

Investigation of Methicillin Resistance of *Staphylococcus aureus* Strains Isolated from Various Sources by Different Methods

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Object of this study is to determine methicillin resistance of 150 strains of *Staphylococcus aureus* isolated and identified from various sources by the methods of oxacillin agar screen, broth microdilution and oxacillin disc diffusion, to compare sensitivity and specificity of these methods by making oxacillin agar screen test reference and to determine multi-antibiotic resistance in these strains. 16 (10.7%) of the 150 strains were determined as Methicillin-Resistant *S.aureus* (MRSA) through the methods of agar screen and microdilution while 17 (11.3%) strains were found as the same through the method of disc diffusion. Agar screen and microdilution methods were found as 100% compatible with each other. Sensitivity of the disc diffusion method was calculated as 100% while its specificity was calculated as 99.2%. Antibiotic susceptibility tests conducted by using the disc diffusion method against 10 antibiotics showed that the highest resistances of MRSA and MSSA (Methicillin-Susceptible *S. aureus*) strains were determined as 100% and 93.3% to penicillin, 81.2% and 5.2% to tetracycline, 62.5% and 1.5% to ofloxacin, 56.2% and 0.7% to rifampin respectively while all strains were found susceptible to linezolid and vancomycin. It was seen that multiple-antibiotic resistance in MRSA strains are higher compared with that in MSSA strains. It has been thought that linezolid may be an alternative for vancomycin in curing MRSA infections.

Key words: Oxacillin agar screen, antibiotic resistance, Methicillin resistance, *Staphylococcus aureus*.

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most virulent organisms and is considered as the most important cause of hospital acquired infections and community acquired infections¹. The most infections caused by MRSA include deep skin and soft tissue infections, endocarditis, and bacteremia, as well as a variety of toxin-mediated diseases such as gastroenteritis, staphylococcal scalded-skin syndrome, and toxic shock syndrome². Since most of these bacteria carry multiple antibiotic resistance genes against commonly used antibiotic, they show multiple antibiotic resistance patterns and

thus cause important treatment problems³. In many cases, glycopeptide antibiotics such as vancomycine and teicoplanine are the only therapeutic alternatives. However glycopeptide resistance is expected to become an important problem in future, because of reduced susceptibility of *S. aureus* strains to these group of antibiotics. Rapid and definite identification of MRSA strains is critical to prevent their spread and to start curing with a suitable antibiotic immediately. Also correct identification enables to avoid economic loss caused by unnecessary infection control precautions⁴. If methicillin resistant strains are identified as susceptible by mistake, the cure will be unsuccessful while similarly if methicillin susceptible strains are identified as resistant by mistake, this will cause unnecessary use of glycopeptide antibiotics⁵.

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Almost all of MRSA isolates form a PBP2a called protein binding additional penicillin. PBP2a shows low affinity to beta-lactam antibiotics and encoded by *mec A* gene⁶. *mec A* is an additional gene formed in methicillin resistance Staphylococci and with no allelic equivalent in methicillin susceptible Staphylococci⁷. Genotypic tests identify *mec A* gene through the PCR method; however, molecular methods have not become workable yet for routine use for many laboratories. The accepted phenotypic methods for identification of MRSA strains are the methods of oxacillin disc diffusion, oxacillin agar screen and broth microdilution or E-test methods identifying MIC (Minimum Inhibition Concentration) values⁸.

The aim of the present study is to determine methicillin resistance in *S. aureus* strains isolated from various sources (Clinical samples, food samples and nasal swap samples of students of Biology Department of Konya Selçuk University) by the methods of oxacillin agar screen, disc diffusion and broth microdilution and to determine in-vitro susceptibility to various antibiotics in these strains.

