Original article:

Performance of different phenotypic tests in detecting *mecA* mediated methicillin resistance in Coagulase negative staphylococci (CoNS)

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Abstract:

Accurate detection of methicillin resistance (MR) in coagulase negative staphylococci (CoNS) is of utmost importance as infections due to CoNS are on the rise. Various phenotypic methods have been described for detecting *mecA* mediated MR with varying performance characteristics. The present study was designed to evaluate the performance of oxacillin and cefoxitin disc diffusion / MIC test and oxacillin screen agar against NAAT for detecting *mecA* mediated MR in 150 strains of CoNS.

An inexpensive test to determine methicillin resistance reliably is of great value as NAAT are not easily available in all settings. In this study, cefoxitin disc diffusion accurately identified MRCoNS and can be used as a reliable test to determine the same.

Key words: Cefoxitin, *mecA*, MRCoNS, Oxacillin

Introduction:

Coagulase negative staphylococci (CoNS) have evolved from being a colonizer to an important healthcare associated (HCA) pathogen especially in high risk settings.[1] With penicillins and later penicillinase resistant penicillins becoming the mainstay of therapy, methicillin resistance (MR) in *Staphylococcus aureus* was first reported in 1960s.[2] Recent reports indicate not only a rising trend in the prevalence of methicillin resistance in staphylococci, but also a shift to the community acquired setting.[3,4] In India, methicillin resistance in coagulase-negative staphylococci (CoNS) varies from 22.5% to 64.8%.[3,5]

Resistance to penicillins is either due to the presence of an altered penicillin binding protein, PBP2a, which has a lower affinity to penicillin, encoded by *mecA* gene carried on the SCCmec element or due to the production of β-lactamases.[6,7] Strains carrying the *mecA* gene have become increasingly prevalent in health care and community associated infections worldwide, compromising treatment options.[1,2,8,9]

Conventional phenotypic methods of detection include disc diffusion / MIC testing with oxacillin and cefoxitin or oxacillin agar screening. Strains that possess *mecA* gene can be heterogeneous or homogeneous in their expression of resistance.[10,11] Routine oxacillin tests may fail to detect heterogeneous methicillin resistant populations, which are consequently reported as methicillin susceptible,[11] thereby directly impacting therapy given. Nucleic acid amplification tests (NAAT) are considered the gold standard for detection of *mecA* mediated methicillin resistance[1] but these molecular methods are expensive as well as not available in most centres. There is need for identifying an accurate phenotypic method which can be a part of the routine antimicrobial susceptibility testing (AMST) protocol in a diagnostic laboratory.[12] There is also a paucity of literature on *mecA* mediated methicillin resistance in coagulase negative staphylococci .

Aims and objectives:

This study was designed to evaluate the performance of oxacillin and cefoxitin disc diffusion / MIC test and oxacillin screen agar against NAAT for detecting *mecA* mediated methicillin resistance in Coagulase negative staphylococci (CoNS).

Materials:

150 consecutive, non- duplicate clinical isolates of Coagulase negative staphylococci (CoNS) were included in the cross sectional study. Identification of the strains was done using standard tests such as Gram's stain characteristics, growth characteristics on blood agar, catalase, tube and slide coagulase, anaerobic mannitol fermentation, urease production and mannose fermentation.

Methods for detection of methicillin resistance:

(A)Conventional

Disk Diffusion (DD): AMST was performed and interpreted as described by CLSI using Kirby-Bauer disk diffusion method.[13] For CoNS, an inhibition zone diameter of >17mm to oxacillin and >25 mm to cefoxitin was considered as resistant.[13, 14]

Oxacillin screen agar (OSA): The test was performed and interpreted as per CLSI standards [13]. After incubation for 24 hrs at 35^0C in ambient air, plates were observed in transmitted light. If any growth was present, the isolate was reported as oxacillin resistant.

The cultures were maintained at -70^0C for nuclei acid amplification tests.

(B)NAAT for *mecA* gene

The test was performed and interpreted as described by Zhang et al [15]

DNA extraction (Heat Extraction):

Frozen bacteria were sub-cultured twice onto 5% sheep blood Columbia agar plates (HiMedia) prior to DNA extraction. For rapid DNA extraction, one to five bacterial colonies were suspended in 50 µl of sterile distilled water and heated at 99°C for 10 mins, followed by centrifugation at 30,000 x g for 1 min. 2 µl of the supernatant (extracted DNA) was used as template in a 25- µl PCR.

