Original Article

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Phenotypic Detection of MRSA from Conventional Methods and CHROM Agar Medium in the Bloodstream Infections: Laboratory-based Cross-sectional Study

Sudeshna Das[1], Kirti Nirmal[2], Avinash Kant Lakra[3], Chandrakshee Kakati[4], Shukla Das[5]

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Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) has currently become an important healthcare concern as its resistant to multiple antibiotics. Currently, it's one of the major hospitals acquired and community acquired pathogen causing blood stream infections(BSI). Therefore, rapid identification of MRSA in clinical specimens is essential for timely decision on effective antimicrobial drug therapy.

Aim: To detect the prevalence of MRSA isolates isolated from blood culture samples. To compare phenotypic methods; Mannitol salt agar (MSA), Cefoxitin disk diffusion (CDD) & Oxacillin resistant screening agar base (ORSAB) with CHROMagar MRSA.

Materials and Methods: An Laboratory based cross-sectional study was carried out in the Microbiology department, UCMS and GTBH from January 2024 to April 2024 were included and further characterized. All the isolates were cultured in MSA, blood agar and MacConkey agar. The phenotypic methods used for confirming MRSA was Cefoxitin disc (30µg) diffusion, CHROMagar™MRSA andORSAB for phenotypic identification.

Results: Out of 328 isolates of *S. aureus*, 151(73. 3%) isolates were MRSA detected by CDD method, CHROMagar™ MRSA and ORSAB. The sensitivity of chromogenic media i. eCHROMagar™ MRSA and ORSAB is 94. 2% and 97. 2% respectively. All MRSA isolated were susceptible to Vancomycin, Linezolid and resistant to commonly used antibiotics.

Conclusion: CHROMagar™ MRSA and ORSAB is found to be accurate for the detection of MRSA. It is reliable, easy to perform, less time-consuming, and cost-effective. It is an affordable alternative to the conventional method of detection of MRSA in resource-poor settings.

Keywords: Cefoxitin disk diffusion; CHROM agar MRSA; ORSAB; Methicillin-resistant *Staphylococcus aureus*.

Author's Affiliation: [1]Senior Resident, [2]Associate Professor, [4]Post graduate, [5]HOD, Director Professor, Department of Microbiology, University College of Medical Science and Guru Teg Bahadur Hospital, Dilshad Garden, Delhi 110095, India, [3]Research Officer, Department of Microbiology, Translational Health Science and Technology Institute, Faridabad, Haryana, India.

Corresponding Author: Kirti Nirmal, Associate Professor, Department of Microbiology, University College of Medical Science and Guru Teg Bahadur Hospital, Dilshad Garden, Delhi 110095. India.

E-mail: doctorkirtinirmal@gmail.com

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INTRODUCTION

In the recent years, *Staphylococcus aureus (S. aureus*) has become a public healthmenace as its causing increased morbidity, mortality, hospitalization duration. MRSA are resistant to majority of beta lactam antibiotics (e. g. -Methicillin) including other drugs such as Vancomycin etc. MRSA strains are one of the major etiological agents causing nosocomial and community acquired infections.[1-3]

The management of both community acquired (CA-MRSA) and Hospital acquired (HA-MRSA) has become tedious due to ineffective infection control measures and novel drugs for the treatment of these cases.[2-4] The determination of the anti-microbial susceptibility is crucial for an optimal therapy, for epidemiological purposes and for infection control measures. [4,5] The treatment of MRSA is Vancomycin while, Methicillin sensitive *Staphylococcus aureus* (MSSA) infections can be managed by Beta lactam drugs. Due to high rates of oxacillin resistance, empirical vancomycin therapy is increasing. The routinely used methods cannot accurately detect the Methicillin and theVancomycin resistance.[6]

