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A COMPARISON OF PCR DETECTION OF *meca* WITH OXACILLIN DISK SUSCEPTIBILITY TESTING IN DIFFERENT MEDIA AND SCEPTOR AUTOMATED SYSTEM FOR BOTH *STAPHYLOCOCCUS AUREUS* AND COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATES

*S Ercis, B Sancak, G Hasçelik*

Abstract

**Purpose:** To evaluate three methods for 406 isolates of *Staphylococcus aureus* and coagulase-negative staphylococci (CNS) for the detection of methicillin resistance (MR) using National Committee for Clinical Laboratory Standards (NCCLS) new interpretive criteria. **Methods:** We used polymerase chain reaction (PCR) as a gold standard method to evaluate three methods [disk diffusion with Mueller-Hinton agar (MHA) and mannitol salt agar (MSA) and Sceptor system (Becton Dickinson, USA)] for the detection of *meca* gene. The isolates that were methicillin-resistant with any of the three tests were evaluated further for MR by E-test. **Results:** MHA, MSA and Sceptor showed sensitivities of 100, 100 and 99% for *S. aureus* and 100, 82.6 and 72.1% for CNS, respectively. The specificities of the same methods were found as 100, 90.1 and 99.3% for *S. aureus* and 79.2, 95.8 and 97.2% for CNS, respectively. E-test showed 100% sensitivity for both *S. aureus* and CNS. Forty-eight CNS and 16 *S. aureus* isolates, which presented discrepancies with the three phenotypic methods (MHA disk diffusion method, MSA disk diffusion method and Sceptor), were correctly classified as resistant/susceptible with the E-test when compared with PCR. Only five CNS isolates, which were *meca*-negative with PCR were resistant with E-test. Analysis of 248 *S. aureus* revealed that MHA is superior to other phenotype-based susceptibility testing methods in detecting MR. When we examined the results of 158 CNS, none of the three methods proved efficient in detecting MR. **Conclusions:** We conclude that although the accuracy of the MHA disk diffusion test for the detection of MR approaches the accuracy of PCR for *S. aureus* isolates, the need for easy and reliable methods of detecting MR in CNS still remains.

**Key words:** Coagulase-negative staphylococci, *meca*, methicillin-resistant *Staphylococcus aureus*

The incidence of nosocomial infections caused by methicillin-resistant staphylococci continues to increase in many countries worldwide. Therefore, rapid and accurate detection of MR strains of staphylococci and differentiating them from susceptible strains by clinical microbiology laboratories have a great importance in the therapy of infectious diseases caused by staphylococci.[1] Several methods for the detection of MR in staphylococci have been evaluated and widely used in many bacteriological laboratories including the following: agar dilution, disk diffusion, MIC determination by broth dilution, oxacillin agar screening test, automated systems such as the Vitek.[2,3]

Methicillin resistance in staphylococci is due to the acquisition of the *meca* gene, which encodes the low-affinity penicillin-binding protein 2a.[4] Presence of the *meca* gene defines the *Staphylococcus* as MR, while absence of the gene from a staphylococcal strain indicates methicillin susceptibility (MS).[5] MR can be difficult to detect because *meca*-positive strains can differ in their level of expression of MR.[4] Resistance is typically heterogeneous with only a few cells, one in 10^4 or 10^6, expressing the phenotype.[6] In the routine laboratories, phenotypical methods are preferred in detecting MR, but it is time consuming and there are some difficulties in detecting all of the resistant isolates. Many factors including inoculum size, incubation time, incubation temperature, beta-lactam antibiotic being tested, pH of the culture medium and NaCl concentration have a major effect on the expression and therefore the detection of resistance.[7] Hence, the tests based on detection of genotype are more accurate than phenotypic methods.[1] The *meca* gene is highly conserved among staphylococcal species, therefore, presently, detection of this gene by polymerase chain reaction (PCR) is considered as “gold standard” for detection of MR in staphylococci.[8-10]

In the present study, we planned to evaluate the methicillin susceptibilities of coagulase-negative staphylococci (CNS) and *Staphylococcus aureus* clinical isolates using various susceptibility testing methods [disk diffusion method with Mueller-Hinton agar (MHA), disk diffusion method with mannitol salt agar (MSA) and Sceptor system] by comparing the results obtained by *meca*-based PCR.
Materials and Methods

Clinical isolates

A total of 406 isolates of staphylococci, isolated from different clinical specimens in Adult Hospital Microbiology Laboratories of Hacettepe University Medical Faculty, were included in the study. All isolates were identified as *S. aureus* (n = 248) or CNS (n = 158) by colony morphology, Gram staining, catalase, coagulase tests and Sceptor automated system (Becton Dickinson, USA).

