

Phenotypical and Genotypical Detection of Methicillin Resistance in *Staphylococcus Aureus* Isolates of Water Buffalo Milk and Dairy Product

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Abstract. The aim of our study was to investigate genotypic and phenotypic characteristics of methicillin resistance in *Staphylococcus aureus* isolates and as well as to determine MIC value. A total of 99 *S. aureus* isolates obtained from water buffalo milk and dairy product was used in the study. Methicillin resistance was measured using oxacillin (1µg) and cefoxitin (30µg) disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI). Oxacillin and cefoxitin MIC value was determined by E test. Genotypic methicillin resistance was evaluated using Polymerase Chain Reaction (PCR) for the *mecA* gene. Out of 99 isolates, 14 (13.8%) were found to be methicillin resistant by oxacillin disc diffusion test, and nine (9%) were found to be resistant with cefoxitin by disc diffusion. Nine (9%) isolates were *mecA* gene positive by PCR.

Keywords: staphylococcus aureus, methicillin resistance, *mecA*, oxacillin, cefoxitin

1. Introduction

Milk and milk products is an important food substance on human nutrition. Water buffalo milk is accepted superior in comparison to cow milk due to containment of less water, having dry matter and fat content in high levels and high calorie content [1]. It is reported that number of milked water buffalo was 51.900 and 51.947 kilogram water buffalo milk was obtained in 2013 in Turkey. Number of water buffalo is 19.360 in Samsun city. This comprises 14 percent of water buffalo population in Turkey [2]. Generally water buffalo milk is consumed as cream and cheese in Turkey. Water buffalo products pose a risk with regard to *S. aureus* during pasteurisation if it is not implemented in appropriate temperature and duration during cheesemaking.

S. aureus is a Gram-positive coccus shaped microorganism. It is located in skin and nose flora of humans and animals [3]. It is reported that foodborne *S. aureus* intoxications occur as a result of consuming different kinds of foods in various regions of world [4], [5]. It is reported that *S. aureus* is a durable microorganism having surviving skills where it is situated at natural flora of humans like nose mucosa and skin [6]. *S. aureus* is an opportunist and commensal pathogen which might cause potential, severe and wide spectrum fatal diseases as well as superficial skin infections [7].

Methicillin-resistant *S. aureus* (MRSA) is a primary epidemiological and clinical problem all over the world in last 10 years [8]. Methicillin-resistant *S. aureus* is the most important reason of hospital infections

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in humans. Treatment becomes difficult due to progressive resistance against antibiotics which is used commonly in treatment. [9]. Oxacillin disc diffusion test is a test used routinely on detecting methicillin resistance. It is reported that cefoxitin disc gives more accurate results on determining methicillin resistance recently. CLSI (Clinical and Laboratory Standards Institute) advises cefoxitin rather than oxacillin on determining *mecA* resistance [8]-[10]. The quality of antimicrobial discs might affect analysis results due to failure on detecting hetero-resistivity [11]. Therefore, detection of *mecA* gene is accepted as gold standard on determining methicillin resistance [12].

The aim of this study was to evaluate *i*) phenotypic characteristics of methicillin resistance in *S.aureus* isolates using oxacillin (1 µg) disks, cefoxitin (30 µg) disks *ii*) genotypic characteristics using *mecA* gene by PCR, *iii*) MICs (minimum inhibitory concentrations) of oxacillin and cefoxitin by E test.

2. Materials and Method

2.1. Isolates

A total of 99 isolates of *S. aureus* obtained from water buffalo milk and dairy products was used in the study. All isolates were collected from November 2012 to May 2013 in Samsun region, Turkey. Among 99 *S. aureus* isolates, 57 of them were obtained from water buffalo milk, nine of them from water buffalo cream, 33 of them from water buffalo cheese. Confirmation of the isolates was done by PCR using 16S rRNA and *nuc* gene according to Maes et al [12]. *S. aureus* ATCC 43300 (16S rRNA and *nuc*) and *S. aureus* ATCC 46300 (*mecA*) were used as positive control. Methicillin resistance among isolates was evaluated both phenotypically and genotypically.

2.2. Detection of the *mecA* gene by PCR

Genomic DNA extraction was performed using proteinase K (Sigma P2308) and lysostaphin (Sigma, L7386, St. Louis, Mo., U.S.A.) as described previously [13]. The *mecA* gene was amplified using the primers (forward 5'-GTAGAAATGACTGAACGTCCGATAA-3' and reverse 5'-GCCAATTCCACATTGTTTCG GTCTAA -3') as described by Geha et al [14]. PCR was performed in a final volume of 50 µL containing 1 X PCR buffer (Sigma), 2 mM MgCl₂ (Sigma), 0.2 mM dNTP (Sigma), 0.4 µM *mecA* primer, 2 U Taq-polymerase (Sigma) and 5 µL of target DNA. PCR amplification conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 2 min, annealing at 52 °C for 2 min, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min in thermal cycler (Bio-Rad MJ Mini-PTC-1148). DNA fragments were separated on 1.5% agarose in Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and stained with ethidium bromide at 0.5 µg/mL. Electrophoresis was carried out at 90 V for 1 h (BioRad Power Pac-Basic, Singapore; BioRad electrophoresis tank, Wide Mini, Singapore). PCR amplicons were visualized under UV illumination (Wise-UV Wuv-L50, DAIHAN Scientific, Seoul, Korea). The *mecA* genes were visualized at 310 bp.