The study aimed to provide an accurate assessment of the prevalence of Methicillin and Vancomycin resistance in *S. aureus* strains in order to inform infection control measures and guide antibiotic therapy. The study involved screening a large number of *S. aureus* strains obtained from both hospital and community-acquired infections. The screening involved analyzing the susceptibility of these strains to methicillin and vancomycin, two commonly used antibiotics. The results of the study will provide valuable information on the prevalence of MRSA strains and inform strategies for infection control and appropriate antibiotic therapy. Rapid and early diagnostic tests with AST is essential in order to prevent resistance to multiple drugs. Due to delayed and incorrect culture reports there has been mismanagement of patients and misuse of antibiotics. In order to improve analytic phase of lab reports, higher diagnostic accuracy of newer tests is required. The methods used to detect MRSA in clinical samples should have high sensitivity and specificity and short turn around time. The most commonly used method in the laboratories is culture and antibiotic sensitivity test (AST) cefoxitin disk diffusion (ODD). Other methods available for diagnosing MRSA include mannitol salt agar (MSA) with oxacillin (agar screening method), minimum inhibitory concentration (MIC) tests, agar dilution tests.[7-9] All these tests are the conventional phenotypic methods of MRSA identification. Genotypic (molecular) method is the polymerase chain reaction based method for detecting *mecA*gene, which remains the "gold standard"for diagnosing MRSA.[7-10] The present study was conducted to compare the performance of Conventional Methods including Oxacillin resistant screening agar base (ORSAB) and CHROMagar™ MRSA for Phenotypic Detection of MRSA in the bloodstream infections (BSIs).

MATERIALS & METHODS

An observational Laboratory based cross-sectional study was carried out in the Microbiology department, UCMS and GTBH from January 2024 to April 2024. In this study, 328 blood samples from IPD/OPDpatients were included and further characterised. All the blood samples were collected in BD BacT/alert under aseptic precautions.

Sample processing: The bottles were inserted in BacT/Alert which is a fully automated blood culture system that detects bacteraemia and fungemia. The blood samples which flagged positive were taken out and subculture on blood agar and MacConkey agar. The total number of 206 Gram positive cocci: *Enterococcus*, CONS (Coagulase negative Staphylococcus), *Staphylococcus aureus*, Micrococcus were isolated. A total of 151 clinical isolates of *S. aureus* from blood stream infections were included in the study. Isolates were identified as *S. aureus* based on conventional methods as per the standard protocol. We tested for the presence of *S. aureus* and if present, determined Methicillin resistant strains. All the isolates were cultured in Mannitol salt agar (MSA), blood agar and MacConkey agar. The phenotypic methods used for confirming MRSA was Cefoxitin disc (30µg) diffusion (CDD), CHROMagar ™ MRSA and ORSAB for phenotypic identification. When isolating a staphylococcus from clinical or screening specimens, it is of the utmost importance to ensure that it is, in fact, *S. aureus* rather than coagulase- negative staphylococcus, as the latter can be an opportunistic pathogen. Round, smooth, creamy white and distinctive yellow colonies on BA were deemed pre-sumptive for *S. aureus*. Gram stain was performed on presumptive isolates, and Gram-positive cocci were dealt with for further biochemical tests, including catalase, Coagulase (slide/tube agglutination) and supplementary tests. The disc diffusion susceptibility testing using cefoxitin impregnated discs (30µg) was performed according to the latest Clinical and Laboratory Standards Institute (CLSI M100 -2024) guidelines.

[12]Each isolate was considered as a single patient. This study was conducted after obtaining ethical approval from the Institutional Ethics Committee (GTBHEC 2024/456-114).

Bacterial identification

The clinical specimens(blood) were inoculated on 5% sheep blood agar and MacConkey's agar (Himedia), incubated at 37°C for 24-48 hours. The screening of*S. aureus* was identified using standard methods like colony morphology on culture plates, Gram stain, catalase test, and coagulase test (tube and slide). [13]The *S. aureus* strains were processed by the following three techniques for diagnosing MRSA:

- Culture in MSA (Mannitol Salt Agar)medium,
- Detect the Oxacillin Disk Diffusion, and
- CHROMagar TMMRSA
- Oxacillin resistant screening agar base (ORSAB)

Culture in mannitol salt agar: The Gram positive cocci isolates were tested for Methicillin resistance by culture in MSA medium and 4% NaCl (Hi Media), incubated at 37°C overnight (24 h). Any yellow colonies growth in the medium is taken as *Staphylococcus aureus* and no growth / pink colonies represented *other staphylococci species* (CONS).

