

An easy-to-use, rapid and inexpensive method to determine methicillin resistance in *Staphylococcus aureus*

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ABSTRACT

Objective: We aimed to report a new turbidimetric method to identify methicillin resistance in *S. aureus* strains just two hours after identification of the microorganism, and to analyze diagnostic and discrimination abilities of this new method.

Methods: A total of 319 *S. aureus* isolates were included. Identification of bacteria was done by the colony morphology, and conventional biochemical methods. The methicillin resistance of the *S. aureus* strains was studied as indicated in Clinical Laboratory Standards Institute 2009. The turbidimetric method we developed is based on different growth rates of *S. aureus* in two media, with or without oxacillin. The growth rates of MRSA and MSSA are similar in normal media, however the MRSA grows significantly faster in the media containing oxacillin. Therefore, after 2 hours of incubation, the difference of turbidity produced by bacteria is less in MRSA, and more in MSSA. The absorbance of the microplates were measured before incubation, and at 2nd and 3rd hours of incubation. The "absorbance rate" was calculated for each bacteria and the bacteria were classified as MRSA or MSSA based on the absorbance rate.

Results: All MRSA and MSSA strains were correctly discriminated via our turbidimetric method, when an absorbance rate of 1.900 was taken as cut-off value. The new method could diagnose MRSA with 100% specificity and 100% sensitivity in just two hours.

Conclusion: The turbidimetric method is a rapid, easy and cheap method that does not require any specific equipment. It can be easily performed in every microbiology laboratory.

Key words: Turbidimetric method, MRSA, MSSA, methicillin resistance, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus, is a Gram positive bacteria with a high virulence, and it is isolated from humans as an infectious agent. Penicillins started to be used in 1945 to treat infections caused by *S. aureus*, and penicillin resistance occurred in a short time due to beta lactamase. Currently, *S. aureus* isolates show a high resistance (95%) to penicillins.¹

Methicillin is a semi-synthetic penicillin resistant to penicillinase, and it started to be used in 1960, and methicillin-resistant *S. aureus* (MRSA) were isolated short after, in one year. After 1980, the infections caused by MRSA constituted a significant proportion of the

nosocomial infections. In staphylococci, methicillin resistance occurs due to production of PBP 2a instead of PBP 2 by a mutant chromosomal gene, *mecA*, and PBP 2a has lower affinity for beta- lactam antibiotics. Resistant bacteria can keep on synthesizing their cell wall. This protein makes MRSA strains resistant not only to methicillin, but also to all beta lactam agents, and this causes a treatment challenge.¹⁻³

Until recently, MRSA caused only nosocomial infections; however recently, severe community-acquired infections were observed in some groups (prisoners, sports teams, military units, homeless people etc.), particularly in the United States.^{1,3} The rates of catheter- related bacteriemia, ventilator-related pneumonia, and surgical site

and cutaneous infections caused by MRSA (10-50%) vary among countries. This rate is 39.9% in Turkey.⁴ MRSA infections cause threefold increase in hospital stay (from an average of 4.5 days to 13.5 days), threefold increase in treatment cost, and fivefold increase in mortality. The mortality rate due to invasive *S. aureus* infections was reported as 19-34%.⁵ English medical data indicated that MRSA was responsible for 0.1% of all deaths, and 0.2% of in-hospital deaths between 2008 and 2012.⁶

Owing to increasing frequency of community-acquired MRSA strains, rapid diagnosis and rapid determination of antibiotic susceptibility, and starting appropriate treatment immediately become important not only in nosocomial, but also in community-acquired infections.^{1,3} Obtaining the result of the susceptibility test with routine methods (disk diffusion, microdilution, gradient strips) takes 24 hours.^{7,8}

This time is quite long for patients in intensive care units, as well as the ones being treated in haematology and oncology clinics. Since urgent and appropriate therapy is essential in many situations, MRSA is targeted in daily practice when there is a suspicion for a staphylococcal infection, and glycopeptide antibiotics are administered empirically. However, those antibiotics cause more adverse effects. In addition, risk of failure is higher, and treatment response is delayed when compared to beta lactam antibiotics if the causative agent is MSSA. Therefore, obtaining the result of the antibiotic susceptibility test is as important as the determination of the causative agent in staphylococcal infections.¹⁻³

In this study, we aimed to report a new turbidimetric method to identify methicillin resistance in *S. aureus* strains just two hours after identification of the microorganism, and to analyse diagnostic and discrimination abilities of this new method.