MATERIALS AND METHODS

Bacterial strains

In our study, 150 *S. aureus* strains in total were used. 100 of them are clinical strains isolated and identified by various hospitals in Konya City as *S. aureus* strains. 20 of them were isolated from various unpackaged foods taking place in the market (ice cream, milk, cream cake and chicken) while 30 strains were isolated from noses of students of Biology Department of Konya Selçuk University. The obtained isolates were identified as *S. aureus* with conventional methods (colonial morphology, Gram staining, catalase test, coagulase test, hemolysis test, lecithinase test and mannitol salt agar fermentation). All isolated strains were planted onto Brain Heart Infusion broth and stored at +4 °C by making passages in certain intervals. Antibiotic susceptibility tests were conducted according to recommendations of the Clinical and Laboratory Standards Institute^{9,10}. *S. aureus* ATCC 25923 and ATCC 43300 strains were used as control.

Antibiotic discs under the use

Commercial antibiotic discs were used for

determination of multi-antibiotic resistance of the strains under study. Discs of penicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), erythromycin (15 µg), tetracycline (30 µg), rifampin (5 µg), amoxicillin/clavulanic acid (30 µg), ofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) and linezolid (30 µg) were supplied commercially from Becton Dickinson (USA) in this study.

Disc diffusion method

Bacteria cell density was adjusted to 0.5 of McFarland turbidity according to recommendations of the CLSI (Clinical Laboratory Standards Institute) in 0.9% NaCl in the form of colonial suspension directly from one-night fresh culture on the bloody agar plaques. The suspension was spread over surface of the Mueller-Hinton Agar (with addition of 4% NaCl) with a thickness of 4 mm with the help of a swab and the disc containing 1 µg of oxacillin was placed after the surface had been dried. The plaques were incubated at 35 °C for 24 hrs. The strains, whose inhibition zone diameter is ≤ 10 mm were determined as resistant while those, whose inhibition zone diameter ranges between 11 and 12 mm were determined as intermediate resistant and those having inhibition zone that is ≥ 13 mm were found as susceptible⁹.

Broth microdilution method

Oxacillin's minimum inhibition concentration value was determined in Mueller-Hinton Broth to which 2% NaCl had been added through Broth microdilution method according to recommendations of the CLSI¹⁰. Microplates having sterile U-base wells were used for this experiment. Final concentration of the inoculums inside the wells was adjusted to 5 x 10⁵ colony forming units (cfu)/ml so that oxacillin concentration was 0.125-128 µg/ml. The plates were incubated at 35 °C for 24 hrs. 20 µl of 2,3,5-triphenyltetrazolium chloride (5%) solution was added to the wells at the end of the incubation period and incubation was repeated at 35 °C for 30 minutes. At the end of this time period, final well in which there was no visible proliferation, in other words, which did not show pink-red color, was assessed as MIC^{11,12}. *S. aureus* strains, whose MIC value versus oxacillin was ≤ 2 µg/ml, were accepted as susceptible while those having MIC value is ≥ 4 µg/ml were accepted as resistant¹⁰.

Oxacillin agar screen method

Oxacillin was added to Mueller-Hinton Agar to which 4% NaCl had been added in a way that the concentration would be 6 µg/ml. The strains were transferred on the surface of the Mueller-Hinton Agar from the direct colonial suspension with the help of a swab in the form of lines. The plaques were incubated at 35 °C for 24 hrs. The strains were recognized as resistant to oxacillin even if only a single colony was produced at the end of the incubation¹³.

RESULTS

In our study, 150 *S. aureus* strains in total were used. 100 of them are clinical strains. 20 of them were isolated from various foods while 30 strains were isolated from noses of students of Biology Department of Konya Selçuk University. Considering all strains, 134 (89.3%) of total 150 *S. aureus* strains, which were examined under the agar screen test that was accepted as reference test, were found susceptible to oxacillin while 16 (10.7%) of them were found resistant to it. According to the broth microdilution test, 134 (89.3%) of the strains were found susceptible to oxacillin while 16 (10.7%) of them were found resistant to it, on

the hand, the disc diffusion test showed that 133 (88.7%) of the strains were susceptible to oxacillin while 17 (11.3%) of them were resistant to it (Table 1).