PCR amplification

Amplification was done using following set of primers, provided by Genetix Biotech, Eurofins Genomics India Pvt Ltd.

Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Specificity
*mec*A147-F *mec*A147-R	GTG AAG ATA TAC CAA GTG ATT ATG CGC TAT AGA TTG AAA GGA T	147	*mecA*

An aliquot of 2µl of extracted DNA was added to 23 µl of PCR mixture containing 12.5 µl of PCR Master Mix (Fermentas), 1 µl forward and reverse Primer (10 pmol/ µl) and water. The amplification was performed in a thermal cycler (Eppendorf Mastercycler gradient) beginning with an initial denaturation step at 94°C for 5 min followed by 10 cycles of 94°C for 45 seconds, 65°C for 45 seconds, and 72°C for 1.5 min and another 25 cycles of 94°C for 45seconds, 55°C for 45 seconds, and 72°C for 1.5 min, ending with a final extension step at 72°C for 10 min and followed by a hold at 4°C. The cycle parameters were confirmed using known positive and negative controls. (Positive Control (*mec A*) - Staphylococcus aureus ATCC 33591; Negative Control (*mec A*) - Staphylococcus

aureus ATCC 25923)

All PCR assay runs incorporated a reagent control (without template DNA). The PCR amplicons were visualized using a UV light box after electrophoresis on a 2% agarose gel containing 0.5 µg/ml ethidium bromide. Amplicons of 147bp were consistent with *mecA* gene amplification.

Analysis:

The results of NAAT for mecA gene detection was considered as the statndard for comparison. The sensitivity, specificity and errors for the three phenotypic tests were calculated as per standard formulae. Errors were ranked as follows: very major error, false-susceptible result by test method; major error, false-resistant result produced by test method; and minor error, intermediate result by test method and a resistant or susceptible category by the reference method . Unacceptable levels were defined as > 1.5% for very major errors, >3% for major errors and 10% for minor errors as recommended in CLSI document M23-A2.[16]

Results:

150 non-duplicate, phenotypically identified, CoNS strains were analysed with the overall prevalence of methicillin resistance as confirmed by PCR for *mecA* gene was 40 %. MR in the four CoNS spp. is given in table(1).

Table 1: Species wise distribution

Species	*mec A* detected	Methicillin resistance (%)	*mec A* not detected	Total
S.hemolyticus	30	42	41	71
S.warneri	22	41	32	54
S.epidermidis	8	38	13	21
S.lugdunensis	0	0	4	4
Total	60	40	90	150

The sensitivity and specificity using cefoxitin DD was 100% and 98.9% respectively (Table 2). All the *mecA* positive strains were accurately detected. The negative predictive value (NPV) was 100%, irrespective of the species of staphylococci.The sensitivity and specificity of oxacillin DD was 80% and 96.67%, giving a very major error of 8% (*mec A* detected, oxacillin sensitive) and a major error of 2% *(mec A not* detected, oxacillin resistant). The sensitivity and specificity of OSA was 90% and 97.78%, giving a very major error of 4% (*mec A* detected, oxacillin sensitive) and a major error of 1.33 *%(mec A not* detected, oxacillin resistant). Concordance between cefoxitin disc diffusion and oxacillin disc diffusion was 90.67 % .Concordance between cefoxitin disc diffusion and oxacillin screen agar was 95.33%.

Table 2 - Comparison of the three phenotypic tests with NAAT

A. Results of NAAT vs Cefoxitin DD (CDD)			
Cefoxitin	*mec A* detected	*mec A* not detected	Total
Resistant	60	1	61
Sensitive	0	89	89
Total	60	90	150

B. Results of NAAT vs OSA			
Oxacillin screen agar	*mec A* detected	*mec A* not detected	Total
Resistant	54	2	56
Sensitive	6	88	94
Total	60	90	150

C. Results of NAAT vs Oxacillin DD (ODD)			
Oxacillin	*mec A* detected	*mec A* not detected	Total
Resistant	48	3	51
Sensitive	12	87	99
Total	60	90	150

Discussion:

CoNS has not been the focus of many studies with respect to identification of an appropriate method for detecting detect *mecA* mediated methicilin resistance. The present study contributes to selecting an appropriate test in settings where molecular methods are not feasible as a routine.