METHODS

1. Bacteria A total of 319 *S. aureus* isolates isolated from various samples were included in the study. The strains were kept in skim milk at -80 °C were passed into Tryptic Soy Agar and 5% Sheep Blood Agar plates, twice. The plates were incubated in the incubator for one night, at 35 °C.⁶

2. Identification of the bacteria: Identification of bacteria was done by the colony morphology and conventional biochemical methods [catalase test, plasma coagulase test, and the effects on mannitol in mannitol salt agar].⁶

3. Determination of antibiotic susceptibility with classical method, discrimination of MSSA and MRSA: The methicillin resistance of the *S. aureus* strains was studied as indicated in Clinical Laboratory Standards Institute (CLSI) 2009:⁷

3.a. Oxacillin screening test: Muller Hinton agar medium containing 6 microgram/L oxacillin (Sigma-USA) and 4% NaCl were prepared. Bacterial colony suspension was equivalent to 0,5 McFarland standard. (MF) (1.5×10^8 bacteria/ml) and 10 μ l

of this suspension was inoculated to an area of 1 cm² on the medium. The petri dishes were inoculated at 35 °C for 24 hours. Presence of growth was evaluated at the end of this period.⁷

3. b. Determination of susceptibility with disk diffusion method: *S. aureus* inoculum was prepared according to 0.5 MF turbidity standards. They were inoculated in Mueller Hinton Agar (Difco-USA) using three- dimensional inoculation method. Oxacillin (1 μ gr) and cefoxitin (30 μ gr) (BD-USA) antibiotic disks were placed onto the surface of the agar. The petri dishes were incubated at 35 °C for 18-24 hours. The diameters of the susceptibility zones were measured. ATCC 25923 and MRSA ATCC 43300 strains were used as the control strains. The threshold zone diameters were regarded as ≤ 10 and ≥ 13 mm for oxacillin, and ≤ 21 and ≥ 22 mm for cefoxitin, and the strains were classified as MSSA or MRSA.⁸

3.c. Determination of susceptibility with microdilution: The MIC values of all isolates for oxacillin and cefoxitin were determined using U-bottom microplates. Cation- adjusted Mueller Hinton broth was used as the medium. Dilutions of the isolates were done to obtain the last bacterial concentration as 5×10^5 bacteria/ml. The medium contained 2, 3, 4, 8 mg/L oxacillin (Ox), or 4, 6, 8, 16 mg/L cefoxitin (Fox). Control of media, antibiotic-added media, and the controls with standard strains were performed in all tests done in all strains. The microplates were incubated at 35 °C for 24 hours. The wells without any visible growth were determined at the end of this period.⁸

4. Antibiotic susceptibility with turbidimetric method; discrimination of MSSA and MRSA: 319 strains were analyzed in flat-bottomed microplates (12x8) using the method we developed.

4.a . The medium (M) in turbidimetric method: A number of different liquid media were used including Mueller Hinton broth, Tryptic Soy Broth, Cation- adjusted Mueller Hinton Broth, and BHIB in order to determine the medium to be used in the experiment. At the end, BHIB (Difco-USA) medium was decided. Twofold more dehydrated medium than indicated on the BHIB package was used ($2 \times 185 = 370$ gr/L), and a twice- concentrated medium was obtained. It was autoclaved at 120 °C for 15 minutes.

4.b. The medium with antibiotic in turbidimetric method (oxacillin): A sterile, twice-concentrated BHIB medium containing 8 mg/L oxacillin (O1002-Sigma-Aldrich-USA) was prepared.

4.c. Bacteria in turbidimetric method: Comparative studies showed that 0.5 MF bacterial density used in the study was the best turbidity. To obtain this concentration after mixing, 1 MF (3×10^8 bacteria /ml) bacterial suspensions were prepared in normal saline for each isolate studied.

4.d. Pipetting in turbidimetric method: The experiment was performed as follows: 3 wells for experiment (M with antibiotic + bacteria), and 3 wells for positive control (M + bacteria) (Table 1). The final concentration after pipetting was 185 gr/L for the medium, 0.5 MF for bacteria suspensions, and 4 mg/L for oxacillin (Table 1).