Comparing findings produced through all three methods, findings from the oxacillin agar screen and broth microdilution methods 100% agreed with each other with respect to sensitivity and specificity. The agar screen and microdilution methods showed 134 strains susceptible to oxacillin while 133 strains were susceptible to oxacillin according to the disc diffusion method. Thus, sensitivity of the disc diffusion method with respect to both of the methods was found as 100% while its specificity was found as 99.2% (Table I).

15 (15%) of the 100 *S. aureus* strains, which had been isolated from clinical samples, were found resistant to methicillin while one (3.3%) of 30 *S. aureus* strains, which had been isolated from noses of students, were found resistant to methicillin. Any methicillin-resistant strain was not seen among the food samples. It was determined that 16 (10.7%) of total 150 *S. aureus* strains under study were methicillin-resistant (Table 2).

In the present study, furthermore, resistance of *S. aureus* strains to certain antibiotics was assessed. Table 3, shows number and

Table 1. Comparison of different laboratory methods for the detection of methicillin resistance *Staphylococcus aureus* (n=150)

Method	MRSA	MSSA	Sensitivity %	Specificity %
Oxacillin Agar screen	16 (%10.7)	134 (%89.3)	100	100
Broth Microdilution	16 (%10.7)	134 (%89.3)	100	100
Disc Diffusion	17 (%11.3)	133 (%88.7)	100	99.2

Table 2. Number and percentage of isolated MRSA from different specimens

Specimen	<i>S. aureus</i> (n=150)	MRSA (n=16)	Percentage
Wound swab	44	8	18.2
Throat swab	23	3	13
Nasal swap	3	-	-
Blood	11	-	-
Body fluids	8	1	14.2
Sputum	6	2	33.3
Urine	5	1	20
Nasal swap			
sample of student	30	1	3.3
Food sample	20	-	-

Table 3. Antibiotic susceptibility patterns of *S. aureus* isolates.

Antibiotics	Resistant		Intermediate		Susceptible	
	n	%	n	%	n	%
Tetracycline	20	13.3	1	0.7	129	86
Rifampin	10	6.7	-	-	140	93.3
Amoxicillin/Clavulanic Acid	9	6	-	-	141	94
Trimethoprim/Sulfamethoxazole	3	2	-	-	147	98
Erythromycin	16	10.7	12	8	122	81.3
Vankomycin	-	-	-	-	150	100
Gentamicin	8	5.3	1	0.7	141	94
Linezolid	-	-	-	-	150	100
Ofloxacin	12	8	1	0.7	137	91.3
Penicillin	141	94	-	-	9	6

Table 4. Antibiotic resistance pattern of MRSA, MSSA and all *Staphylococcus aureus* isolates

Antibiotics	All isolates (n=150) Resistant		MRSA (n=16) Resistant		MSSA (n=134) Resistant	
	n	%	n	%	n	%
Tetracycline	20	13.3	13	81.2	7	5.2
Rifampin	10	6.7	9	56.2	1	0.7
Amoxicillin/clavulanic Acid	9	6	9	56.2	-	-
Trimethoprim/Sulfamethoxazole	3	2	3	18.7	-	-
Erythromycin	16	10.7	8	50	8	5.9
Vankomycin	-	-	-	-	-	-
Gentamicin	8	5.3	8	50	-	-
Linezolid	-	-	-	-	-	-
Ofloxacin	12	8	10	62.5	2	1.5
Penicillin	141	94	16	100	125	93.3

percentage of strains, which are resistance, intermediate resistant and susceptible to the used antibiotics, for all strains under study. According to resistance in percentage, highest resistance occurred against the following antibiotics respectively: Penicillin, Tetracycline, Erythromycin while highest susceptibility occurred against Vankomycin, Linezolid, Trimethoprim/Sulfamethoxazole, Amoxicillin/Clavulanic Acid, Gentamicin, Rifampin and Ofloxacin.

Resistance of MRSA and MSSA strains under study was found as 81.2% and 5.2% to tetracycline, 56.2% and 0.7% to rifampin, 56.2% and 0% to amoxicillin/clavulanic acid, 18.7% and 0% to trimethoprim/sulfamethoxazole, 50% and 5.9% to erythromycin, 50% and 0% to gentamicin, 62.5% and 1.5% to ofloxacin, 100% and 93.3% to

penicillin respectively. Resistance to vancomycin and linezolid was not found in MRSA and MSSA strains (Table 4).

