COMPARISON OF THE PCR WITH THE CEFOXITIN DISC DIFFUSION TEST FOR DETECTION OF METHICILLIN RESISTANCE IN OXACILLIN RESISTANT COAGULASE-NEGATIVE STAPHYLOCOCCI (CONS)

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ABSTRACT
Nosocomial infections are a prevalent problem all over the world. Bacteraemias are the commonest types of infections and have high mortality and morbidity rates. S. epidermidis and S. aureus are the most frequently associated infectious agents with bacteremia-acquired in hospital. It is common that these pathogens are resistant to methicillin that limits treatment alternatives. Several methods are developed to detect methicillin resistance rapidly and accurately. This study was aimed to compare the oxacillin and cefoxitin disc diffusion test with PCR for detection of methicillin resistance. Thirty-two CNS strains isolated from blood culture samples that were collected from hospitalized patients in ICUs of our hospital between October-December 2007 and determined to be resistant by oxacillin disc diffusion test, were included. Three strains were sensitive to cefoxitin and the rest of them were resistant. A total of 59.3% of oxacillin resistant strains and 55.2% of cefoxitin resistant strains were mecA positive. The detection of methicillin resistance by disc diffusion test is often false in a high percentage and therefore, especially in ICUs where critical patients are hospitalized, the usage of molecular techniques is more suitable.


Keywords: coagulase-negative staphylococci, methicillin resistance, PCR, cefoxitin disc diffusion test

Introduction
Coagulase negative staphylococci have been frequently isolated as an agent of nosocomial infections in last twenty years although they are pathogens which have low virulence rate (12, 13). They are colonized in hospital environment and hospitalized patients’ skin. Immunosuppressive therapies, invasive procedures, common usage of broad-spectrum antibiotics tend to result in bacterial infections. NNIS (National Nosocomial Infections Surveillance) and SCOPE studies in ICUs in USA reported that CoNS was an important agent that may lead to hospital acquired bacteremia (7, 10). EPIC (Europe Prevalence Infection Committee) study also reported that CoNS was the fourth most common cause of nosocomial infections (32). CoNS oxacillin (methicillin) resistant strains are endemic all over the world (13). CoNS also plays an important role such as reservoir out of which antibiotic resistance genes might be transferred to other Staphylococcus species. All this turns beta-lactam antibiotics ineffective for MRSA infection treatment and causes failure of therapy (23, 24).

In recent years, it was reported that MRSA strains also vastly developed an increased resistance to aminoglycosides and quinolons (9, 19, 23). In this case, the unique choice is glycopeptides such as vancomycin and teicoplanin.

Usage of molecular techniques for detection of mecA gene in methicillin resistant bacteria is accepted as “a gold standard”.

The heterogeneous resistant strains with microorganism population can be detected by PCR. However, the application of these techniques requires certain conditions and so far it is really hard to utilize such tests in the clinical laboratories. Several methods for detection of methicillin resistance in CoNS were improved, and oxacillin and cefoxitin disc diffusion test is a frequently used one (4, 5, 20).
DNA isolation
PCR procedure was performed according to standard DNA extraction protocol (18). Rapid lysis procedure was applied: 0.1 ml buyon culture (=10⁸ bacteria cells) was taken for 24 hours after incubation, and bacteria cells were taken through buyon by centrifugation at 16 000 x g in microcentrifuge (Eppendorf Microfuge 5415) for 30 sec, and then 50 µl lysostaphin (100µg/ml, Sigma Chemical Co., St. Louis, Mo.) was added. Bacterial suspension was incubated at 37°C for 10min and 50 µl Proteinase K solution (100µg/ml, Sigma) and 150 µl buffer (0.1 M tris [pH 7.5]) were added. The mixture was boiled for 5min followed by incubation for 10min and 10 µl of the obtained DNA was used for the PCR procedure.