4.e. **The assessment in turbidimetric method:** The absorbance of the microplates (wells) were measured at hours 0, 2, and 3, at 450 nm. The microplates were incubated at 35 °C after measurement at hour 0, until the measurements at hours 2 and 3.

4.f. **Calculation in turbidimetric method:** The calculations were done separately for hour 2 and 3. The difference for absorbance was obtained by subtracting 0 hour value from 2nd and 3rd hour values for each well. The mean absorbance difference of 3 positive control and experiment wells was calculated for every isolate. Then, those means were used to calculate absorbance rate for each isolate

$$\text{Absorbance rate} = \frac{\text{The absorbance difference of positive control}}{\text{The absorbance difference of experiment}}$$

5. **Devices:** MF adjustments were done with MF device (Biomérieux-France), and absorbance measurements were done with µQuant microplate reader (BioTek-USA).⁸⁻¹¹

6. **Statistical analysis:** The sensitivity and specificity of the turbidimetric method was calculated and ROC analysis was performed with a statistical package program (SPSS for Windows 13.5).

RESULTS

The oxacillin screening test, and oxacillin and cefoxitin disk tests performed according to CLSI standards revealed that 171 of 319 staphylococcus strains were MRSA, and 148 of them were

MSSA. All of 171 MRSA strains had oxacillin MIC values > 8 mg/L, and cefoxitin MIC values were > 16 mg/L. The new turbidimetric method identified 171 strains as MRSA, and 148 strains as MSSA, identical to aforementioned results.

The 2nd and 3rd hour absorbance rates of the turbidimetric method are shown in Table 2. Maximum 2nd hour absorbance rate was 1.783 among the MRSA strains while minimum 2nd hour absorbance rate was 2.000 among MSSA. The difference between those values was 0.217 at 2nd hour, and 1.136 at 3rd hour.

The 2nd hour absorbance rates of MRSA and MSSA are presented in Figures 1a and 1b, and it is clearly seen that there are no intersections between MRSA and MSSA strains. The cut off value for 100% sensitivity and 100% specificity of the test was determined as 1.900. At this cut off value, the area under the curve in ROC analysis was found as 1.

Table 1. Study plan of the experiment.

Content	Experiment n=3*	Positive control n=3	Negative control** n=3
Medium	-	150 µl	150 µl
Medium with antibiotic (oxacillin)	150 µl	-	
Bacteria ***	150 µl	150 µl	
Normal saline	-	-	150 µl

* The experiment was done in 3 wells for each isolate

** Negative controls (without bacteria) were also tested in 3 wells for Medium used in every series

*** In addition to examined bacteria, control strains of MRSA and MSSA (ATCC 25923 and MRSA ATCC 43300) were also tested in every series.

Table 2. The absorbance rates of MRSA and MSSA at 2nd and 3rd hours.

	Absorbance rate at 2 nd hour		Absorbance rate at 3 rd hour	
	MRSA	MSSA	MRSA	MSSA
Mean	1.090	3.604	1.183	7.476
SD	0.197	0.959	0.225	2.209
Minimum	0.584	2.000	0.825	3.587
Maximum	1.783	5.806	2.451	12.900

MRSA: Methicilline resistant Staphylococci, MSSA: Methicilline sensitive *Staphylococci*

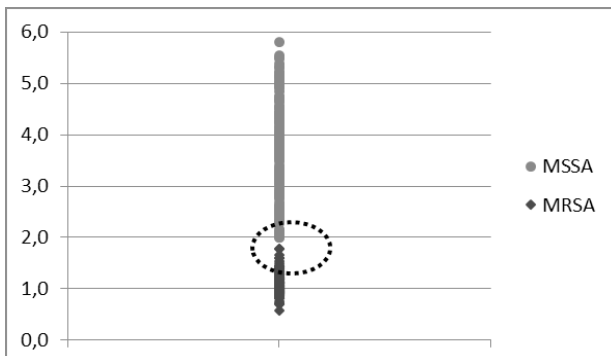


Figure 1a. 2nd-hour absorbance rates of MRSA and MSSA.

