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A Comparative Study On Different Methods For MRSA Detection In Staphylococcus *Aureus* Isolates From Bacteraemia Patients.

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Abstract

Introduction: Methicillin resistant Staphylococcus aureus bacteraemia cases are distributed throughout the globe. There is increased mortality and morbidity in cases of MRSA bacteraemia. MRSA strains are resistant to all antibiotics related to penicillin and beta lactam groups. Detection of MRSA is challenge as there are multiple way with different sensitivity and specificity. Therefore, this study is planned to compare different ways for MRSA detection.

Material & Methods: For a period of two years June 2017 to May 2019, 162 Staphylococcus aureus isolates from blood cultures received from different critical care units were studied. MRSA was phenotypically detected by Cefoxitin Disc Diffusion, oxacillin broth microdilution, oxacillin agar diffusion methods. Genotypic detection was done by mec A, mec C PCR.

Results: Out of 162 Staphylococcus aureus isolates screened, methicillin resistance was detected in 93 (57.4%) isolates by cefoxitin disk diffusion method, 84 (51.8%) isolates by the oxacillin broth microdilution 82(50.61%) isolates by Oxacillin Agar Diffusion method. mec A was detected in 94 strains. mec C was not detected.

Conclusion: MRSA detection by Cefoxitin Disk diffusion testing is reliable as genotyping detection is difficult to be done in the resource constraint setting.

Keywords: MRSA, Bacteraemia, Staphylococcus aureus, mecA

Introduction

Antimicrobial resistance has become a global issue. Staphylococcus aureus that is resistant to methicillin is a global concern. Methicillin resistant SA has emerged due to overuse of antibiotics. Staphylococcus aureus is a common cause of hospital acquired infections. Staphylococcus aureus bacteraemia accounts for 23% cases of bacteremia in the ICUs and methicillin resistance is increasing in the hospitals as they are easily acquired from the health care workers.[1] As MRSA isolates are resistant to the entire group of the β-lactum antibiotics, infection caused by them are difficult to treat, especially in ICU settings.

Detection of MRSA is carried out by phenotypic and genotypic. Both phenotypic and genotypic methods are widely available. In the earlier ways to detect MRSA, oxacillin disc diffusion was done

which phenotypically detected Methicillin resistance. It was replaced by cefoxitin disc diffusion as cefoxitin is considered stable as compared to Oxacillin.[2] Other methods like MIC detection using broth microdilution of oxacillin was considered as gold standard in MRSA diagnosis. Cefoxitin screen agar method is also employed in various laboratories for routine diagnosis. Genotypic detection of MRSA is done by molecular detection of mecA gene. Several genes are present that are related to expression of resistance to methicillin in Staphylococcus aureus including mecB and mec C.

Phenotypic detection of methicillin resistance is difficult, as there is presence of heterogenous resistance in case of S. aureus resistance to penicillinase stable Penicillin-oxacillin. Heterogenous resistance means that there are 2 types of colonies in a culture of Staphylococcus aureus , one is susceptible to oxacillin & the other type of colony is resistant. So, phenotypically there is discrepancy in detection. MRSA resistant strains have an additional protein i.e. PBP2a encoded by mecA gene.[1] Moreover phenotypic expression oxacillin resist may vary due to external factors in culture plate such as osmolarity, pH, salt concentration, temperature of the media used in testing.

Phenotypic detection is used in daily diagnosis of MRSA in a hospital based setting. Molecular based methods are time taking, labour intensive, but are considered the most reliable gold standard in the diagnosis of MRSA. Molecular based methods are done in reference laboratories only.

As, we can understand that there are several shortcomings in diagnosis of MRSA, which method to be used for reliable diagnosis, our study is therefore planned to compare different methods of MRSA detection and provide a reliable, accurate and easy to use test for MRSA diagnosis.

Material and methods

The present prospective study was undertaken out in our hospital which caters a large group of population of Northern India. The period of study was 2 years. Ethical clearance was taken from the institutional ethics committee. All admitted patients on critical care units were included in the study. Consent on a pre-approved format was taken from the patients and their relatives whereever available. 9-10 ml blood from adult and 2-5 ml blood from children was taken and subjected to blood culture. Where-ever available paired blood cultures were obtained for patients included in the study.

All blood cultures were incubated on automated system (Bac-T-Alert system) and positive blood culture bottles were identified for the growth of Staphylococcus aureus using manual as well as MALDI-Tof technology . A total 162 samples positive for Staphylococcus aureus were collected and further tested for phenotypic and genotypic detection of Methicillin resistance.