Cefoxitin and oxacillin disc diffusion test: Cefoxitin disc diffusion test was carried out using a 30-µg disc of Cefoxitinon Muller-Hinton agar plate(MHA), and Oxacillin disc diffusion test was carried out using a 1 µg disc on Muller-Hinton agar plate containing 2% Naclon all isolates of *S. aureus*. Lawn culture of the bacterial suspension standardized to 0. 5 McFarland standards was done on the agar plates. The plates were incubated at 37°C for cefoxitin and 35°C for oxacillin disc for 18 to 24 hrs. Zone diameters were measured. Zone diameters≤19 mm was reported as Methicillin-resistant, and zone diameters≥22 mm was considered as Methicillin sensitive for cefoxitin disc. Whenzone diameters ≤10 mm was reported as Methicillin-resistant and zone diameters ≥13 mm was considered as Methicillin sensitive for oxacillindisc.[12-14]

Isolates of S. aureus were identified as MRSA using cefoxitin disc as surrogate marker. *S. aureus* ATCC 25923 and ATCC 43300 strains were used as negative and positive controls, respectively, for standardization of procedure and quality control.

Oxacillin resistant screen agar base (ORSAB): ORSAB is a selective media developed to detect MRSA in clinicalspecimens. The medium uses aniline blue to detect mannitolfermentation in *S. aureus*. The antibiotic supplements (oxacillin, 2. 0 µl; polymyxin B, 50, 000 IU/I) of 5. 5% NaCl reduce the growth of non-staphylococcal organisms and helps in the selection of MRSA. The test was carried out as per the manufacturer's instructions. Colonies form each culture was taken in a loop and mixed in peptone water to bring it to 0. 5 McFarland standards. The plates were subsequently inoculated by spot inoculation method and incubated at 37°C for 48 hrs. When blue coloured colonies are seen within 24-48 hrsit was considered for MRSA strains and Zone diameters were measured by following Clinical and Laboratory Standards Institute guidelines. [12-13]

CHROMagar TM MRSA: CHROMagar MRSA screening agar plates were allowed to attain room temperature before inoculation. Screening swab specimens were processed by direct streaking on the agar. The agar plates were incubated at 35°C ± 2°C for 18-24 hours in aerobic conditions. After incubation, plates were examined for the presence of mauve colonies, and the results were obtained. The presence of mauve colonies was indicative of a positive result (MRSA), and colonies with any other colour or no growth were considered negative (no MRSA). *Figure 4.1* depicting the colonies characteristic of MRSA in MSA, ORSAB and CHROMagarTMMRSA.

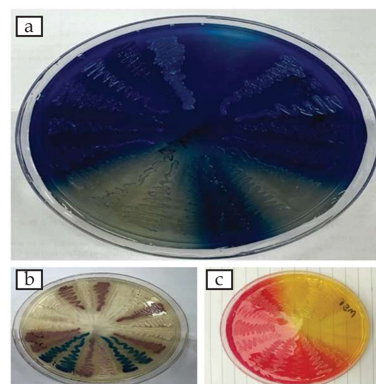

Fig. 1: (a) Oxacillin resistant screening agar base (ORSAB) shows growth of MRSA as deep blue colonies, **(b):** CHROMagarTM MRSA shows mauve colonies interpreted as MRSA **(c)**: Mannitol Salt agar showing *Staphylococcus aureus*

Antibiotic susceptibility testing: Isolates of *S. aureus* to various antimicrobial discs was carried out by using Kirby-Bauer disc diffusion method. All antimicrobial discs were obtained from Hi-media Laboratories Pvt. Ltd., Mumbai, India. In this study, all testing was done according to the CLSI as well as the manufacturer's recommendations. Conformed

strains of *S. aureus* were identified as MRSA using Cefoxitin disc as surrogate marker and oxacillin disc diffusion method as per CLSI guidelines. *S. aureus* ATCC 25923 positive controlsused for standardization of procedure and quality control. The inoculums were prepared, and density was adjusted to obtain semi-confluent growth after incubation. Sterile swabs were used to uniformly inoculate the bacterial suspension on Mueller Hinton agar plates. The Cefoxitin impregnated discs (30 µg) were dispensed onto the same agar plates and incubated at 35°C ± 2°C for 24 hr. The interpretation of measured diameters of zones of inhibition was made using interpretative criteria of *S. aureus* given in the latest CLSI 2024.[12 14]

Data management and statistical analysis: Data recording was carried out using the MS Excel spreadsheet program. Categorical variables were summarized using frequencies and percentages. To compare groups in terms of categorical data, the Chi-square test was employed. A p-value of less than 0. 05 was considered as the threshold for statistical significance.