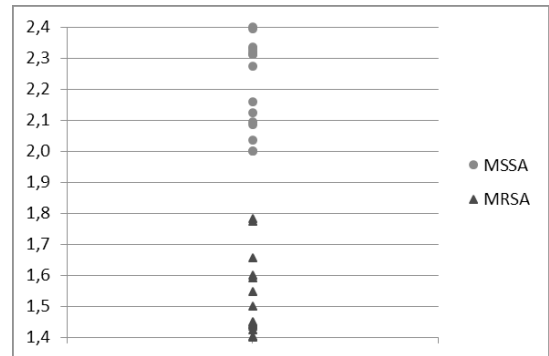


Figure 1b. 2nd-hour absorbance rates of MRSA and MSSA (close up view).

DISCUSSION

It is possible to identify MRSA and MRSE strains by disk diffusion, agar screening, and agar dilution and microdilution methods according to recommendations of CLSI. In 2009, CLSI reported that methicillin resistance could be determined by cefoxitin disk susceptibility.⁸⁻¹⁰ In aforementioned tests, the sample obtained from the patient is cultivated, the bacteria isolated from the primary culture are used, and susceptibility test takes 24 hours. Some studies focused on follow up of bacterial growth, determining the metabolic activity, and use of turbidimetric and spectrophotometric methods for determination of susceptibility.¹¹⁻¹⁴

In our turbidimetric method, the turbidity rate produced by bacteria in experiment and positive control groups at 2nd hour showed 100% concordance with MRSA-MSSA discrimination determined by oxacillin and cefoxitin disk diffusion methods. Although the cut off value was found as 1.900 in ROC analysis, and we did not find any intermediate value in our study, we suggest a two-step analysis to decrease the probability of error. Consequently, the bacteria with an absorbance rate <1.800 at 2nd hour will be regarded as MRSA, and the bacteria with an absorbance rate ≥ 2.000 at 2nd hour will be regarded as MSSA. In case of the values in between, 3rd hour values will be used. Our data indicates that 3.000 should be taken as the 3rd hour cut off value. The bacteria with an absorbance rate below 3.000 can be regarded as MRSA, and the ones with a value >3.000 as MSSA (Table 2).

Molecular methods are the most reliable ones for differentiation of MRSA and MSSA. Determining presence of *mecA* gene or PBP2a with molecular methods are regarded as gold standard tests in detection of methicillin-resistance among staphylococcus strains. However, special laboratory equipment and setup should be available for this method. On top of that, the cost for per unit test is quite high.¹⁵⁻²⁶ Therefore, the molecular methods are not widely used in Turkey except for the university hospitals, and laboratories of some education and research hospitals offering routine care.

Apart from those methods, some commercial and experimental techniques, and automatic, semi- automatic, and turbidimetric antibiotic susceptibility tests give reliable results for determining methicillin resistance. However, these methods require specific device and consumable materials of the manufacturer. Some of those tests have multiple steps. For instance, “TNF as Biomarker for Rapid Quantification” method is a multi-step test, it is still experimental, and it requires a well- equipped laboratory.²⁷ Some of the aforementioned methods enable direct use of the sample, but some need bacteria grown in the primary culture (Table 3).

Isolating the bacteria and obtaining the susceptibility test results takes approximately 48 hours when classical methods are

used. The results of commercial tests that discriminate MRSA and MSSA easily and rapidly are obtained between 35 minutes and 3 days. Determining methicillin resistance shortly after isolation of the bacteria is important for treatment planning. However, methicillin resistance should be determined by an inexpensive method that could be carried out in a laboratory with the basic equipment.

Table 3 shows the basic techniques, steps of performance, need for using the patients' samples directly or the primary culture, the equipment required, and the time needed to obtain results of the current methods used for identification of MRSA/MSSA and our new method.²⁵⁻³³

Our method is based on the growth rate difference of *S. aureus* in two environments, with or without oxacillin. MRSA and MSSA grow at similar rates in normal media, however MSSA grows significantly slower than MRSA in presence of oxacillin. Therefore, the turbidity difference that appears after two hours in media with or without oxacillin is smaller in MRSA, and greater in MSSA. Choosing the bacterial colony to be studied is very important, as in other similar methods. The study ends in a perfect way when a pure colony is sampled, however the study may give uncertain results if a mixed colony is sampled.

The consumable material and the microplate reader (450 nm) are present in every microbiology laboratory. The cost per unit test is very inexpensive (~0.36\$ per strain). In addition, the test is easy-to-perform, and may be performed by every laboratory technician that had basic laboratory training.

