSSION and CONCLUSION

In this study, we used automatized systems to determine the antibiotic sensitivity phenotypically. Conventional PCR was used to identify the resistance genes. The results were compared. In addition, we identified the plasmid content of the isolates.

The aac(6')/aph(2") gene provides the organism with the resistance against to gentamicin. Also it contributes to the resistance against tobramycin and kanamycin and it encodes the AME. The aac(6')/aph(2") gene is also the most commonly found resistance gene in *Staphylococcus*. (Hauschild et al, 2008).

When the relationship between the presence of the *aac (6')/aph (2")* gene and gentamicin resistance was checked, 2% of *S. aureus* isolates were found to possess the gene. Some researchers reported an excellent correlation between the phenotypic method and the genotypic method they employed using the *aac(6')/aph(2")* gene (Vanhoof et al., 1994; Pfaller et al., 1998; Martineau et al., 2000).

Likewise, in our study, the results of the PCR tests were in accordance With the phenotypic antibiotic-resistance determination results. Aminoglycoside resistance mechanisms have become more complicated by the increased useof aminoglycoside. Moreover, there is still no rapid and reliable method to determine aminoglycoside resistance. Therefore, the PCR technique allows the effective treatment to start faster and produces therapeutic success by reducing the empiric treatments with high-range antibiotics (Dessouky et al., 2013).

In Staphylococcus penicillin resistance occurs through two distinct mechanisms. The first and most important one is the production of the beta-lactamase protein encoded by the *blaZ* gene, which inactivates penicillin by hydrolyzing the beta-lactam circle. The second one is the translation of a penicillin-binding protein PBP2a, which is primarily related to human isolates, encoded by the *mecA* gene (Zmantar et al., 2013).

It was determined that out of 100 isolates used in the study, all carried the *blaZ* gene. This result was found to be compatible with some studies (Zmantar et al., 2013; XuanThiep et al., 2014). Some additional studies proved a decent correlation between phenotypical and genotypical properties (Martineau et al., 2000; Gaoet al., 2011), while the results obtained through PCR and Phoenix also showed a correlation with our study.

Increased resistance to methicillin among staphylococci poses great challenges in managing infections (David et al., 2010). There are many phenotypic methods developed to determine MRSA, including disc diffusion, MIC measurements (in broth or by E-test), chromogenic agar, latex agglutination, automated methods. However, these methods are quite slow and their specificities may vary (Datta et al.,

2011). Additionally, due to the heterogen expression of the *mecA* gene, it is not always possible to detect methicillin resistance correctly. Currently, determination of the *mecA* gene through PCR is the gold standard for MRSA identification (Pillai et al., 2012). The fact that PCR-based *mecA* detection is entering routine laboratories is rather important, especially for cases with no response to clinical treatments or for those who require a quick result.

Nineteen percent of the isolates were found to be methicillin resistant by the three methods used in our study. Similarly, some reports have stated that there is a convincing correlation between phenotypes and genotypes (Oliveira et al., 2007; Ekrami et al., 2010). However, there have been some studies in which differences between phenotypic and genotypic methods were observed (Pillai et al., 2012; Zmantar et al., 2013). Some researchers indicated that the differences in their studies might be caused by the amount of inoculation, incubation time, PH and salt concentration of the environment (Pillai et al., 2012; Pournajaf et al., 2014).

MecA is not the only gene that manifests methicillin resistance. It has been shown that certain supportive products, such as *femA/B/X*, are essential in the expression of methicillin resistance in addition to *mecA* (Chikkala et al., 2012). The *femA* gene, which is located away from the *mecA* gene on the chromosome, encodes a 48 kDa protein. Analysis of the *femA* product revealed that this protein is responsible for expressing a high rate of methicillin resistance without affecting PBP-2 production. The importance of *fem* genes in the methicillin resistance mechanism has been proven in *S. aureus* isolates and inactive *femA* due to the lost their resistance against methicillin. On the other hand, this resistance could be restored by the transduction of *fem* genes. Other biochemical analyses proposed that the product of the *femA* gene may have a role in cell wall synthesis (Kobayashi et al., 1994).