PCR primers and amplification conditions
Two oligonucleotide primers were designed based on sequence of mecA gene that belongs to S. aureus T784. The used primers were as follows: 5'-GttGt AGttGtcGGGtttGG-3' and 5'-ccAcccAAtttGtctGccAGtttctcc-3' where the first primer was targeted at position 37-57 within the mecA gene and the second primer was a reverse transcript corresponding to position 1827-1854 (18, 27). These primers are located on an open reading frame (ORF) of mecA. PCR was performed on the DNA thermal cycle (Model 9810) and GeneAmp Kit (Perkin Elmer Cetus, Norwalk, Conn) was used. The cycling profile consisted of denaturation step at 94°C for 30 sec, followed by cycles of annealing at 55°C for 30 sec, extension at 72°C for 2 min for a total of 30 cycles. A total of 12 µl from the amplification mixture was mixed up with 2 µl loading dye and 2% agarose gel was prepared (2 gr agarose, 100ml 1XtAe (1mM tris acetate, 1mM eDtA with distilled water to final volume of 100ml) and Etidium bromide was added. After electrophoresis (at 100 volts for 1 hour) the gel was visualized under UV light (≈304 nm). HaeII XH174 DNA size marker was used as control. 1107 bp band was evaluated as positive. Statistical analysis was not performed because of included OX resistant isolates in this study.

Results and Discussion
In our study, 32 oxacillin resistant CoNS isolates were tested. Strains were isolated from blood cultures that were obtained from patients hospitalized in ICUs. Thirteen isolates (40.6%) from internal ICU (iICU), 11 (34.4%) from surgical ICU (SICU), 7 (21.8%) from pediatric ICU (PICU) and 1 (3.2%) from neonatal ICU (NICU) were isolated. Eighteen S. epidermidis (56.1%), 5 S. haemolyticus (15.1%), 4 S. auricularis (12.5%), 2 S. cohnii (6.3%) and 1 S. warneri (3.1%) were identified from the detected 32 CoNS isolates. Oxacillin and cefoxitin sensitivities of these isolates and comparison of disc diffusion test with mecA gene positivity were shown at Table 1. Three oxacillin resistant cefoxitin sensitive CoNS isolates were identified as S. epidermidis (Fig. 1).

Three cefoxitin sensitive of the 32 oxacillin resistant CoNS isolates were detected as mecA positive. Thirteen of 29 cefoxitin resistant isolates were mecA negative. Of 32 oxacillin resistant CoNS, 19 mecA positive and 13 mecA negative were determined. mecA gene positivity of isolates was shown at Table 2. Of 18 S. epidermidis isolates, 3 were cefoxitin sensitive and 6 were mecA negative. All of the 5 S. haemolyticus isolates were resistant to oxacillin and also cefoxitin and 1 isolate was mecA negative. All 4 S. auricularis isolates were resistant to oxacillin and cefoxitin and 2 isolates were mecA negative. Results of mecA positivity by PCR was shown at Fig. 2.

**TABLE 1** Comparison of Disc Diffusion Test with mecA Gene Positivity

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Disc Diffusion</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>32 R</td>
<td>19 (59.3)</td>
</tr>
<tr>
<td></td>
<td>0 S</td>
<td>13 (40.7)</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>29 R</td>
<td>16 (55.2)</td>
</tr>
<tr>
<td></td>
<td>3 S</td>
<td>13 (44.8)</td>
</tr>
</tbody>
</table>

Fig. 1. Disc Diffusion Test

In spite the advance of antibiotic therapy and usage of support and protective precaution, frequently infections in patients from ICUs are the most significant cause of mortality (30). ICUs are high risk areas for nosocomial infections. Furthermore, 20-25% of nosocomial infections have developed in ICUs. The risk of nosocomial infection is higher 5-7 fold in ICUs than other areas within a hospital (26, 32). This frequency differs among the hospitals. In a study that included 1747 ICUs from Europe, the rate of nosocomial infections was detected as 20.6% (2, 21). According to studies from several centers of Turkey, the rate of nosocomial infection in ICUs varied between 5.3-56.1% (1). The remarkable differences among the hospitals in Turkey, point to the renewal of surveillance standards at time to time, and also whether the centers adopted themselves appropriately to the given standards.

Nosocomial infections are most frequent in ICUs because of invasive procedures such as ventilation, tracheostomy and catheterization, and common usage of broad-spectrum antibiotics leading to development of resistant pathogens (12,
In USA, a nosocomial infection has been developed in over 2 million patients annually and of 5-35% of them have been in ICUs. According to Washington Medical Institute, nosocomial infections cause to 44 000-98 000 mortality cases and 17-29 billion dollars in costs per year (8). National and international guides, local data, microbial agents and resistance profiles and the principles of antibiotic usage are important factors for antibiotic therapy (17). For the antibiotic appropriate and sufficient usage, the knowledge of local antibiotic resistance profile is necessary both to choose empirical antimicrobial therapy and reduce resistance rate (15, 18).