In conclusion, the turbidimetric method we developed enables determination of methicillin resistance in 2 hours with 100% specificity and 100% sensitivity, using a single sample obtained from one colony after isolation of the bacteria. In addition, it is a cheap and easy method. Our method will enable the laboratories to give results approximately one day earlier when other fast and reliable methods cannot be used due to their costs or due to technical incapacity. In this way, quite precious hours will be gained in terms of treatment planning, use of wide-spectrum antibiotics will be avoided, and undesirable results such as a high cost, adverse effects, and development of resistance will be prevented.

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Patent: The method of the study, in 22.3. 2016 was granted a patent from the Turkish Patent Institute (no: TR 2012/10505 B).

Table 3. Comparison of the methods that determine methicillin resistance of *S. aureus*.

Methods	The basic technique	Chemicals or other materials required	Production method	Device requirements	Test result time	The method to obtain results	Sensitivity/ specificity	Price
Velogene Rapid MRSA Identification Assay³².	The Velogene Rapid MRSA utilizes isothermal CyclingProbe™ technology for the detection of the <i>mecA</i> gene	DNA extraction + ELISA-like reagents	Manufacturer's instructions	Incubator (55°C)	" Velogene Rapid Identification Assay" is applied, 2 hours	A colored reaction: methicillin-susceptible isolate (mecA negative); a colorless reaction: methicillin-resistant strain (mecA positive)	99.3 % compared to conventional susceptibility tests 100 % compared to Polymerase Chain Reaction	Velogene Rapid MRSA Kit/16 test/ price could not be found
PBP2' test MRSA-Screen³²	Latex agg	Classic Latex agglutination reagents	Manufacturer's instructions	Centrifuge Water bath Shaker	After a primary culture, 15 min.	Agglutination	98.5% / 100%.	1.5 \$
BBL Crystal MRSA ID.^{25, 32}	The inoculated tray	Special kit reagent	Evaluation of the BBL Crystal MRSA ID System	Incubator Spectrofluorometer	After a primary culture, 4-5 hours	Fluorescence in wells is observed by illuminating the panel	Sensitivity 86.5% (negative predictive value 92.2%), specificity 97.6% (positive predictive value 95.7%) 1,20	Not in market
Oxacillin agar screen and oxacillin susceptibility testing²⁵.	Culture (Antimicrobial susceptibility Testing)	Culture media	Manufacturer's instructions	Incubator	Culture from direct sample, 18-24 hours	Oxacillin susceptibility or resistance	100 % / 99%	2 \$
Chromagar MRSA^{25, 30,31}	Culture	Culture media	Manufacturer's instructions	Incubator	Culture from direct sample, 18-24 hours	Oxacillin susceptibility or resistance	Sensitivity after 24 h of incubation is 95.4%, increasing to 100% after 48 h. The specificity is already 100% after 24 h. ^{2,3,20}	2.25 \$
Multiplex PCR²⁹.	Molecular	PCR reagents	Manufacturer's instructions	Cooler centrifuge Thermocycler Electrophoresis Imaging system	After a primary culture, 4 -6 hours	Simultaneous detection of <i>mecA</i> and <i>nucA</i>	PCR for the detection of the <i>mecA</i> gene is used as the "gold standard"	Commercial kits or manual method, the cost changes when PCR is used
CytAMP assay for MRSA³³	Molecular [The CytAMP assay is based on isothermal signal mediated amplification of RNA technology (SMART)]	Prototype CytAMP assay	Prototype CytAMP assay kit	Centrifuge Heat block Microplate reader	From direct sample 3-5 hours	Results are obtained in approximately 3.5 h as a color signal in 96-well microplate	In the test phase	Not yet known
TNF as Biomarker for Rapid Quantification²⁷	Immunological assays	Specific reagents	Manufacturer's instructions	BD OptEIA mouse TNF ELISA Set 5% CO2 incubator Spectrophotometry	From direct sample, 2-3 days	Intracellular staining of TNF with an APC anti-TNF antibody is performed following manufacturer's protocol	In the test phase	Not yet known
Our Method	Turbidimetric assay	Brain heart infusion broth Oxacillin suspension	Described in the article	Mac Farland device Microplate incubator (37 C°) Microplate reader	After a primary culture, 2-3 hours	Calculated according to the cut-off value	100 % / 100 %	For one strain, the operating cost is ~0.36\$

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