To Study the Phenotypic and Genotypic Characterization Method for the Detection of Methicillin Resistant Staphylococcus aureus Isolates at a Tertiary Care Hospital, Uttar pradesh.

NashraAfaq[1], R. Sujatha[*2]

Abstract:

Introduction: MRSA strains have become a severe clinical and epidemiological problem in recent years, as resistance to this antibiotic suggests resistance to beta-lactam antibiotics. The aim of the present study was to compare the Phenotypic and Genotypic Characterization Method for the Detection of Methicillin Resistant Staphylococcus aureus.

Aim and Objective: To Study the Phenotypic and Genotypic Characterization Method for the Detection of Methicillin Resistant Staphylococcus aureus Isolates at a Tertiary Care Hospital, Uttar pradesh.

Material and Methods: This was a cross sectional study conducted in the Department of Microbiology at RMCHRC, Mandhana, Uttar Pradesh for a period of 1 year i.e, November 2021 to November 2022. A total of 965 clinical isolates was studied in which 210 isolates of S. aureus were identified using the biochemical test from the clinical samples such as pus, swab, blood, wound and urine etc. The Comparison of Different Phenotypic Methods including Cefoxitin and Oxacillin Disc Diffusion test and the genotypic method including MecA gene detection for Methicillin Resistant Staphylococcus aureus Isolates was performed.

Results: A total of 210 S.aureus isolates were identified from a total of 965 clinical samples out of which 58 isolates were identified as the MRSA isolates. The gold standard method was chosen to be the genotypic method as well as the Cexofitin disc diffusion method for the phenotypic detection. The cefoxitin and the Mec A gene by PCR detected all the 58 isoaltes whereas oxacillin was found to be less sensitive. The MRSA isolates were highly susceptible to teicoplanin, vancomycin and linezolid.

Conclusion: The Genotypic method as well as the Cefoxin disc diffusion method was observed to be equally susceptibility for testing of MRSA as comparative to the oxacillin method. Therefore Cefoxitin is better than the other phenotypic method and is highly recommended to be used as a surrogate marker for the detection of Methicillin resistance in S.aureus, in resource constraint setups that cannot afford PCR testing for mecA as a confirmatory test.

Keywords: MRSA, Beta-lactam, Oxacillin, Cefoxitin disc diffusion method, PCR

Introduction

S. aureus is an important pathogen of many nosocomial and community-related infections leading to high morbidity and mortality. Staphylococcus aureus have become a serious problem as it is resistant to methicillin and the infections caused by them are often fatal in nature and are associated resistance to several beta-lactam antibiotics used in hospitals [1]. These strains are known as MRSA (methicillin resistant S. aureus), which consequently becomes difficult in managing infections [2].

The increasing antibiotic resistance is a worrisome problem observed worldwide. Among Gram-positive cocci, Staphylococcus aureus is a well-known cause of community acquired as well as hospital acquired infections. Beginning with the emergence of methicillin-resistant Staphylococcus aureus (MRSA) in UK within two years of Methicillin launch, this organism is notorious to develop resistance against majority of empirical antibiotics targeted against it. [3, 4]

The methicillin resistant itself means that a S. aureus isolate will not be sensitive to Penicillins, Cephalosporins, β- lactamase inhibitors, and Carbapenems and can further exhibit resistance to other classes of antibiotics.[5,6] MRSA have been implicated in serious skin infections, necrotizing fasciitis, deep tissue abscesses, and their hematogenous spread can result in bone and joint infections, sepsis and endocarditis.[7] Drug of choice to treat these multidrug-resistant MRSA are glycopeptide antibiotics such as vancomycin.[8]

Methicillin resistance is due to harboring of mec-A gene, resulting in synthesis of an altered penicillin binding protein (PBP)-2a by the organism having low affinity for β-lactam antibiotics. The prevalence of MRSA strains has increased worldwide. [9-11]

Presently, the Gold standard test for detecting MRSA is identification of the mecA gene using polymerase chain reaction (PCR) [12]. Among phenotypic methods,

Research Associate[1], Dept of Microbiology Rama Medical College Hospital and Research Centre, Mandhana Kanpur
Professor & Head[2], Dept of Microbiology Rama Medical College Hospital and Research Centre, Mandhana Kanpur.*

cefoxitin disc diffusion (CDD) test, oxacillin disc diffusion (ODD) test, are recommended by CLSI for detection of methicillin resistance. [13].