These 406 clinical isolates were tested for MR by disk diffusion with MHA and MSA and Sceptor automated system. From this collection, all isolates were evaluated further with mecA-based PCR. All of the isolates were stored at −20 °C until use. Reference strains included clinical MRSA strain COL,[11] S. aureus ATCC 29213 and 25923. Positive (clinical MRSA strain COL) and negative (S. aureus ATCC 29213) target DNA controls were included in every set of PCR.

Disk diffusion test with MHA and MSA

The disk agar diffusion was performed by following the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS).[12] A sterile cotton swab was dipped in a suspension, which was adjusted to 0.5 McFarland turbidity. After the swab was streaked uniformly onto both MHA and MSA, 1 µg oxacillin disks (BBL) were applied onto each plate. After incubation at 35 °C for 24 hours, the zone diameters were documented in millimeters for both *S. aureus* isolates and CNS.

For MHA disk diffusion method, *S. aureus* ≤10 mm zone size was accepted as resistant and for CNS, zone size was accepted as ≤17 mm.[12] For MSA disk diffusion method, both for *S. aureus* and CNS, zone sizes were accepted as ≤16 mm.[13]

Sceptor automated system

Automated testing with Sceptor was performed according to manufacturer’s instructions. The same suspension, which was prepared for disk diffusion test was also used for Sceptor automated system. The reading of the results was done according to manufacturer’s recommendations. For the Sceptor system, MIC category breakpoints used for determining MS were those recommended by the NCCLS.[12]

MIC of oxacillin by E-test

Oxacillin MICs were measured only for the isolates that were found resistant either with any of the phenotypic tests or PCR. E-tests (AB Biodisk, Solna, Sweden) were performed according to the manufacturer’s instructions. A sterile swab was dipped into an inoculum suspension (McFarland standard 0.5) prepared in Mueller-Hinton broth. The entire surface of an MHA plate (2% NaCl supplement) was swabbed by rotating the plate to ensure an even distribution of the inoculum. An E-test strip was placed aseptically onto the agar plate. After incubation at 35 °C, MIC values were read at 24 hours for *S. aureus* and at 48 hours for CNS. The MIC was read at the point of intersection between the zone edge and the E-test strip. The isolates that had MIC value ≥4 µg/mL for *S. aureus* and ≥0.5 µg/mL for CNS were accepted as methicillin-resistant.

Detection of mecA by polymerase chain reaction

Bacterial DNA was obtained by the rapid cell lysis method as described by Ünal et al.[14] For DNA extraction, 0.1 mL of an overnight culture of bacteria in Mueller-Hinton broth was harvested by centrifuging in a microcentrifuge tube at 16,000×g for 30 seconds. The precipitates were resuspended in 50 µL lysostaphin (100 µg/mL; Sigma) and incubated at 37 °C for 10 minutes. Following the addition of 50 µL proteinase K (100 µg/mL; Sigma Aldrich) and 150 µL 100 mM Tris (pH 7.5), the suspension was incubated for 10 minutes at 37 °C and then boiled for 5 minutes. After centrifugation at 13,000×g for 2 minutes, the supernatant containing the extracted bacterial DNA was used in the PCR assay.

PCR was performed using the previously described mecA gene-specific primer pairs.[14] The primer pair of mecA1 (primer 1) and mecA2 (primer 2) is complementary to sequences within the mecA gene open reading frame and yields an amplimer of 1.8 kb. The reaction mixture (25 µL) contained 100 pmol of each primer, Tag polymerase (2.5 U), Mg (2.5 mM), PCR buffer and 3 µL of template DNA. Cycling conditions consisted of 3 minutes at 94 °C, followed by 40 cycles of 30 s at 94 °C, 45 s at 60 °C, 1.5 minutes at 72 °C, followed by 72 °C for 3.5 minutes. Amplification products were separated by electrophoresis through 0.8% agarose gels in TAE for 2 hours at 100 V, stained with ethidium bromide and photographed under UV illumination.

Results

We studied 406 clinical isolates consisting of 248 *S. aureus* and 158 CNS. Of these, 106 (42.7%) *S. aureus* and 86 (54.4%) CNS were methicillin-resistant by PCR. The sensitivity and specificity of all phenotypic methods against PCR as a gold standard are given in Tables 1 and 2.