2.3. Phenotypical detection of methicillin resistance

2.3.1. Disc Diffusion Test

Methicillin resistance on the *S.aureus* isolates was phenotypically evaluated using disc diffusion method on Mueller-Hinton agar (MHA, Oxoid CM 337) as recommended by the Clinical and Laboratory Standards (CLSI) [10] with oxacillin (1 µg) and cefoxitin. The turbidity of bacterial suspension (*S.aureus*) was adjusted to 0.5 McFarland Standard with a densitometer (Biosan, DEN-1, Latvia). Then 1 mL of suspension was inoculated on Mueller-Hinton agar (MHA, Oxoid CM 337). Plates were incubated at 35 °C for 18-24 h and inhibition zones were measured. Diameters of the inhibition zones were interpreted according to the CLSI guidelines. Oxacillin inhibition zone diameter of ≥13 mm was considered as susceptible, 11-12 mm was considered as intermediate and ≤10 mm as resistant. Cefoxitin inhibition zone diameter of ≥22 mm was considered as susceptible, ≤21 mm was resistant.

2.3.2. E-Test

The oxacillin and cefoxitin MICs (minimum inhibitory concentration) were determined by E test (AB Biodisk, Solna, Sweden). E test for oxacillin was performed using Mueller-Hinton agar plates supplemented with 2% NaCl. E test for cefoxitin was performed without NaCl. The plates were incubated at 35 °C for 24 hours. Oxacillin MIC testing results interpreted as susceptible (S) if MIC was ≤ 2 $\mu\text{g/mL}$ and as resistant if MIC was (R) ≥ 4 $\mu\text{g/mL}$ for *S.aureus*. Cefoxitin MIC testing results interpreted as susceptible (S) if MIC was ≤ 6 $\mu\text{g/mL}$ and as resistant (R) if MIC was ≥ 8 $\mu\text{g/mL}$ using Clinical and Laboratory Standards (CLSI) guidelines [10].

3. Result and Discussion

3.1. Confirmation of isolates

A total of 99 *S. aureus* isolates was used in the study. Of them, 57 isolates were obtained from water buffalo milk, nine from water buffalo cream and 33 from water buffalo cheese. All isolates were found positive for 16S rRNA and *nuc* genes by PCR.

3.2. Detection of the *mecA* gene by PCR

The *mecA* gene is highly conserved among methicillin resistant *S. Aureus* isolates [15]. Detection of *mecA* gene is considered as the gold standard for identifying of MRSA [16]. In our study, the *mecA* gene was found positive in nine of 99 (9%) *S. aureus* isolates. Among *mecA* gene positive isolates, eight of them were obtained from water buffalo milk and one of them was obtained from water buffalo cheese. None of the water buffalo cream samples was found to be positive for *mecA* gene. The *mecA* genes were visualized at 310 bp on a UV transilluminator (Fig. 1). The results are in agreement with Vanderhaegen et al. [17] as they were also isolated 9% MRSA positive *S.aureus* isolates from milk samples in Belgium. Contrary to this, higher than our results WanXia et al. [18] reported that *mecA* gene was positive among 49 of 103 *S.aureus* isolates (47.6%) obtained from cows with mastitis in China. On the other hand, Haran et al. [19] reported 1.3% in milk of MRSA positivity which is at lower percentages than our results in Minesota. Virgin et al. [20] found that 5 of 190 milk samples were *mecA* positive *Staphylococcus* spp. (2.6%) in USA, but methicillin resistant *S. aureus* was not present.