The study included following phenotypic and genotypic methods for detection of MRSA in the isolates:

- **Cefoxitin Disc Diffusion method** – Cefoxitin disc diffusion (CDD) method was done using 30 µg Cefoxitin disc. A 0.5 Mc Farland suspension of Staphylococcus aureus isolate was lawn cultured on in-house Mueller Hilton agar plate & 30 µg cefoxitin disc was applied. It was further incubated at 37ºC. (Fig 1) After 24 hrs zone of inhibition was read and interpreted using CLSI-2017 guideline i.e. zone size <22 is resistant. [3]
- **Oxacillin Broth microdilution** – (Fig 2) Serial dilution of oxacillin were made - 0.25µg/ml, 0.5 µg/ml, 1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml. S. aureus isolates was inoculated in all concentrations as per Andreus et al[4]. Results were interpreted as per CLSI M 07-A9.[5] Isolates with MIC of ≥ 4 ug/ml was reported as MRSA.
- **Oxacillin Agar diffusion (Oxacillin Screen Agar)**- In house Mueller Hinton agar (MHA) plates with 4% NaCl and 6 µg/ml of oxacillin solution were prepared and checked by quality control. Plates were inoculated with 0.5 Mc Farland suspension of Staphylococcus aureus isolate and incubated at 35^0 C for 24 h. According to NCCLS guidelines any growth after 24 h was considered oxacillin resistant.[6] (fig 3)
- **Detection of MRSA by PCR** - mec A and mec C were detected using preformed primers (Table1) by conventional PCR. DNA was extracted from the isolates. Conventional PCR was performed on a reaction volume of 25µl having universal PCR master mix 12.5 µl, 1 µl of both primers, 5.5 µl of nuclease free water and 5 µl extracted DNA template).[7]

The DNA thermocycler was set for denaturation at 95 °C for 5 min followed by 35 cycles of amplification (denaturation at 94 °C for 45 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min); and last extension phase at 72 °C for 10 min. Post PCR a gel electrophoresis was performed using 5 µl of the PCR amplicon. It was loaded in 1.5 % agarose gel containing 0.5µl/ml of ethidium bromide dye along with 100 bp DNA molecular weight ladder. Gel electrophoresis was done at 80 V for 2 h and amplified DNA was analysed by 264nm wavelength UV transillumination and gel was documented. (fig 4) Fragment length of 533bp were for mecA gene and 454 were for mecC gene.[8]
Statistical analysis was done for calculating sensitivity, specificity, positive predictive value & negative predictive value of all the tests to compare various methods of MRSA detection mec A detection with PCR was considered as gold standard**.**

Table 1: Primer sequence used for mecA and mec C detection[8]

Genes	Primers	Primer sequence	PCR product length
mecA	Mec A -F	AAA AAA GGT GGT ATC GAT TGG C	533bp
	Mec A - R	AGT TCT GCA GTA CCG GAT TTG C	
mecC	MecC - F	GTC CCT AAC AAA ACA CCC AAA GA	454bp
	Mec C - R	GAA GAT CTT TTC CGT TTT CGA C	

Results
162 Staphylococcus aureus isolates were isolated from blood cultures of suspected bacteraemia patients from different critical care units of our tertiary care hospital. (as shown in table 2).
Using conventional PCR, mec A was detected in 94 strains, making samples labelled as MRSA. mec C was not detected in our study.
On cefoxitin disk diffusion method 93 (57.4%) isolates showed zone of inhibition < 22mm. some of these strains showed breakthrough colonies also. By oxacillin broth microdilution 84 (51.8%) of isolates had turbidity in =/> 4µg/ml, making them methicillin resistant. Oxacillin Agar Diffusion method reported methicillin resistance in 82 (50.61%) isolates. (fig 5)
Using statistical formulas, sensitivity, specificity of all the test in our study was calculated and compared. PPV and NPV were calculated keeping mecA detection as gold standard. All the calculations and results are shown in table 3.

