

Evaluation of Five Different Phenotypic Methods for Detection of Methicillin Resistance in Coagulase-Negative *Staphylococci* (CoNS) Isolated from Clinical Specimens

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Rapid and accurate detection of methicillin resistant coagulase -negative staphylococci (MRCoNS) is an important role of clinical microbiology laboratories to avoid treatment failure. The aim of this study was to compare conventional phenotypic methods for detection of methicillin resistance in CoNS isolates. Methicillin resistance was studied among clinical isolates of CoNS in Milad hospital of Tehran. Five different phenotypic methods including E-test MIC, oxacillin screening agar, oxacillin disk diffusion, cefoxitin disk diffusion and CHROMagar MRSA methods were used for detection of methicillin resistant CoNS. During our study, between January and December 2010 in total 114 CoNS strains were isolated from clinical specimens. The majority isolates were from blood culture. Oxacillin and cefoxitin both showed 98 methicillin resistant CoNS. The sensitivity and specificity for these two methods was 95.37% and 100% respectively. The sensitivity and specificity of oxacillin screening agar was similar to those disk diffusion methods. CHROMagar MRSA had low sensitivity in comparison to other methods All isolates including methicillin resistant CoNS were susceptible to vancomycin. Nearly 96% isolates were resistant to penicillin Rate of resistance to oxacillin was 90.35% using E-test MIC method. Our study revealed that all phenotypic methods have high sensitivity and specificity for detection of methicillin resistant CoNS. However the cefoxitin disk test is easy to perform and rapidly becoming the preferred method for detection of oxacillin heteroresistance in CoNS.

Keywords: Methicillin Resistant, Coagulase-Negative *Staphylococci*.

Coagulase- negative *Staphylococci* (CoNS) are normal flora of human body especially in skin and mucous membranes. CoNS have been the cause of both nosocomial and community acquired infections. The most important infection caused by CoNS including nosocomial blood stream infections especially in patients with indwelling catheters or other medical devices¹⁻³. According to the National Nosocomial Infections Surveillance System of National Center for Infectious Disease, 58% of neonatal nosocomial

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bacteremia cases are caused by CoNS. Resistance to methicillin among isolates of CoNS is very important due to cross-resistance to virtually all β -lactam drugs and other antibiotics as a result therapeutic protocol are restricted to vancomycin and new agents such as linezolid⁴. There are two essential ways responsible for the resistance of staphylococci to β -lactam antibiotics. The first mechanism includes production of β -lactamase that destroys antibiotics. The second mechanism is alteration of proteins located in cell wall of bacteria. These proteins are called penicillin binding proteins (PBPs). Most resistance to oxacillin by staphylococci is mediated by the *mecA* gene, which codes for production of additional penicillin binding protein named PBP2a. These proteins expressed either homogeneously or heterogeneously. Homogeneous resistance is easily detected with standard testing methods, whereas heterogeneous is more difficult to detect with some methods, because a fraction of the population of cells express the resistance phenotypic⁵.

There are many different laboratory methods including phenotypic and genotypic methods for detection of methicillin resistance in CoNS^{2,5-12}. Therefore correct detection of methicillin resistance in CoNS may allow selecting the better antimicrobial therapy and avoiding the selection of vancomycin and other expensive antibiotics.

Studies evaluating the accuracy of methods emphasizing on the phenotypic detection of methicillin resistance in CoNS in Islamic republic of Iran are scarce and limited to oxacillin disk diffusion method. In present study we aimed to evaluate methicillin resistance in CoNS isolated from clinical specimens using various phenotypic susceptibility testing methods, including disk diffusion method using both oxacillin and cefoxitin disks, oxacillin screen agar, MIC-E test and chromoagar MRSA methods^{2,5-11}.

MATERIAL AND METHODS

Clinical isolates of CoNS from different specimens including blood cultures, and other specimens between January and December 2010 in Milad hospital of Tehran were subject of our study. Milad hospital is a 1000-bed non-teaching tertiary care hospital. In total, one hundred and

fourteen strains of CoNS were isolated from specimens of patients admitted to our hospital. Out of 114 CoNS 107 (93.8%) strains were isolated from blood cultures. All of patients was hospitalized patients. Briefly the specimens were cultured aerobically in blood and MacConky agar. The plates were incubated overnight at 35°C. All isolates were identified using gram stain, biochemical tests including catalase, coagulase and DNase and other conventional biochemical tests⁹⁻¹⁰.