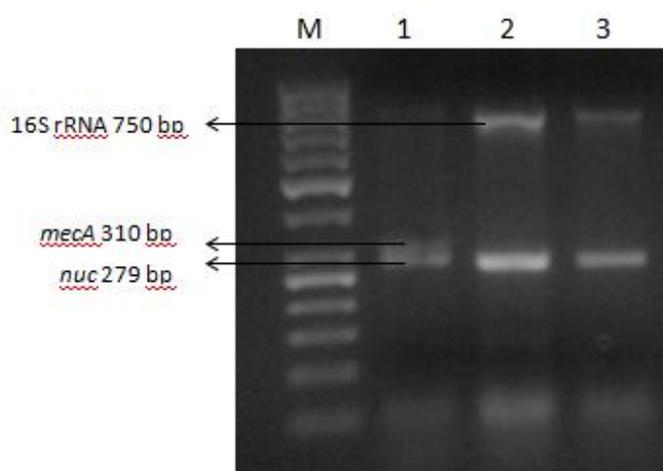


Fig. 1: Interpretation of PCR results of the *mecA* gene positive *S.aureus*

M: 50bp DNA Marker, Line 1: *mecA* (310 bp) positive *S.aureus*; Line 2-3: *nuc* (279 bp) and 16S rRNA (750bp) positive *S.aureus*

3.3. Phenotypical detection of methicillin resistance

Phenotypical detection of methicillin resistance of *S.aureus* isolates was performed using oxacillin and cefoxitin disc diffusion test. Oxacillin and cefoxitin MIC value was determined by E test. In present study,

according to disc diffusion tests, 10 of 99 *S. aureus* isolates (10%) were resistant to only one antibiotics, seven of 99 (7%) were resistant to double antibiotics and 82 of 99 isolates did not show any resistance to antibiotics. Out of 99 isolates, 14 (13.86%) were found resistant to oxacillin and nine (8.9%) were found resistant to cefoxitin by disc diffusion. Table 1 shows the results for the phenotypic methods used for detection of methicillin resistance in the present study.

Table 1. The result of disc diffusion test from *S. aureus* isolates for oxacillin and cefoxitin

No. of <i>S. aureus</i> (+) isolates	Oxacillin			Cefoxitin		
	S	I	R	S	I	R
Water buffalo milk (n=57)	49	-	8	54	-	3
Water buffalo cream (n=9)	6	-	3	6	-	3
Water buffalo cheese (n=33)	30	-	3	30	-	3
Total (n=99)	85	-	14	90	-	9

R: Resistant; S: Susceptible; I: Intermediate

As shown in Table 1, among oxacillin resistant isolates, eight of them were obtained from water buffalo milk, three of them from water buffalo cream and three of them from water buffalo cheese. Among cefoxitin resistant isolates, three of them were obtained from water buffalo milk, three of them from water buffalo cream and the remaining from water buffalo cheese. Phenotypical methicillin resistance of *S. aureus* isolates was found lower in water buffalo cream and cheese sample than raw milk. Phenotypic determination of methicillin resistance was affected by many factors such as prolonging the incubation period, incubation at low temperature, addition of NaCl and pH of environment [21]. Problems for detecting MRSA may be the reason of being low level expression of methicillin resistance in some strains of *S. aureus*, therefore, phenotypic methods for detecting MRSA strains are not usually correctly identified [22]. Therefore it is possible to be obtained different result for phenotypical methicillin resistance in raw milk and milk products. CLSI has recommended cefoxitin instead of oxacillin using the disk diffusion method for methicillin-resistance [10]. In our study we detected that number of cefoxitin resistant isolates was found lower than oxacillin resistant isolates.

Using E test MIC, it was found out that 16 of 99 strains of *S. aureus* were resistant to methicillin. MRSA interpretive breakpoint of oxacillin MICs (susceptible (S) ≤ 2 $\mu\text{g/mL}$ and resistant (R) ≥ 4 $\mu\text{g/mL}$) was found out that of 11 isolates were resistant and of 88 isolates were susceptible. For cefoxitin MICs value (susceptible (S) ≤ 6 $\mu\text{g/mL}$ and resistant (R) ≥ 8 $\mu\text{g/mL}$) was found out that of five isolates were resistant and 94 isolates were susceptible. Oxacillin and cefoxitin MIC results are presented in Table 2.

Table 2: Oxacillin and cefoxitin MICs for *S. aureus* isolates by E test

No. of isolates	Number of isolates with oxacillin and cefoxitin MIC ($\mu\text{g/ml}$)																	
	≤ 0.25	0.32	0.50	0.64	0.75	1.0	1.5	2	3	4	6	8	12	16	24	32	64	
Oxacillin	99	56	13	6	1	2	6	3	1	-	3	1	3	1	1	-	1	1
Cefoxitin	99	16	9	9	-	7	9	15	16	7	4	2	1	3	-	1	-	-

3.4. Phenotypical and genotypical comparison of methicillin resistance

Comparison of methicillin resistance with discs diffusion test and PCR results are shown in Table 3. In our study nine isolates were found positive for *mecA* gene by PCR. Three of 14 phenotypically resistant isolates detected by oxacillin disc diffusion test were positive for *mecA* gene. Two of nine phenotypically resistant isolates detected by cefoxitin disc diffusion test were positive for *mecA* gene. On the other hand, three of nine *mecA* gene positive isolates were found to be oxacillin resistant. Two of nine *mecA* gene positive isolates were found to be cefoxitin resistant. The remaining five *mecA* gene positive isolates did not show any resistance to antibiotics.

