CLINICAL STUDY

Methicillin-resistant *Staphylococcus aureus*: Comparison of susceptibility test methods with *mecA* gene analysis for determining oxacillin (Methicillin) resistance in our clinical isolates

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Abstract

The aim of the study was to determine which of the following susceptibility test methods, using recommended or modified NCCLS, best detects oxacillin resistance: disk diffusion, agar screen, and broth dilution. PCR for mecA was used as „gold standard". We studied 120 *Staphylococcus aureus* isolates received from different patients hospitalized at the Clinical center in Skopje from May 2001 to November 2003. There were no two isolates from the same patient. Methicillin resistance in *Staphylococcus aureus* strains was performed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS). PCR appears to be promising. Since variations among the methods exist and no acceptable guidelines are formulated, a combination of conventional methods, including either 3 μg of oxacillin/ml. in Mueller Hinton broth or one of the screen agar plates (6 μg/ml), alone or with PCR should be the method of choice for the detection of MRSA. *(Tab. 4, Ref. 12.)*

Key words: methicillin resistance, *Staphylococcus aureus,* antibiotics, methods.

The commonly used methods for the detection of methicillin resistance rely on modified culture conditions to enhance the expression of resistance. Modifications include the use of oxacillin, incubation at 30 or 35 °C instead of 37 °C, and incubation for 24 h instead of 16 to 18 h. Susceptibility tests using agents other than oxacillin or methicillin (e.g., cephalosporins or imipenem) are unreliable (1). Methicillin-resistant strains may appear falsely susceptible to some beta-lactam antibiotics in vitro. The National Committee for Clinical Laboratory Standards periodically reviews test methods for the detection of methicillin-resistant staphylococci, and these standards should be consulted on specific test recommendations (2).

The heterogeneous nature of methicillin resistance is an inherent limitation of the accuracy of susceptibility testing. *MecA* detection tests based on PCR or DNA hybridization correctly identify the most heterogeneous strains and should be considered the gold standard for methicillin resistance (2, 3).

The oxacillin disk diffusion method is the least reliable method for the detection of methicillin resistance. The disk diffusion test has lower specificity compared to other methods, displaying specificity of about 80 % (4).

Under appropriate conditions, ≥95 % of resistant strains are detected by broth microdilution method. Current National Committee for Clinical Laboratory Standards recommends to use Muller-Hinton broth supplemented with 2 % NaCl, an inoculum of 5.10^5 CFU/ml, and a 24-h incubation at 35 °C. Agar dilution test performed by using Mueller-Hinton agar containing 2 % NaCl and incubation at 30 to 35 °C for 24 h gives the results similar to the broth dilution method (4, 5).

The agar screen test is performed by inoculating 10^4 CFU onto Mueller-Hinton agar supplemented with 4 % NaCl containing 6 μg/ml of oxacillin. After 24-h incubation at 35 °C, the agar is inspected for growth. The presence of even one colony is indicative of resistance. The sensitivity of this method approaches 100 % for the detection of methicillin-resistant *Staphylococcus aureus* (4, 5, 6).

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Tab. 1. Susceptibility test methods used in the study.

Method	Media	Oxacillin concentration	Inoculum	Incubation	Interpretive guidelines
Disk diffusion	Muller Hinton agar (MH) with 2 % NaCl	1 µg disk	Swab, a McFarland standard equal to 0.5 to 1	35 °C for 24 and 48 h	Susceptible, zone diameter of ≥13 mm; Intermediate, zone diameter of 11 to 12 mm; Resistant, zone diameter of ≤10 mm
Agar screen	MH agar so 4 % NaCl	6 µg/ml	Swab, McFarland standard of	35 °C for 24 to 48 h	Resistant: >1 colony growth at 6 µg/ml 0.5 to 1
Broth dilution	Broth with 2 % NaCl	1 and 2 µg/ml	McFarland standard of 0.5 to 1	35 °C for 24 to 48 h	Susceptible: ≤2 µg/ml, Resistant: ≥4 µg/ml

Aim

The aim of the study was to determine which of the following susceptibility test methods, using recommended or modified NCCLS best detects an oxacillin resistance: disk diffusion, agar screen, and broth dilution. PCR for *mecA* was used as "gold standard".

Materials and methods

Bacterial isolates

We studied 120 MRSA isolates received from different patients hospitalized at the Clinical center in Skopje from May 2000 to November 2002 and 50 methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates received from different patients in the same time period. There were no two isolates from the same patient.

Methods

1. Methods for the detection of resistance to methicillin

Methicillin resistance in *Staphylococcus aureus* strains was performed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) with the following methods:

Screen agar: The screen agar plates of Mueller-Hinton agar with 4 % NaCl containing 6 µg/ml oxacillin were used. The bacterial inoculum 10^7 CFU/plate as recommended by NCCLS guidelines was used. Any growth after incubation at 35 °C for 24 h was interpreted as a positive oxacillin agar screen result for MRSA (Tab. 1).