Fig 5 – Line diagram showing various methods of detection of MRSA

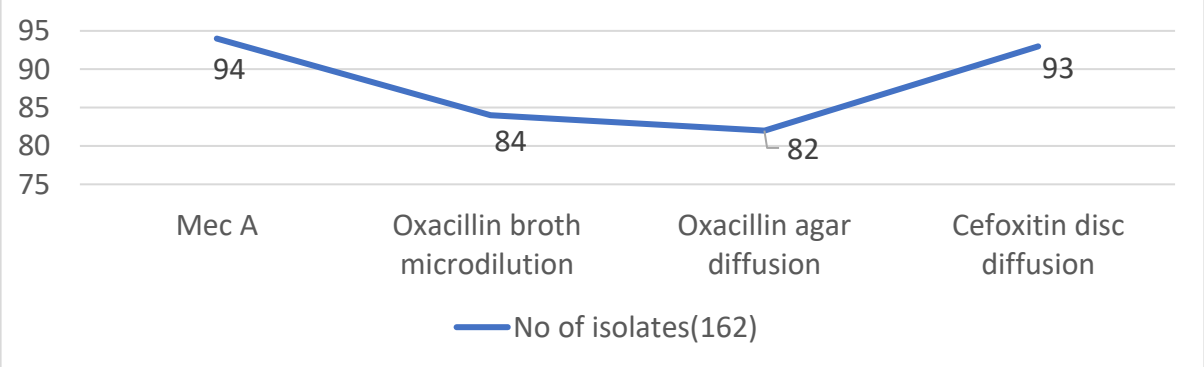


Table 2 – Distribution of various Staphylococcus aureus isolates in different Critical care units of our tertiary care hospital.

S. No	Critical care units	Isolates	MRSA by mecA detection
1	CCM	5	2
2	PICU	42	28
3	MICU	38	25
4	NICU	36	23
5	SICU	19	8
6	TVU	22	8

- CCM – Critical care medicine, PICU – paediatric intensive care unit, MICU -Medicine ICU, NICU – Neonatal ICU, SICU – Surgical ICU, TVU – Trauma ventilator unit.

Table 3 – Statistical analysis of different methods of MRSA detection.

Method	Specificity	Sensitivity	Positive predictive value	Negative predictive value
Mec A detection	100%	100	100	100
Oxacillin broth microdilution	100%	89.3	100	87
Oxacillin agar diffusion	100%	87.2	100	85
Cefoxitin disc diffusion	100%	98.9	100	98.5

Discussion

MRSA infections are a major concern in context to critically ill bacteraemia patients. Patients harbouring MRSA strains act as a reservoir of infection and spread it to health care providers and further to patients. Therefore, early and accurate MRSA detection should be considered as a tool for better infection prevention practices in a health care setting. Early detection of MRSA, is also important clinically for treatment and prognosis of Staphylococcus aureus patients.

CLSI guidelines recommend, mecA detection by PCR as the gold standard for MRSA detection. [3,5] As mecA PCR is time consuming, needs expertise and is costly method, in the present study different phenotypic methods like cefoxitin disc diffusion, oxacillin agar diffusion and MIC detection by oxacillin broth microdilution were evaluated, to find out reliable, easy, cheap and accurate method for MRSA detection. Various statistical methods like sensitivity, specificity positive predictive value(PPV) and negative predictive value (NPV) were used to compare the results with the gold standard test.

In our study 58.02% of Staphylococcus aureus isolates were mec A positive. No mec C was detected in our study. Considering mecA detection as the gold standard, Cefoxitin disc diffusion method is most specific (100%) and sensitive(98.9%), nearing MRSA detection by MecA PCR method. This is similar to studies done by Anant K et al.[6] Based on CLSI recommendation, cefoxitin should be used for disc diffusion testing as it helps in forming a clear zone around the disc as compared to oxacillin disc. This prevents misinterpretation through oxacillin diffusion test.[7]

MRSA detection using Oxacillin agar diffusion and broth microdilution are cheap, and easy to be done. On the other hand, we can get different concentrations and report MIC values also. Sensitivity and specificity of oxacillin agar diffusion test using 6µg oxacillin in the medium in our study was 100% and 87.2% respectively. Raza et al reported 99.5% sensitivity and 98.5% specificity for the

same.[8] Studies report that Oxacillin agar diffusion does not detect heterogenous strains and borderline cases of MRSA.[9]

The sensitivity of oxacillin broth microdilution and agar diffusion in our study was 89.3 and 87.2%. These were not as accurate as MRSA detection by cefoxitin disc diffusion method, so MRSA detection by CDD can be considered as an alternative to detection of resistance by PCR.

Studies indicate the use of cefoxitin in disc diffusion method as a very sensitive and specific as mecA mediated marker to recognise MRSA.[1, 7,9] This is comparable to our study, so cefoxitin disc diffusion testing can be considered as an alternative method of MRSA detection by mecA PCR. Also use of cefoxitin in disc diffusion testing is encouraged as there is clear zone of visibility surrounding the disc on the isolate inoculated agar that cannot be misinterpreted compared to oxacillin diffusion test.[2]

Conclusion

Our study provides significant data on Cefoxitin Disk diffusion testing method as a reliable alternative to genotypic detection of mecA by PCR in the resource constraint setting. This test had sensitivity and specificity comparable to mecA PCR. We recommend this method to be used in various clinical laboratories as it reliable, accurate, easy to perform and interpret and moreover cost effective.