DISCUSSION

Rapid and accurate determination of resistance to methicillin and consequently to other beta-lactam antibiotics in staphylococci ensures that the proper cure is started and prevents expensive and toxic-effect cures significantly. However, heterotypic emergence of this resistance and the requirement for optimized test conditions in susceptibility tests reduce reliability of routine tests. Therefore, various methods for determination of resistance to methicillin like dilution, disc diffusion and agar screen in which specific

conditions are met for bacterial cell have been described¹⁴. The selected method for determining resistance should be practicable in routine laboratories, should be reliable and should yield result in a short while⁵.

Because methicillin-resistance is heterogeneous in certain strains, phenotypic methods sometimes fail in identification of MRSA strains. Therefore, identification of *mecA* gene through PCR is accepted as the gold standard in determination of methicillin resistance in staphylococci¹⁵.

Because searching *mecA* gene, which is accepted as the gold standard in determination of methicillin resistance, was not preferred in the present study due to the reasons like high costs, difficulties in practicing it in routine laboratories and impracticability of the method, sensitivity and specificity of the microdilution and disc diffusion tests were determined by making oxacillin agar screen test reference in determination of methicillin resistance in *S. aureus* strains. The agar screen method has been accepted as reference test in some other studies, which has been conducted in our nation, also^{5,13,15,16,17}. Razlighi and Derbentli¹³, found broth microdilution and agar screen methods 100% consistent while sensitivity of the disc diffusion method was found as 100% and specificity as 97.5% in comparison of the disc diffusion method with other two methods. Similarly, in our study, microdilution and agar screen methods were found 100% consistent while sensitivity of the disc diffusion method was found as 100% and specificity as 99.2% in comparison of the disc diffusion method with other two methods. Sumbul *et al.*¹⁶, compared the disc diffusion method with the agar screen method and found sensitivity as 95.3% and 95.2%. In other similar study, Hasbek *et al.*,⁵ made the agar screen method reference and determined sensitivity of the disc diffusion method as 98.2% and specificity as 97.8%. Thus, they recommended the agar screen method. Ögunc *et al.*,¹⁷ made the agar screen method reference and determined sensitivity of the disc diffusion method as 95.5% and specificity as 93.4% and sensitivity of the microdilution method as 95.5% and specificity as 99.1%. Comparison of our results with findings of other researchers showed that our results are reliable from the point of view of sensitivity and specificity. Çetinkol *et al.*,¹⁸,

compared the agar screen, oxacillin disc diffusion, cefoxitin disc diffusion, microdilution and PBP2a latex agglutination methods to determine methicillin resistance and concluded that the agar screen should be used additionally. This comment of the researchers is consistent with our results.

Practicing disc diffusion method is easy and inexpensive; however, its consistency with the agar screen test is low. Specificity and consistency with the agar screen disc for the microdilution method is high. However, practicing it in routine laboratories is difficult and it takes significant time¹⁷. Based on our findings, it may be foreseen that the agar screen method may be used as reference for further studies.

15 (15%) of the 100 *S. aureus* strains, which had been isolated from clinical samples, were found resistant to methicillin while one (3.3%) of 30 *S. aureus* strains, which had been isolated from noses of students, were found resistant to methicillin. Any methicillin-resistant strain was not seen among the food samples (Table 2).

Nasal carriage of hospital personnel was studied in certain studies conducted in our nation and it was reported that MRSA ratio in the isolated strains ranges between 2.4% and 5.6%^{19,20}. This ratio was determined as 3.3% in our study. Askarian *et al.*,²¹ determined nasal MRSA carriage of hospital personnel as 5.3% in their study conducted in a hospital in Iran. Yameen *et al.*,¹ isolated 236 *S. aureus* from noses of hospitalized patients in their study and reported that 59 (25%) of them are MRSA. The researchers reported that MRSA incidence may vary between regions (33-43%) based on the studies conducted in various cities of South Africa^{22,23}.