### TABLE 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Rates of mecA positive strain (%)</th>
<th>Rates of mecA negative strain (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis</td>
<td>12 (66.6)</td>
<td>6 (34.4)</td>
<td>18</td>
</tr>
<tr>
<td>S. haemolyticus</td>
<td>4 (80)</td>
<td>1 (20)</td>
<td>5</td>
</tr>
<tr>
<td>S. auricularis</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td>4</td>
</tr>
<tr>
<td>S. hominis</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>2</td>
</tr>
<tr>
<td>S. cohnii</td>
<td>0 (0)</td>
<td>2 (100)</td>
<td>2</td>
</tr>
<tr>
<td>S. warneri</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>19 (59.4)</td>
<td>13 (40.6)</td>
<td>32</td>
</tr>
</tbody>
</table>

![Fig. 2. mec A Gene Positivity](image)

All of the oxacillin (methicillin) resistant strains of CoNS are prevalent all over the world (13). CoNS in this group are resistant to most other antibiotics (29). Even if it is quite low, resistance to glycopeptides that are frequently used to treat MRSA infections, was also reported. In SENTRY study, while only 9 (0.4%) of 2068 CoNS strains collected from European centers were resistant to teicoplanin, none of them was resistant to vancomycin (6). In a study from Turkey by Uzun and colleagues, CoNS were most sensitive to vancomycin and teicoplanin and most resistant to oxacillin and amoxicillin/clavulanate (30).

In our country, Koksal and colleagues found that methicillin resistant CoNS rate was 67.5% in Istanbul (16). In Chest ICU of Konya Selcuk University, Uzun et al. reported that the rates of oxacillin (meticillin) resistance was 80% of CoNS and 66.7% of S. aureus. Furthermore, both CoNS and S. aureus were 100% sensitive to vancomycin and teicoplanine (30).

Heterogeneity of methicillin resistance has complicated the detection and identification of these strains at the clinical microbiology laboratories. The usage of beta-lactam antibiotics stored under improper circumstances, the short incubation period (18 instead of 24 hours), incubation at 37°C or low inoculum quantity may cause false results (5, 11).

Oxacillin was suggested to detect resistance to PSPs (penicillinase stable penicillin) as phenotyping test by CLSI. Several studies reported that stability and sensitivity of oxacillin were superior than the other methods used to detect sensitivity to PSPs (27, 28). Nevertheless the difficulties of evaluation have often been reported although such methods to discriminate between sensitivity at oxacillin tests were developed (27, 28). Both MIC panels and disc diffusion plates with oxacillin should be carefully tested in order to detect any resistance (27, 28). CLSI suggested oxacillin agar screening in addition to dilution or disc diffusion tests (27). Furthermore oxacillin agar screen test has been recommended only for S. aureus but the evaluation of hetero-resistant strains was reported to be complicated (27).

As it is well known, the detection of mecA gene by PCR is “a gold standard” to determine the resistance to methicillin of S. aureus isolates. However it should not be forgotten that sensitive strains which have mecA gene might not express it (3,22).

### Conclusions

In our study, mecA was detected in 19 (59.3%) and Cefoxitin resistance in 29 (90.6%) among the 32 CoNS resistant strains. Oxacillin resistant isolates were included and 3 of them were sensitive to cefoxitin. 59.3% of oxacillin resistant and 55.2% of cefoxitin resistant strains were mecA positive. Three cefoxitin sensitive isolates were mecA positive. This result can be explained by the fact that detection with cefoxitin is less sensitive in heterogeneous strains. Disc diffusion test has been seen to be capitious for detection of methicillin resistance. Several studies in parallel with our results reported that detection of methicillin resistance by oxacillin and cefoxitin disc tests may be faulty (25, 31). According to the result of this study, both cefoxitin and oxacillin disc diffusion tests may not reveal definite results or may reveal incorrect results to detect resistance via mecA in CoNS. The determination tests of mecA based on PCR are widespread but their applications are considerably hard for routinely use because of lack of equipment and experienced technical staff. We suggested that molecular techniques are beneficial in departments where critical patients are hospitalized such as ICUs.

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