RESULTS

A total of 328 blood samples were collected during this duration. A total of 206 isolates of *Staphylococcus aureus* species were reported from tertiary institute in the department of Microbiology Laboratory during period of January to March 2024. Among these isolates, 151(73. 3%) were MRSA and 55 (36. 4%) were MSSA detected by routine disc diffusion test using Cefoxitin disk. The prevalence of MRSA strains was more in males than females(F:M=1:1. 3). Age wise distribution results showed that the infections by MRSA were more common in paediatrics and adolescent age group i. e<20 years; most probably hospital acquired –MRSA as shows in *Figure 4.2*. This was statistical not significant (p>0. 08, chi-square test). The maximum number of blood culture samples positive for MRSA were from Medicine followed by Paediatrics department as shows in *Figure 4.3* (This was statistical significant from paired-t test (p< 0. 02).

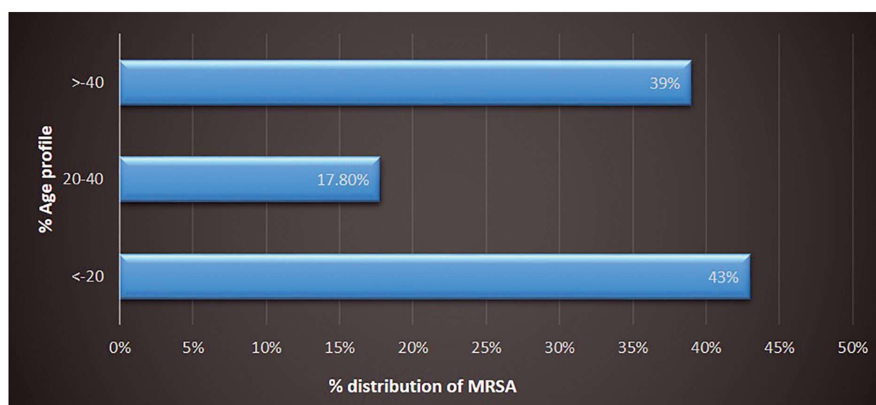

Fig. 2: Age-group based distribution of Methicillin resistant *Staphylococcus aureus* isolates in the blood stream infection (n=151)

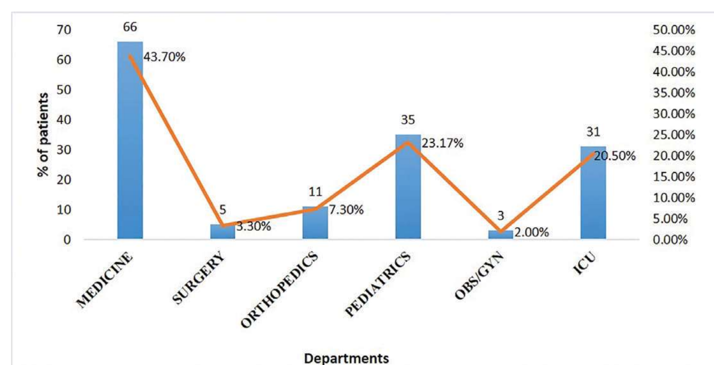

Fig. 3: Location wise distribution of Methicillin resistant *Staphylococcus aureus* isolates in the study group population (n=151)

Comparison of CDD and ORSAB: Out of the 206 isolates, 151 with a zone of inhibition ≤21mm(73. 3%) showed Methicillin resistance *Staphylococcus aureus* (MRSA) using the Cefoxitin disc diffusion method and 157(76. 2%) showed Methicillin resistance using the ORSAB chromogenic medium. Three of the isolates that were Cefoxitin resistant demonstrated resistance using the ORSAB Medium. (*Table 1*). This was statistical significant. (p<0. 00001, chi-square test).