Celik²⁴, isolated 92 *S. aureus* from 870 food samples in his study and determined methicillin resistance as 1.1%. Dilsiz²⁵, searched methicillin resistance of 148 *S. aureus* strains with different methods and identified methicillin resistance in 24 strains (16.2%). In our study, methicillin resistance was not observed in any of 20 *S. aureus* strains isolated from various foods (Table 2). Similarly, Ünal and Yildirim²⁶ and Spanu *et al.*,²⁷ did not report methicillin-resistant *S. aureus* strain. Normano *et al.*,²⁸ determined resistance to methicillin in 6 (3.75%) of 160 *S. aureus* strains isolated from animal-origin foods. Kumar *et al.*,²⁹ determined resistance to methicillin in 10.2% of *S.*

aureus strains isolated from milk samples collected from animals with mastitis. It is seen that methicillin resistance is at low ratios in *S. aureus* strains isolated from foods. Our finding that methicillin resistance was not observed in any of strains isolated from foods is consistent with findings of other researchers.

In addition to the fact that methicillin resistance may vary between regions, different resistance ratios may be observed in different departments of even the same hospital³⁰. According to the studies conducted in Turkey, methicillin resistance in *S. aureus* strains isolated from various clinical samples varies between 10.9% and 58%³¹⁻³⁴. Among these studies, the lowest MRSA ratio (10.9%) is seen in the study of Aydin *et al.*,³¹. In the present study, 15 (15%) of the 100 *S. aureus* strains, which had been isolated from clinical samples, were found resistant to methicillin. Diekema *et al.*,²³, reported methicillin resistance ratios in *S. aureus* strains collected between 1997 and 1999 as 1.8% in Switzerland, 2% in Netherlands, 4.9% in Germany, 5.7% in Canada, 9.4% in Austria, 19.3% in Spain, 21.4% in France, 25.6% in Belgium, 27.5% in England, 50.5% in Italy, 54.4% in Portugal, 61.1% in Taiwan, 71.6% in Japan and 73.8% in Hong Kong. Average of methicillin resistance ratios reported in the studies conducted in Turkey between 1996 and 1999 was calculated as 47.5% and it was reported that average of the results of the studies conducted between 2000 and 2003 is very close to this ratio (46.6%)⁵. The lowest ratios for methicillin resistance in *S. aureus* strains were reported as 0.5% in Iceland, 0.6% in Denmark, 0.6% in Netherlands, 0.8% in Sweden and 0.9% in Estonia while the highest ratios as 40.9% in Italy, 41.2% in Ireland, 41.5% in England, 43.8% in Malta and 44.4% in Greece in another study conducted across 26 European countries except Turkey in 2004^{36,35}. This ratio was reported as 36% in the studies conducted with clinical samples between 2003 and 2009 in our nation^{30,33,34,37,38}. MRSA ratio isolated from the clinical samples was found low compared the recent averages. Our findings are similar to those found by Aydin *et al.*³¹.

In the present study, resistance of staphylococcus strains to various antibiotics was tested. In the assessment covering all strains, the highest resistance was observed to penicillin (94%). This ratio is 100% in MRSA strains while it

is 93.3% in MSSA strains (Table 4).

It is observed that resistance to penicillin reaches 100% in MRSA strains in various studies conducted in our nation while this ratio is up to 91% in MSSA strains^{31,39}. It has been reported in certain studies conducted at abroad that this ratio varies between 94 and 100% in MRSA strains while it reaches 83% in MSSA strains^{40, 41}.

Tetracycline resistance was found as 81.2% in the isolated MRSA strains while it occurred as 5.2% in MSSA strains. The ratios reported for Turkey varies between 22.2 and 80% in MRSA strains and between 18.2 and 47% in MSSA strains^{5,30,39,42}. It has been reported in certain studies conducted at abroad that tetracycline-resistance ratio varies between 33 and 87.5% in MRSA strains while it takes place between 8.1 and 34.3% in MSSA strains^{2,43}.