Table 3: Result of disc diffusion test and E test from *mecA* gene positive isolates

No. of <i>mecA</i> (+) isolates	Sample	Disc Diffusion Test		E Test	
		Oxacillin	Cefoxitin	Oxacillin	Cefoxitin
1	Buffalo milk	S	S	0.19	1.5
2	Buffalo milk	S	S	0.38	1.5
3	Buffalo milk	S	S	0.50	1.0
4	Buffalo milk	S	S	0.38	0.38
5	Buffalo milk	R	S	12	2.0
6	Buffalo milk	S	S	0.50	1.5
7	Buffalo cheese	S	R	2	8
8	Buffalo milk	R	R	4	24
9	Buffalo milk	R	S	16	0.094

R:Resistant; S: Susceptible

Among the 99 strains included in our study, nine were *mecA*-positive and 90 were *mecA*-negative. In four of nine isolates, *mecA* gene was observed compatible and resistant with E test. Among *mecA* gene negative isolates, 11 isolates were found resistant with E test. The results of the phenotypic tests are shown in Table 4.

Table 4: Results of the MRSA positive isolates with phenotypic tests and their correlation with *mecA*

<i>MecA</i>	Disc Diffusion Test				E test			
	Oxacillin		Cefoxitin		Oxacillin		Cefoxitin	
	R	S	R	S	R	S	R	S
<i>mecA</i> (+) n=9	3	6	2	7	3	6	2	7
<i>mecA</i> (-) n=90	11	79	7	83	8	82	3	87

R: Resistant; S: Susceptible

Methicillin-resistant *S. aureus* (MRSA) is described as *S. aureus* showing a minimum inhibitory concentration (MIC) of oxacillin higher than 4 mg/mL or containing the *mecA* gene. In addition, some clinical isolates are oxacillin-susceptible and *mecA*-positive [23]. Supportively, we found out the occurrence of *S. aureus* containing the *mecA* gene and an MIC of oxacillin of less than 2 mg/mL (oxacillin-susceptible MRSA; OS-MRSA) in a total five of nine strains of *S. aureus* isolated. The reason of resistance for oxacillin not having *mecA* gene is may be the overproduction of penicillinase, or by alteration of other penicillin-binding proteins (PBPs) [24]. It has been reported that these types of strains can be treated with beta-lactam antibiotics in proper doses and new MRSA having high antibiotic resistance strains can occur [23]. Although *mecA* is positive in our study, due to oxacillin MIC below than 2 µg/mL, features of 5% MRSA strain to be evaluated as OS-MRSA is summarized in Table 5.

Table 5: Result of OS-MRSA positive isolates with phenotypic and genotypic tests

Isolates	O MIC	<i>mecA</i>	ODD	CDD	Sample
1	<2	+	S	S	Buffalo milk
2	<2	+	S	S	Buffalo milk
3	<2	+	S	S	Buffalo milk
4	<2	+	S	S	Buffalo milk
6	<2	+	S	S	Buffalo milk

OS-MRSA: Oxacillin susceptible *mecA* positive *S. aureus*; O MIC: Oxacillin minimum inhibitory concentration; ODD: Oxacillin disc diffusion; CDD: Cefoxitin disc diffusion; S: Susceptible

In this study, five isolates were determined by phenotypic method as oxacillin and cefoxitin sensitive although they had *mecA* gene. This may be related to the heterogeneity of resistance or oxacillin sensitive *mecA* positive *S. aureus* isolates (OS-MRSA). Similarly, WanXia et al. [18] found that 37 of 49 isolates with *mecA* positivity were oxacillin sensitive phenotypically. They defined these isolates as oxacillin sensitive *mecA* positive *S. aureus* (OS-MRSA). It was also reported that methicillin resistance was heterogeneous among MRSA populations and some isolates might be phenotypically oxacillin sensitive due to heterogeneous expression of resistance although they had *mecA* gene [25]. Cekovska et al. [26] compared oxacillin disc diffusion, microdilution, and oxacillin agar screening tests for the determination of MRSA. Their results indicate similarity with our study which were variable due to heterogeneous resistance and suggested that these tests should be used together with another conventional method.

4. Conclusions

The data which was obtained in our study showed that sensitivity tests which were carried out for the purpose of determining methicillin resistance were affected by phenotypical changes. Genotypic methods were more sensitive and reliable in determining methicillin resistance. Incorrect determination of the methicillin resistance may cause various negative effects such as ineffective implementation of treatment, extension of treatment periods and use of new and more costly antibiotics. In order to prevent increased resistance against antibiotics, unconscious and random drug usages should be prevented.

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6. References

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