Unlike *mecA* gene, *fem* factors can be found in both resistant and sensitive isolates (Henze et al., 1993). In our study, *femA* gene was detected in all isolates that were both sensitive and resistant to methicillin. Our study was found to be compatible with other studies (Braios et al., 2009; Manikandan et al., 2011; Al-Talib et al., 2014).

Vancomycin is a preferred drug for the treatment of MRSA, however this drug may be problematic because of the fact that some *S. aureus* isolates that are moderately sensitive or resistant to MRSA (VISA and VRSA) may occasionally appear (Pe´richon et al., 2009). Phenotypic *vanA* is the most commonly seen type of this resistance, and it manifests a strong endurance against vancomycin and teicoplanin (Fluit et al., 2001).

In some studies on *S. aureus* antibiotic resistance, no vancomycin resistance was found in any of the isolates (Aghazadehet al., 2009; Ogbolu et al., 2015). Although one studies in which VISA was detected through the E test, the *vanA* gene was not observed (Denis et al., 2002). In some studies isolates identified as VRSA with MIC values have been reported to carry *vanA* gene by PCR method. (Thati et al., 2011; Abdul-Hameed et al., 2014; Saadat et al., 2014; Farhadian et al., 2014). In the present study, we have not observed the *vanA* or *vanB* gene in our isolates. Our PCR results nicely correlated with phenotypic antibiotic resistance determination results. Yet, in light of the findings collected through the study, we strongly support the notion that molecular techniques are not only faster but also more reliable in detecting the genes responsible for resistance or reduced sensitivity towards antimicrobial agents than phenotypic methods.

Kehrenberg and Schwartz (2006) reported that none of the 302 chloramphenicol –resistant *S. aureus* strains isolated in humans carry the *cfr* gene but only one of the 188 resistant isolates from animals carried the gene. In their global surveillance reports, Jones et al. (2008) declared an *S. aureus* linezolid resistance < 1. Of 215 clinical MRSA isolates they investigated by PCR. Sierra et al. (2013) found the *cfr* gene in only one specimen.

Linezolid resistance was not detected in any of the isolates in our study.

Surveillance studies have already shown that the resistance mechanism is extremely rare in MRSA. However, it has the potential to spread to humans from animals due to its ability to form horizontal transfer potential. When the phenotypic method is used to detect the linezolid resistance, the suppressed *cfr* gene can be missed.

The most studyies aimed at determining tetracycline resistance. These studyies reported that the *tetK* gene has been found to be the dominant gene (Schmitz et al., 2001; Jones et al., 2006; Ullah et al., 2012). There are also studies that have identified the *tet (M)* gene more frequent (Strommenger et al., 2003; Lim et al., 2012). These studies proved that the *tetK* gene is predominantly more common than other resistance genes such *as tetM, tetL, tetO.* In literature, it has been stated that *S. aureus* tetracycline resistance is manifested by efflux coded in the *tetK* gene or a ribosomal change encoded by *tetM*, and *tetK* can be found more commonly in the organisms (Strommenger et al., 2003).

In our study, the *tetK* was detected as the most common gene, and it was found to be present in 17% of the isolates. The most common tetracycline resistance gene found in studies is *tetK*. Therefore, it should be investigated first in studies.

The *tetM* gene, both *tetM* and *tetK* genes were detected

in 3% and 2% of the isolates respectively in our study. Only one of the 3 isolates carrying the *tetM* gene did not have the *tetK* gene.

Of all the isolates detected as being tetracycline-resistant through the Phoenix automatized system, but one of them could not be determined molecularly. This particular isolate had two different-sized plasmids: one around 4000 bp, the other over 10000 bp. In the isolate whose resistance of tetracycline could not be confirmed molecularly, the resistance might have been developed through plasmid transfer. On the other hand, there was one isolate for which no resistance was detected through the Phoenix automatized system, and this isolate had the *tetK* gene. The reason of this situation may be that the gene is suppressed.

As a result, the laboratory techniques used in order to determine bacteria with multiple resistance must be highly sensitive and specific. More reliable and accurate results can be obtained when conventional and PCR-based methods are used in combination.

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Ethical approval

The use of patient samples for the investigation of *Staphylococcus aureus* antibiotic resistance has been approved by the ethics committee of Kahramanmaras Sutcu Imam University (Kahramanmaras, Turkey, Decision no: 2013/14-03).

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