The MHA disk diffusion test fully agreed for 106 mecA-positive and 142 mecA-negative *S. aureus* strains (sensitivity 100%, specificity 100%). All of 106 mecA-positive *S. aureus* isolates were correctly determined as methicillin-resistant by disk diffusion on MSA (sensitivity 100%) and 128 of the 142 mecA-negative isolates were found to be methicillin-susceptible (specificity, 90.1%). Sceptor identified 105 of 106 mecA-positive *S. aureus* isolates and yielded 141 of 142 mecA-negative isolates corresponding to a sensitivity of 99% and a specificity of 99.3%.

All of 86 mecA-positive CNS were identified as resistant by MHA disk diffusion test (sensitivity 100%), but 57 of
Sensitivity Specificity
MHA 100% (106/106) 100% (142/142)
MSA 100% (106/106) 90.1% (128/142)
Sceptor 99% (105/106) 99.3% (141/142)

E-test was performed only on the isolates that were resistant either with phenotypic tests or with PCR. The results of the E-test for MR agreed with 106 of 106 mecA-positive for S. aureus isolates and 85 of 86 mecA-positive CNS isolates, which presented discrepancies with three phenotypic methods (MHA disk diffusion method, MSA disk diffusion test and Sceptor), 34 were correctly classified as resistant and 14 were classified as susceptible with E-test. Only five CNS isolates that were mecA-negative with PCR were resistant with E-test. All 16 S. aureus isolates that were resistant with any of the three phenotypic methods were correctly classified by E-test when compared with PCR (15 isolates susceptible; 1 isolate resistant).

Discussion

Currently available phenotypic methods for the detection of MR in S. aureus and CNS are problematic because of the heterogeneous resistance displayed by many clinical isolates.[3] To overcome this problem, many phenotypic and genotypic methods have been used. Genotype-based methods of detecting methicillin-resistant staphylococci are more accurate than susceptibility testing. While PCR is considered as the gold standard assay for the detection of MR,[9,10] it remains a time-consuming and expensive method; besides, it is not available for most of the routine laboratories. The automated systems are appealing because results can be obtained in a short time. Unfortunately, for many of these systems, the sensitivity for detection of MR is low.[1,15,16]

In the present study, we evaluated different phenotypic tests for the detection of MR in staphylococci with mecA PCR using new NCCLS criteria.[12] For MHA disk diffusion method, our results were compatible with other studies.[17-19] In our study, the sensitivity and specificity of detecting MR were 100 and 100% for S. aureus, 100 and 79.2% for CNS, respectively, compared to mecA-based PCR.

Although MHA disk diffusion test has a high sensitivity and specificity of detecting MR for S. aureus isolates, it could not show the same success for CNS isolates. Therefore, we intended to evaluate the performance of MSA disk diffusion test that was used earlier for detection of MR of S. aureus. Though Kampf et al.[13] found high sensitivity and specificity for S. aureus, in our study we found that MSA showed less specificity for both S. aureus and CNS isolates. One of the reasons for that could be the breakpoints that we used in this study. To our knowledge, this is the first study that evaluates the methicillin susceptibilities of CNS using MSA disk diffusion method. Therefore, we used the breakpoints determined by Kampf et al.[13] for S. aureus but not for CNS.

The E-test has the advantage of being easy to perform as a disk diffusion test and approaches the accuracy of PCR for mecA. There are several reports comparing E-test with dilution and PCR methods with generally good results, depending on the particular combination of medium and incubation conditions used.[2,13,20]
results are comparable to those of other studies that have evaluated the E-test. It showed sensitivity of 100% both for S. aureus and for CNS. Forty-eight CNS and 16 S. aureus isolates, which presented discrepancies by three phenotypic methods (MHA disk diffusion method, MSA disk diffusion method and Sceptor), were correctly classified as resistant/ susceptible with E-test when compared with PCR. Only five CNS isolates that were mecA-negative with PCR were resistant with E-test. Unfortunately, we could not perform E-test on all mecA-negative isolates. Thus, detection of the specificity of this test was not possible. Our results suggest that E-test, which is available in most of the clinical microbiology laboratories, could be an accurate method to detect MR in staphylococci.

In conclusion, our results showed that for detection of MR in S. aureus, MHA disk diffusion test is a reliable method and can be easily performed in routine microbiology laboratories. For CNS isolates, detection of MR with phenotypic methods still seems to be a big problem and needs to be confirmed with molecular methods. Though recent NCCLS modifications to the oxacillin MIC breakpoints and the disk diffusion zone sizes have improved the accuracy of detecting resistance in CNS, new studies showed that the new NCCLS criteria are not as specific for certain species of CNS such as S. cohnii, S. warneri, S. lugdunensis and S. saprophyticus. Therefore, in order to improve the detection of MR of disk diffusion method, determination of new breakpoints for certain species of CNS may prove to be better in the future.

References