Ofloxacin resistance for MRSA and MSSA strains respectively were found by Hasbek *et al.*,⁵ as 72%, 4.6, by Zeyrek *et al.*,⁴⁴ as 43%, 7% by Aridogan *et al.*,³³ as 76, 7%, by Sirmatel *et al.*,⁴⁵ as 38%, 7%, by Hafeez *et al.*,⁴⁶ as 52.68%, 33.18%, by Perwaiz *et al.*,⁴¹ as 92.2%, 2%. This ratio was found as 62.5% and 1.5% in the present study.

Rifampin resistance varies between the conducted studies. Rifampin resistance for MRSA and MSSA strains isolated from clinical samples respectively were found by Duman *et al.*,³⁴ as 61.9%, 15.5%, by Eksi *et al.*,³⁸ as 80.8%, 6.5%, by Kurutepe *et al.*,³⁰ as 31.4%, 16%. This ratio was found as 56.2% and 0.7% in the present study. Rifampin resistance for MRSA is around above 40% in European countries, like our findings while it has been reported as below 10% in America and Canada^{23,47}.

In the present study, the lowest resistance was observed to trimethoprim/sulfamethoxazole in MRSA strains. This is 18.7% for MRSA strains while resistance in MSSA strains was not observed. This ratio was reported for MRSA and MSSA strains respectively by Kurutepe *et al.*,³⁰ as 23.1% and 6.9%, by Gürsoy *et al.*,³⁹ as 15% and 2%, by Duman *et al.*,³⁴ as 20.6% and 1.8%. Resistance ratios in MRSA and MSSA strains have been reported as 26% and 2.6% in America, as 16% and 1.2% in Canada, as 65.4% and 1.4% in South America, as 23% and 2.1% in Europe, as 35.8% and 2.2% in Asian nations⁴⁸. This low resistance to this antibiotic should not be ignored because it may be

an alternative in curing non-complicated staphylococci infections.

In our study, all of *S. aureus* strains were found susceptible to vancomycin. Vancomycin resistance has not been reported in other studies conducted in our nation; however, Gülay *et al.*,⁴⁹ identified intermediate resistant to vancomycin at a ratio of 5.3% in their study for the first time. Vancomycin resistance has not been observed in studies conducted by Kurutepe *et al.*,³⁰, Eksi *et al.*,³⁸, Duman *et al.*,³⁴, Gürsoy *et al.*,³⁹. However, Olowe *et al.*,⁴³, determined resistance at a ratio of 6.3% in MRSA strains and 2.9% in MSSA strains. Hafeez *et al.*,⁴⁶, Perwaiz *et al.*,⁴¹, Brown and Ngeno² and Sanjana *et al.*,⁵⁰ have not reported any vancomycin-resistant strain.

All of *S. aureus* strains under study were found susceptible to linezolid antibiotic and any resistant strain was not observed (Table 4). Similarly, linezolid resistance has not been determined in various studies conducted in our nation⁵¹⁻⁵³.

In the present study, susceptibility of *Staphylococcus aureus* strains to various antibiotics was searched and it was observed that multi-antibiotic resistance in MRSA strains are very higher compared with that in MSSA strains. Variations are seen in antibiotic resistance ratios between regions in which the studies have been conducted. It is noteworthy that any strain resistant to vancomycin and linezolid has not been encountered. Although glycopeptides are the gold standard cure for MRSA infections, in recent years, MRSA/MSSA strains, whose susceptibility to glycopeptides has decreased, have begun to emerge. Linezolid, which has been started to be used in our country in recent times due to potential of MRSA strains for developing resistance to glycopeptides, is a suitable alternative in curing MRSA infections and resistance situation should be monitored. Because the agar screen and microdilution methods yielded results, which have been consistent with each other completely, in the present study, they were assessed as more precious methods than the disc diffusion method was. We believe that the agar screen method may be seen as a useful method in determining methicillin resistance due to the advantages like cost-efficiency, practicability and easiness in the use.

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