Comparison between CHROM agar MRSA and Cefoxitin disc diffusion: 51(73. 3%) showed MRSA using the Cefoxitin disc diffusion method and 144(69. 9%) showed MRSA using the CHROM agar MRSA medium. Using the Chi-square test, the variation between the two testsis statistically significant with a P value of <0. 05. (*Table 2*).

The evaluation between sensitivity, specificity, positive predictive value and Negative predictive value were calculated amongst CHROM agar MRSA and ORSAB in the Methicillin resistance *Staphylococcus aureus* isolates. (*Table 3*).

The antimicrobial resistance pattern amongst the Methicillin resistant Staphylococcus aureus blood culture isolates was showing resistant in erythromycin (89%) and ciprofloxacin (78%). The Linezolid and Vancomycin were not resistant to any isolates of MRSA (*Table 4*).

Table 1: Comparison between Cefoxitin Disc Diffusion and Oxacillin Resistance screening agar based medium for the detection of Methicillin Resistant *Staphylococcus aureus* in the study group population

Cefoxitin Disc Diffusion(CDD)	Oxacillin Resistance screening agar based (ORSAB)		Total (n=206)	Statistics
	Susceptible	Resistant		
Susceptible	140	11	151	(p < 0.00001) Chi-square test
Resistant	4	51	55	
Total (n=206)	144	62	206	

Table 2: Comparison between Cefoxitin Disc Diffusion and CHROM agar MRSA for the detection of Methicillin resistant *Staphylococcus aureus* in the study centres

Cefoxitin Disc Diffusion (CDD)	CHROM agar MRSA		Total (n=206)	Statistics
	Susceptible	Resistant		
Susceptible	148	3	151 (73%)	(p < 0.00001) Chi-square test
Resistant	9	46	55 (17%)	
Total	157	49	206 (100%)	

Table 3: Sensitivity, Specificity, PPV & NPV amongst CHROM agar MRSA and ORSAB in the Methicillin resistance *Staphylococcus aureus* isolates

Variable (%)	Oxacillin Resistance screening agar based (ORSAB)	CHROM agar MRSA
Sensitivity	97.2 %	94.2 %
Specifictiy	82.2 %	93.8 %
Positive Predictive Value (Ppv)	92.7 %	98 %
Negative Predictive Value (Npv)	93V%	83.6 %
Diagnostic Accuracy	92.7 %	94.2 %

Table 4: Antibiotic resistance pattern of the Methicillin resistant *Staphylococcus aureus* isolates in the study group population.

Antibiotics	No of MRSA isolates n=151 (%)
Clindamycin (10 µg)	100 (66.20)
Erythromycin (15µg)	135 (89.40)
Ciprofloxacin (5µg)	118 (78.10)
Cotrimoxazole (1.25/23.75 µg)	106 (74.40)
Chloramphenicol (30 µg)	43 (28.40)
Linezolid (30 µg)	0 (0)
Vancomycin (MIC: 0.5 -2 mg/L)	0 (0)

DISCUSSION

MRSA bacteria have become increasingly resistant to antimicrobial therapies, hindering effective treatment and increasing patient morbidity. To curb the emergence and transmission of this pathogen, surveillance of individuals at high, regular monitoring can limit the unnecessary misuse of antibiotics and enable timely implementation of infection control measures. In this study, we explored a practical MRSA detection method that can be easily performed in laboratories with limited resources and expertise. The reference and gold standard method for MRSA identification is detecting the mecA gene by PCR.[15] Our study aimed to find a easy to perform, rapid and accurate method for MRSA detection for microbiological laboratories where molecular assays are unavailable, and the prevalence of MRSA is high. Our findings suggest that chromogenic media provides a reliable and cost-effective means of MRSA screening.[16]

The main objective of this study was to evaluate cefoxitin disc diffusion test(CDD), CHROMagar™ MRSA and ORSAB. Timely detection of MRSA is still problematic with the majority of techniques taking longer than 48-72 hrs.