Susceptibility testing of all isolates was performed with oxacillin (1 μ g) and cefoxitin (30 μ g) disks, using Mueller Hinton agar with a suspension equivalent to 0.5 McFarland standards of the CoNS isolates. All plates were incubated at 35 °C for 24h. Zone of inhibition were measured in millimeter and interpreted as guideline recommended by CLSI. For MHA oxacillin disk diffuse on \leq 17mm zone of inhibition size was accepted resistant and zone \geq 18mm considered as susceptible. This figures for resistance and susceptibility to cefoxitin was \leq 24mm and \geq 25mm respectively. There was not any intermediate in this category.

The E-test method (AB Biodisk Solna Sweden) was used to determine MIC as recommended by manufacture. Briefly using Muller-Hinton agar supplemented with 2% NaCl and an inoculum density equivalent to 0.5 McFarland standards. A sterile swab was dipped into an inoculum suspension. The entire surface of Mueller-Hinton agar was swabbed by rotating the plate to ensure an even distribution of the inoculum. An E-test strip with sterile pans was placed aseptically onto the MHA plate. Plates were incubated at 35°C for 24h. The MIC was read at the point of intersection between the zone edge and the E-test. Isolation with MIC value $>$ 0.5mg/L were accepted MRCoNS¹². We used also oxacillin screening agar which was performed by inoculating a direct colony suspension (0.5 McFarland standard) with a swab spotting an area 10 to 15mm in diameter. Oxacillin screening agar was performed on Mueller-Hinton agar supplemented with 4% NaCl and 6 μ g/ml oxacillin as recommended by CLSI. After incubation at 35°C for 24 hours. Any growth was interpreted as positive for methicillin resistance¹². For detection of CoNS on CHROMagar MRSA a suspension of 0.5 McFarland was prepared and 10 μ L of bacterial suspension was streaked on above mentioned

medium as recommended by manufacture. All plates were incubated at 35°C for 24h. Strains growing on CHROMagar MRSA and yielding colonies with rose to mauve color were considered methicillin resistant CoNS as recommended by manufacture¹³.

S.aureus ATCC25923, ATCC25922 and *Pseudomona aerugiosa* ATCC 27853 were used as a control strains for quality control of susceptibility testing antibiotics disks. *S.aureus* ATCC 29213 was used as a control strains for detection of methicillin resistance¹².

RESULTS

During our study in total 408 staphylococcus spp were isolated from clinical specimens. Of 408 Stapylococcal isolates 114(28%) isolates were CoNS. *S. warnery* with 74 isolates

were the predominant isolates. Of 114 isolates 107(93.8%) strains were isolated from blood cultures, patient age ranged between nine month to 88 years old and the majority of patents were children. We observed a high rate of resistance among CoNS isolates. Of 114 isolates 103 (90.35%) were resistant to methicillin by E-test MIC method. By using oxacillin and cefoxitin disk diffusion method 98(86%) were resistant to methicillin. Results of oxacillin screening agar was the same as disk diffusion methods. Chromoagar showed just 87 MRCoNS. The sensitivity and specificity all methods were used for detection coagulase-negative methicillin resistant staphylococci are shown in Table 1.

Results of methciliin resistance among CONs isolate using different phenotypic methods are shown in Table 2.

Table 1. Sensitivity and specificity of phenotypic methods for detecton Methicillin resistance in CoNS

Method	Sensitivity	Specificity	PPV	NPV
E-Test MIC	100	100	100	100
Oxacillin disk	95.37	100	100	68.75
Cefoxitin disk	95.37	100	100	68.75
Oxacillin screening agar	95.37	100	100	68.75
Chromoagar	100	86.55	100	40.74

MIC=Minimum Inhibitory Concentration ,PPV=Positive Predictive Value ,NPV= Negative Predictive Value

Table 2. Result of susceptibility testing of CoNS for different antibiotics

Co-trimoxazole	Ampicillin	Ceftriaxone	Clindamycin	Erythromycin	Penicillin	Antibiotics
66%	67%	81%	84%	83%	97%	Resistance

Table 3. Results of methciliin resistance among CONs isolate using different phenotypic methods

Method	Susceptible	Intermediate	Resistant
E-Test MIC	11	0	103
Oxacillin disk	16	0	98
Cefoxitin disk	16	0	98
Oxacillin screening agar	16	0	98
Chromoagar	27	0	87

All isolates were susceptible to vancomycin. However two strains showed reduced susceptibility to vancomycin by E-test MIC method. Resistance to other antibiotics were also prevalent. Resistance to penicillin, erythromycin, clindamycin, ceftriaxone, ampicillin and cotrimoxazole were 97%, 82.5%, 84%, 82.5% and 66.66% respectively. Rifampin and chloramphenicol were the most effective antibiotics against methicillin resistant CoNS in comparison to other routinely used antibiotics. The result of susceptibility testing of CoNS are shown in table-3.