Broth dilution method: (for determining the MICs) with specific modifications: the addition of 2 % NaCl to broth used for oxacillin testing; direct preparation of inoculum from growth on an overnight agar plate; and incubation at 35 °C for 24 h (tab. 1).

Detection of mecA gene by PCR: As a template for PCR amplification, 5 µl of purified DNA was diluted 10 times in deionized distilled water, denatured at 95 °C for 5 min, and chilled on ice. The primer pair used were: 5'-AAA ATC GAT GGT AAA GGT TGG C-3' and 5'-AGT TCT GCA GTA CCG GAT TTG C-3'. The reaction mixture contained 200 µM of each nucleotide triphosphate, 0.25 mM, each primer 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, and 0.1 % Triton. To a 100 µl reaction volume, 0.5 µl of Taq polymerase (5 U/ml) (Promega) was added. Amplification was carried out using a thermal cycler under the following conditions: 40 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min were followed by 5 min at 72 °C. A positive result was inferred by detection of a 533-bp band representing part of the *mecA* gene by electrophoresis on a 2 % agarose gel for 1 h at 100 V.

The control organism included *Staphylococcus aureus* ATCC 29213 (Tab. 1).

Results

We studied 120 strains of *S. aureus*. Out of this number, 61 strain were *mecA*-positive and concomitant manifest resistance to methicillin was confirmed by disk diffusion method. Only eight strains, resistant using disk diffusion method, had no *mecA* gene (were *mecA* negative). Thus, specificity and sensitivity of this method, in comparison with PCR were 86.2 and 98.4 %, respectively in our examined strains (Tab. 2).

The study using agar screen test showed that 60 *mecA*-positive strains were oxacillin agar screen positive and only two *mecA*-positive strains were oxacillin agar screen negative. Accordingly, specificity and sensitivity of this test, compared to PCR as a "gold standard", were 91.3 and 96.8 %, respectively.

Tab. 2. Evaluation of disk diffusion, broth dilution and oxacillin screen method for the detection of oxacillin resistance in 120 *Staphylococcus aureus* isolates.

MecA results	PCR No of isolates tested	Disk diffusion method		Oxacillin agar screen		Broth dilution method	
		Pos.*	Neg.**	Pos.	Neg.	Pos.	Neg.
Positive	62	61	1	60	2	62	/
Negative	58	8	50	5	53	8	50
Specificity		86.2%		91.3%		86.2%	
Sensitivity		98.4%		96.8%		100%	

* positive, ** negative

Tab. 3. Phenotypes and genotypes of isolates showing different results by one or more tests (*mecA*-positive isolates).

Isolate	Origin	Source	MecA PCR result	Result of MRSA using disk diffusion test	Oxacillin screen test result	MIC (mg/l susceptibility) using broth dilution method
SA*1	ICU**	Blood	+	+	-	128
SA2	Surgery	Wound	+	+	+	128
SA3	Pediatric surgery	Wound	+	-	+	64
SA4	ICU	Tubus	+	+	+	2
SA5	Abdominal surgery	Pus	+	+	+	1
SA6	ICU	Tubus	+	+	+	1

* *Staphylococcus aureus*, ** Intensive Care Units

The third test (broth dilution method) revealed that all *mecA*-positive strains (a total of 62) were resistant to methicillin. The eight *mecA*-negative strains were also oxacillin susceptible and consequently, specificity and sensitivity of this test, in relation to PCR test, were 86.2 and 100.0 %, respectively.

Although existence of *mecA*-positive, oxacillin susceptible strains is reported in the literature data, we had no such findings. Though in vitro studies have shown that exposure of several *mecA*-positive, phenotypically methicillin – susceptible *S. aureus* isolates to beta-lactams resulted in an increased MIC of oxacillin above the established breakpoint for resistance (oxacillin MIC, >4 mg/l), initial susceptibility testing had revealed these isolates were susceptible (4, 6, 7) (Tabs 3, 4).

Discussion

The strain marked as SA1 was isolated from a hemoculture of a patient hospitalized at the Intensive Care Unit for a longer period of time. The patient suffered from polytrauma after a traffic accident and the same strain was detected in three consequent hemoculture with the absolutely same antibiogram. Although the agar screen test did not show a colony, the remaining three tests confirmed that it was MRSA strain: *mecA*-positive with an increased MIC of oxacillin (128 mg/l).

The isolate named as SA_2 originated from a wound of a patient caused by a dog bite, which was additionally infected with *Staphylococcus aureus* strain. Only one sample was sent to our Institute for examination and using disk diffusion method *Staphylococcus aureus* was detected, resistant to oxacillin. The additional examinations using PCR and broth dilution method confirmed the resistance of the strain to oxacillin: *mecA* positive and high MIC: 128 mg/l. The agar screen test showed poor growth and therefore, we labeled the strain as moderately susceptible. It was deduced that it was methicillin resistant *Staphylococcus aureus* that was successfully treated with vancomycin therapy.