Fig 1 – Cefoxitin disc diffusion test

Fig 2 – Cefoxitin broth microdilution test

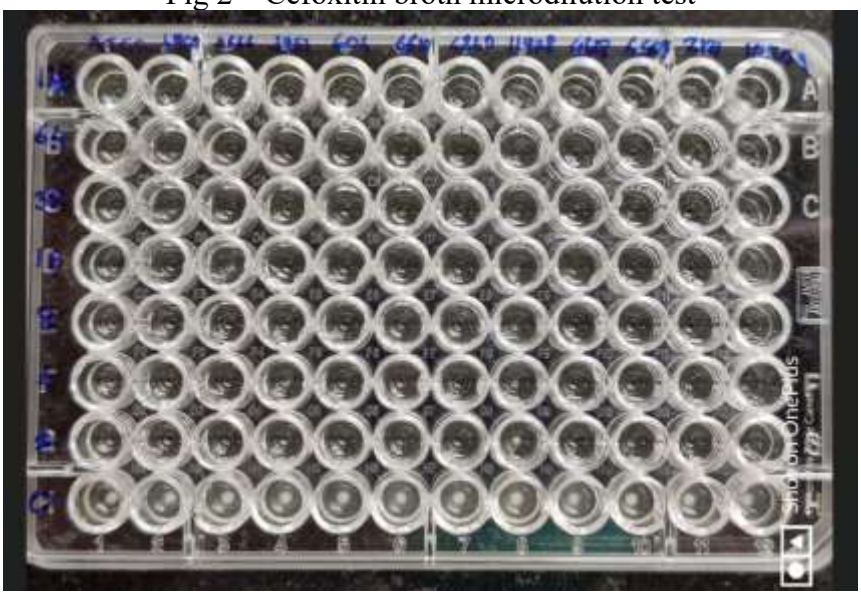
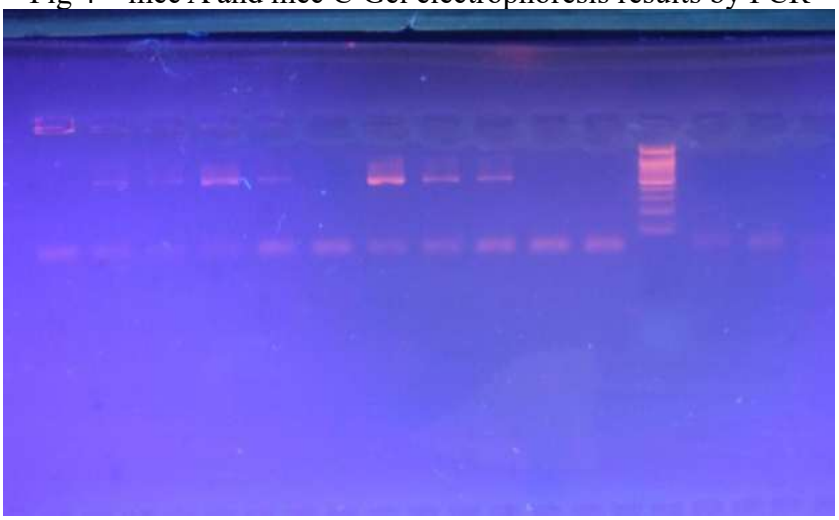


Fig 3 – Cefoxitin Agar diffusion testing

Fig 4 – mec A and mec C Gel electrophoresis results by PCR



Declarations:
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Conflict of Interest
All authors declare that there is no conflict of interest or any affiliation or involvement in any organization whether it is academic, commercial, financial, personal and professionally relevant to the work under consideration to avoid the potential for bias and we accept responsibility for what is said in the manuscript.

Authors' Contribution.
Dr Aditi Garg - Conception and design of study, acquisition of data, evising the manuscript critically for important intellectual content
Dr Rani Kumari Jaiswal - acquisition of data, revising the manuscript critically for important intellectual content
Dr. Dhruv Agarwal – Statistical analysis
Dr. Anuragini Verma - acquisition of data
Dr. Prashant Gupta - Conception and design of study, Approval of the version of the manuscript
Dr. Vimala Venkatesh - Conception and design of study, revising the manuscript critically for important intellectual content, Approval of the version of the manuscript

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Ethics Statement
Ethical approval was taken from the institutional ethical committee.

Informed Consent
The privacy rights of human subjects must always be observed. Informed consent is taken from patients in a preformed quiestionaire.

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