DISCUSSION

Staphylococci are the most commonly pathogens isolated from clinical specimens especially blood culture bottles¹⁴⁻¹⁷. Antibiotic susceptibility pattern of CoNS is unpredictable and for this reason, the recommendation now is to perform antimicrobial susceptibility testing to all isolates. Detection of Methicillin resistance in CoNS is the subject of many studies. Methicillin resistance in staphylococci is coded protein (PBP)PBP2(5). Currently there are some problems for detection of MRCoNS because of the heterogeneous resistance displayed by many clinical isolates. In recent years several studies have showed that molecular methods such as PCR are gold standard method for detection of *mecA*. However, most laboratories in our country are not in position to perform molecular methods. In addition molecular methods are not cost benefit in many laboratories especially in developing countries¹⁹⁻²⁰.

In the present study, we evaluated different phenotypic methods for detection of methicillin resistance in CoNS. In our study oxacillin and cefoxitin disk diffusion methods had 95.37% sensitivity and 100% specificity for detection MRCoNS. Although disk diffusion methods have a high sensitivity and specificity for MRSA but it could not show the same results for CoNS. Like other investigators we found high sensitivity and specificity for routine phenotypic methods for detection of MRCoNS. In our study we found that disk diffusion methods both with oxacillin and cefoxitin have a comparable results with OSA for detection of MRCoNS. Many studies have been showed that cefoxitin disk test is the preferred

method for the detection of oxacillin heteroresistance with high sensitivity and specificity. In our study the sensitivity of cefoxitin disk diffusion method was lower than other studies. The false –negative results in our study may be due to an extremely heterogeneous expression of resistance.

In our study sensitivity and specificity of oxacillin screening agar was the same as disk diffusion methods. Even though some recent studies have showed that oxacillin screening agar test is not efficient towards CoNS but some studies have pointed that oxacillin screening agar is more sensitive for detection of MRCoNS. In study carried out by Adalia et al they showed a good performance of all of the tests used. Comparative studies also by other researchers to assess the OSA and disk diffusion method have shown that OSA has good sensitivity and specificity for detection of MRCoNS.

At present study we used CHROMagar MRSA as a recently introduced method for detection methicillin resistance. Our study revealed a 100% sensitivity and 86.55 specificity for this method which is not comparable with other studies. One reason may be due that CHROMagar MRSA is designed for detection of MRSA and its application for MRCoNS has not standardized by manufacture.

The E-test has the advantage of being easy to perform as disk diffusion test and approaches the accuracy of PCR for *mecA*. There are several reports comparing E-test with dilution and PCR method with generally good results, depending on the particular combination and incubation condition used. In our laboratory PCR method is not available and for this reason we selected E-test method as a gold standard method and results of other phenotypic methods were compared with this method.

Because of increasing importance clinically significant CoNS should be identified to the species level and antimicrobial susceptibility testing especially against methicillin should be performed routinely for all isolates.

Methicillin resistant CoNS are traditionally resistant to a variety of antimicrobial agents including tobramycin, clindamycin, trimethoprim-sulfamethoxazole tetracycline and erythromycin. In our study resistance rate of

CoNS to, ceftriaxone, ampicillin and co-trimoxazole was respectively. It has also been reported that in vitro susceptibility of CoNS to fluoroquinolones drugs is higher for the MRCoNS than for the methicillin susceptible CoNS. In our study 60 (52.63) isolates of CoNS were resistant to ciprofloxacin.

CONCLUSION

Our results showed that E-test MIC is a reliable phenotypic method for detection of MRCoNS. Other methods including oxacillin, cefoxitin disk diffusion methods as well as OSA have a high sensitivity. These methods can be easily performed in routine microbiology laboratories. However the cefoxitin disk test is rapidly becoming the preferred method for detection of oxacillin heteroresistance.