The third strain (SA_3) was also a wound isolate discovered in a child after physical injury and open fracture of the leg. The standard microbiological processing on saline agar has shown its resistance to oxacillin, but its multi-antibiotic resistance (susceptible only to cotrimoxazole and vancomycin, but resistant to cefixime, ceftriaxone, cefuroxime, ampicillin, midekamicyne, azytromicine, cirofloxacine, clindamycin, amoxicillin-clavulinic acid and amykacyne) led us to examine this strain with the remaining three methods. As we have presumed, it has shown resistance to oxacilline. This fact encouraged us to mark this strain as MRSA: present *mecA* gene, resistance with agar screen test and high MIC to oxacillin with broth dilution method: 64 mg/l.

Tab. 4. Phenotypes and genotypes of isolates showing different results by one or more tests (mecA-negative isolates).

Isolate	Origin	Source	MecA PCR result	Result of MRSA using disk diffusion test	Oxacillin screen test result	MIC (mg/l susceptibility) using broth dilution method
SA7	Plastic surgery	Wound	-	+	+	4
SA8	Nephrology	Catheter	-	+	+	4
SA9	Cardiology	Blood	-	+	+	2
SA10	Thoracic surgery	Canula	-	+	+	2
SA11	Orthopedic surgery	Wound	-	+	+	2
SA12	ICU	Tubus	-	+	+	4
SA13	Trauma	Wound	-	+	-	128
SA14	ICU	Tubus	-	+	+	≥256

The fourth and the sixth strains were from different patients hospitalized in the same time at the Intensive Care Unit, but in two different rooms. Both strains were isolated from tubus and had many identical characteristics: same antibiotypes (susceptible only to cotrimoxazol and vancomycin and resistant to all other routinely treated antibiotics, resistant to oxacillin using agar screen test, *mecA* positive, with small difference in MIC of oxacillin: strain SA_4 with MIC = 2 mg/l and strain SA_6 with MIC = 1 mg/l. In spite of their common characteristics, it is necessary to make additional examinations that would result in their final typing and would confirm the identical strain transferred from one patient to the other.

The fifth strain (SA_5) was isolated from a pus of a patient with abscess in the abdomen previously surgically treated. *E. coli* and *Bacteroides fragilis* were found, too. This strain showed a resistance to oxacillin using three methods PCR (*mecA* positive), oxacillin agar test and disk diffusion method, whereas broth dilution method showed that MIC of oxacillin towards this strain was 2 mg/l. In spite of this discrepancy, vancomycin was introduced in the therapy which resulted in negative MRSA in the sample of the patient and the patient felt clinical relief.

The strains from 7 through 12 were oxacillin resistant, but they lack the intrinsic resistance – had no *mecA* gene. According to their characteristics (values of their MICs of oxacillin), we can classify them as the groups of BODSA and MODSA strains. For more precise defining, it is advisable to examine the production of beta-lactams, too.

Three isolates (SA_7, SA_8 and SA_{12}) had entirely same characteristics, but their origin was completely different (Tab. 4). The remaining three strains had the same MIC of oxacillin (2 mg/l), however, the strain SA_9 showed poor growth on oxacillin screen test allowing it to be defined as intermediary strain susceptibility to oxacillin using this test. These examined strains were from different samples and different clinics, which excludes their mutual association.

Numerous studies have been conducted to determine optimal methods for phenotypic detection of oxacillin (methicillin) resistance among clinical isolates of staphylococci. Several studies have demonstrated that 100 % of oxacillin resistant *S. aureus* test isolates were detected by either broth dilution, agar dilution, agar spot screen, gradient diffusion (Epsilometar test), or disk diffusion method or by automated API-Plus system (bioMerieux). For these methods, different concentrations of NaCl in media (0 to 4 %) or varying incubation times (24 or 48 h) had little effect (5–9).

Susceptibility testing of methicillin resistance in *S. aureus* may be problematic due to heterogeneous resistance displayed by many clinical isolates. Some isolates may contain *mecA* gene, but however they may appear phenotypic susceptibility if they are exposed to antistaphylococcal penicillins. After exposition, they become methicillin-resistant. Furthermore, standard susceptibility testing requires an additional 24-h incubation period compared to time required for assays for *mecA* or PBP2a (8, 9, 10).

There are no available data dictating the optimal therapy for infections with *S. aureus* isolates that are phenotypically susceptible to oxacillin but carry *mecA*. In vitro data demonstrate that such isolates are heteroresistant, with only 1 in 10^8 or fewer cells expressing high-level resistance. Because the inoculum sizes used in standard susceptibility testing are orders of magnitude lower than the numbers of isolates, they may not be detected as methicillin resistant. Incubation of these heteroresistant isolates in gradually higher levels of a beta-lactam can yield highly resistant cells resulting in treatment failure (7, 10, 11).

In conclusion, for the rapid detection of MRSA, *mecA* gene based PCR appears to be promising. Since variations among the methods exist and no acceptable guidelines are formulated, a combination of conventional methods, including either 3 μg of oxacillin/ml. in Mueller Hinton broth or one of the screen agar plates (6 μg/ml), alone or with PCR should be the method of choice for the detection of MRSA (11).

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