CLSI proposed use of cefoxitin in 2004, to predict resistance mediated by *mecA* gene in CoNS. Oxacillin continued to be recommended. Standards for interpretation of oxacillin and cefoxitin disc diffusion were revised in the year 2007, since testing with oxacillin showed a high false susceptibility, directly impacting treatment. Current standards include using cefoxitin discs or oxacillin MICs for reprting methicillin resistance in *CoNS.*

In the present study, results of disc diffusion with oxacillin (1µg) and cefoxitin (30 µg) and oxacillin agar screening (6 µg/ml) were compared with NAAT for detection of *mecA* mediated methicillin resistance . Of the 60 MR strains detected by NAAT, cefoxitin DD identified all correctly. Oxacillin DD and OSA had very major error rate of 8% and 4% respectively, thus being unacceptable. For detection of methicillin resistance in CoNS, recommendations differ. Perazzi *et al.* conclude that oxacillin screen agar is better while Palazzo *et al.* conclude that a combination of cefoxitin disc diffusion and oxacillin agar dilution was better.[17,18] Though, OSA gave fewer very major errors as

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compared to ODD, the values were in the unacceptable range. Rostami *et al*. recommend cefoxitin disk to be used for detection of MRSA especially heterogeneous strains and the oxacillin agar screening and *mecA* gene PCR for verifying of the results [19]. Affolabi, *et al.* recommend TPBP 2a as the best test compared to diffusion disks tests for CoNS.[20]

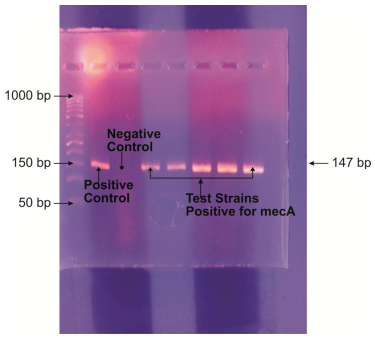
Some of the reasons proposed for the suitability of cefoxitin over oxacillin are as follows. Oxacillin disk diffusion is more prone to the effects of environmental factors as compared to cefoxitin with phenotypic expression of resistance varying as per the incubation conditions especially temperature and concentration of NaCl.[17,21] ODD is also known to give hazy zones making interpretation difficult and requires transmitted light for reading.[22]Also, cefoxitin is a more potent inducer of *mec*A than oxacillin.[23]

Multiple standard reference documents for susceptibility testing are available such as CLSI (USA), British Society for Antimicrobial Chemotherapy (BSAC) and European Committee for Antimicrobial Susceptibility Testing (EUCAST). The interpretative criteria derived from use of specific antimicrobial concentrations for testing and the corresponding inhibition zone diameters vary between these standards and have been developed by testing hundreds of strains that are mainly derived locally. In the Indian subcontinent, similar standards have not yet been developed. The reliability of applying these international standards to determine resistance to antimicrobials in India remains an area for further research. In the present study, the CLSI standards have been used and it was observed that the criteria for detecting *mecA* mediated resistance by using cefoxitin disks correlated well with the occurrence of *mecA* gene detected by NAAT. Hence, the CLSI standards for interpretation of methicillin resistance can be used to reliably predict *mecA* mediated resistance in the Indian setting.

We believe that, the strengths of the present study are the inclusion of CoNS and a comparison between the results of the three commonly used phenotypic methods with NAAT. As CoNS are now an upcoming cause of both HCA infections and CA infections, detecting methicillin resistance in CoNS gains more importance.

Conclusion:

In this study, cefoxitin disc diffusion testing accurately identified methicillin resistance in the different CoNS species despite the variable expression of mecA mediated methicillin resistance reported among CoNS. Also, a strain classified as methicillin sensitive by cefoxitin disc diffusion affirms the methicillin sensitive nature. Cefoxitin disc diffusion has a better performance characteristic in comparison to ODD and OSA and validates the current recommendations of CLSI.



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