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REFERENCES

- Jarløv JO, Busch-Sørensen C, Espersen F, Mortensen I, Hougaard DM, Rosdahl VT. Evaluation of different methods for the detection of methicillin resistance in coagulase-negative staphylococci. *J Antimicrob Chemother.* 1997; **40**: 241-9.
- Caierão J, Musskopf M, Superti S, Roesch E, Dias CG, d'Azevedo PA. Evaluation of phenotypic methods for methicillin resistance characterization in coagulase-negative staphylococci (CoNS). *J Med Microbiol.* 2004; **53**: 1195-9.
- Tenover FC, Jones RN, Swenson JM, Zimmer B, McAllister S, Jorgensen JH. Methods for improved detection of oxacillin resistance in coagulase-negative staphylococci: results of a multicenter study. *J Clin Microbiol.* 1999; **37**: 4051-8.
- Hussain Z, Stoakes L, Lannigan R, Longo S, Nancekivell B. Evaluation of screening and commercial methods for detection of methicillin resistance in coagulase-negative staphylococci. *J Clin Microbiol.* 1998; **36**: 273-4.
- Oliveira AD, d'Azevedo PA, de Sousa LB, Viana Niero C, Francisco W, Lottenberg C, Martino MD, Höfling-Lima AL. Laboratory detection methods for methicillin resistance in coagulase negative Staphylococcus isolated from ophthalmic infections. *Arq Bras Oftalmol.* 2007; **70**: 667-75.
- Ammerlaan H, Seifert H, Harbarth S, Brun-Buisson C, Torres A, Antonelli M, Kluytmans J, Bonten M; European Practices of Infections with Staphylococcus aureus (SEPIA) Study Group. Adequacy of antimicrobial treatment and outcome of Staphylococcus aureus bacteremia in 9 Western European countries. *Clin Infect Dis.* 2009; **49**: 997-1005.
- Brown DF. Detection of methicillin/oxacillin resistance in staphylococci. *J Antimicrob Chemother.* 2001; **48**Suppl 1:65-70.
- Merlino J, Watson J, Funnell G, Gottlieb T, Bradbury R, Harbour C. New screening medium for detection and identification of methicillin/oxacillin-resistant Staphylococcus aureus for nosocomial surveillance. *Eur J Clin Microbiol Infect Dis.* 2002; **21**: 414-6. Epub 2002 May 8.
- Mahon, C., GManuselis, Textbook of diagnostic microbiology, 3rd ed. Philadelphia, WB Saunders USA 2006.
- Isenberg, Henry D., editor., Clinical Microbiology Procedures Handbook, American Society for Microbiology, Washington DC 2004.
- Woods W, Ramotar K, Lem P, Toye B. Oxacillin susceptibility testing of coagulase-negative staphylococci using the disk diffusion method and the Vitek GPS-105 card (small star, filled). *Diagn Microbiol Infect Dis.* 2002; **42**: 291-4.
- Performance Standards for Antimicrobial Susceptibility Testing: Sixteenth Informational Supplement M100-S16. Wayne, PA, USA: CLSI; 2006. Clinical Laboratory Standards Institute
- Rahbar M, Islami P, Saremi M. Evaluation of a new CHROMagar medium for detection of methicillin-resistant *Staphylococcus aureus*. *Pak J. Biological.* 2008; **11**: 496-498.
- Ba° AY, Demirel N, Zenciroglu A, Göl N, Tanir G. Nosocomial blood stream infections in a neonatal intensive care unit in Ankara, Turkey. *Turk J Pediatr.* 2010; **52**: 464-70
- Kondo S, Misawa S, Oguri T, Yamada T, Ohsaka A, Miida T. The number of blood culture bottle sets and the clinical significance of Staphylococcus spp. isolated from the blood culture. *Rinsho Byori.* 2010; **58**: 437-41.
- Ferreira RB, Iorio NL, Malvar KL, Nunes AP, Fonseca LS, Bastos CC, Santos KR. Coagulase-negative staphylococci: comparison of

- phenotypic and genotypic oxacillin susceptibility tests and evaluation of the agar screening test by using different concentrations of oxacillin. *J Clin Microbiol.* 2003; **41**: 3609-14
17. Rahbar M, Gra-Agaji R, Hashemi S. Nosocomial blood stream infections in Imam Khomeini Hospital, Urmia, Islamic Republic of Iran, 1999-2001. *East Mediterr Health J.* 2005; **11**: 478-84.
18. Caierão J, Muskopf M, Superti S, Roesch E, Dias CG, d'Azevedo PA. Evaluation of phenotypic methods for methicillin resistance characterization in coagulase-negative staphylococci (*CNSJ Med Microbiol.* 2004; **53**: 1195-9.
19. Kohner P, Uhl J, Kolbert C, Persing D, Cockerill F 3rd. Comparison of susceptibility testing methods with mecA gene analysis for determining oxacillin (methicillin) resistance in clinical isolates of *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. *J Clin Microbiol.* 1999; **37**: 2952-61.
20. Rahbar, M , Safadel N. Evaluation of cefoxitin disk diffusion test for routine detection of methicillin resistant *Staphylococcus aureus*, *Iranian J Patholol.* 2006; **1**: 145-148.