In India, the prevalence of MRSA among *S. aureus* isolates ranges from 40% to 70% as seen in Verma *et al.*[17] with variations in community and hospital acquired. Our study found a similar prevalence of 73. 3%.

Usually, routine lab tests for MRSA detection had variable specificity and PCR was difficult to perform in routine diagnostic laboratories.[18] However, our study revealed that MSA with oxacillin had a sensitivity of 91.3% and a specificity of 69.1%. These findings are similar to those reported by Kateete *et al.*[19], who found a sensitivity of 94% and a specificity of 79% for MSA with oxacillin.

Several previous studies have examined the efficacy of various commercial chromogenic media for detecting MRSA. In the study by Nahima *et al.*[20], after an 16-18 hour incubation period, the sensitivity of CHROM agar™ MRSA was found to be 59% and ORSAB was found to be 47% lower than our study. Patil *et al.*[14] demonstrated 100% sensitivity and specificity for the CDD test, 100% sensitivity and 98. 66% specificity for the oxacillin disc diffusion test, and 98. 66% sensitivity and specificity for ORSAB. However, the low specificity of the oxacillin disc diffusion test and ORSAB medium make them unsuitable for predicting MRSA on their own. Similar to our study, Patil *et al.*[14] and Umar Ai *et al.*[21] found that CDD was the most effective method for phenotypic MRSA detection, with better sensitivity and specificity compared to oxacillin and ORSAB.

Both our study and Kluytmans J *et al.*[22] demonstrated that CHROM screen agar outperformed ORSAB in identifying *S. aureus*. Ahmad *et al.*[23] and Cherkaoui *et al.*[24] also reported that primary plating on CHROMagar™ MRSA was more specific (91%) than screening with ORSAB, which aligns with our findings. ORSAB exhibited higher sensitivity, making it a potential alternative in settings with a high prevalence of MRSA. CHROM agar™ MRSA available in both agar base form and pre-prepared agar plates. The higher diagnostic accuracy makes it better than ORSAB medium. The agar base can be stored for up to 2 years at temperatures between 2°C to 8°C. These To ensure accurate results, the MRSA screen should be implemented as a routine procedure, starting with identifying a suspected MRSA strain to the species level to exclude coagulase-negative staphylococci. Subsequently, the potential MRSA strain should undergo the MRSA screen-test. A positive result confirms the identification as MRSA, while a negative result requires repeating the assay after exposing the strain to Methicillin to induce a response.

Bhoi *et al.*[25] discovered that commonly used antibiotics such as Erythromycin and Imipenem had high resistance, while Vancomycin and Linezolid remained effective, which is consistent with our study.

Limitation. The longer incubation time and the use of broth enrichment can impact the results obtained from chromogenic media. The exposure to light before and during incubation can cause the destruction of chromogens and result in inaccurate findings. It is important to note that certain strains of Coagulase-negative *Staphylococci* may produce colonies with a mauve color, which can further complicate the interpretation of the results. Prolonged incubation for more than 24 hours may also increase the likelihood of false-positive results. Furthermore, this study did not evaluate the presence of coagulase-negative staphylococci, which could potentially affect the sensitivity and specificity of CHROM agar™ MRSA in clinical samples. Another limitation of this study was that the initial inoculation was restricted to only one MRSA screening agar, which may have influenced the overall findings. According to Becker *et al.*[29], the

ORSAB medium has limitations for surveillance purposes not only due to its lower sensitivity but also because certain coagulase-negative staphylococci, particularly *Staphylococcus hemolyticus*, may appear blue on this plate. Therefore, it is recommended to perform confirmatory tests for MRSA identification when using this plate and to consider its use primarily in high prevalence settings.

CONCLUSION

The CHROM agar™ MRSA test is a dependable and efficient method for detecting MRSA. It is user-friendly, saves time, and is cost-effective. This test has demonstrated both high sensitivity and specificity, greatly enhancing our ability to identify MRSA quickly and accurately from screening swab samples. The routine use of CHRO Magar™ MRSA is recommended as it aids in infection prevention and control efforts. Additionally, it serves as a suitable substitute for traditional MRSA detection methods, such as Cefoxitin disk diffusion. In resource-limited settings, it can also serve as an affordable alternative to molecular detection methods for MRSA.

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