Cefoxitin is taken into consideration as it is a more potent inducer of mec-A gene expression than oxacillin or methicillin and the results obtained are comparable with detection of mec-A gene using PCR and also can be used in the constraint setups that cannot afford PCR testing for mecA as a confirmatory test [14, 15]

Therefore the present study was undertaken to study the phenotypic and genotypic characterization method for the detection of methicillin resistant Staphylococcus aureus isolates at a Tertiary care Hospital, Uttar pradesh.

Material and Methods

This was a cross sectional study conducted in the Department of Microbiology at RMCHRC, Mandhana, Uttar Pradesh for a period of 1 year i.e, November 2021 to November 2022. A total of 965 (inpatients and outpatients) clinical isolates was studied in which 210 isolates of S. aureus were identified using the biochemical test from the clinical samples such as pus, swab, blood, wound and urine etc. The Comparison of Different Phenotypic Methods including Cefoxitin and Oxacillin Disc Diffusion test and the genotypic method including MecA gene detection for Methicillin Resistant Staphylococcus aureus Isolates was performed

Isolation and Identification of Staphylococcus aureus

The clinical samples were inoculated on blood agar and mannitol salt agar (HiMedia laboratories private limited, India) and incubated aerobically at 37°C for 48 hours. The strains of Staphylococcus aureus were identified on the basis of colony morphology, Gram's stain, and different biochemical tests. [16]

The antimicrobial susceptibility testing was performed by modified Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (HiMedia laboratories private limited, India) following Clinical and Laboratory Standards Institute (CLSI) guidelines. [13] Antibiotic discs used were ciprofloxacin (5µg), clindamycin (2µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), tetracycline (30µg), cotrimoxazole (25µg), rifampin (5µg), mupirocin (200µg), and penicillin G (10 units).

Phenotypic Detection Method for MRSA

All isolates of *S.aureus* were tested by oxacillin disc diffusion and cefoxitin disc diffusion method. A standard strain of MSSA (ATCC 29213) and a PCR-positive control strain (ATCC43300) (MecA-positive) were used as controls for all methods. The results were interpreted according to CLSIguidelines 2021 [13].

Identification of MRSA through Oxacillin disc diffusion method

All the strains were tested with the 1mg oxacillin discs (Hi-media) on Mueller-Hinton agar plates. A bacterial suspension adjusted to 0.5 McFarland was used for each of the strain. The zone of inhibition was determined after 24 h incubation at 35°C. Zone size was interpreted according to the two CLSI (2021) [13] criteria: susceptibility > =13mm; intermediate, 11-12mm; and resistance < =10mm.

Identification of MRSA Cefoxitin disc diffusion method

All strain was tested with 30mg cefoxitin discs (Hi-Media) on Mueller-Hinton agar plates. For each strain, a bacterial suspension adjusted to 0.5 McFarland was used. The zone of inhibition was determined after 16-18h incubation at 37 °C. Zone size was interpreted according to CLSI (2008) [13] criteria: susceptibility > =22mm; resistance < =21mm.

Identification of MRSA by E-test strip method

Muller Hinton Agar plate with 2% NaCl was prepared. The dried plates were lawn cultured with test strain using sterile cotton swab using standardized inoculum (0.5 McFarland). The Ezy MIC of mic by e-test and cefoxitin disc diffusion for detection. Oxacillin strips (EM-065, HiMedia, India) were applied on the inoculated plates as per manufacturer's instruction. The plates were incubated at 35oC±2oC for 24 hours and read when sufficient growth is seen and MIC is noted where the ellipse of zone of resistance intersected the MIC scale on the strip. The strains were considered to be MRSA when MIC of ≥ 4 µg/ml was observed and Methicillin sensitive S.aureus if MIC was ≤ 2.0 µg/ml.

Genotypic Detection Method of MEC A Gene of Mrsa by PCR

Detection of mec a gene by Polymerase Chain Reaction where the Bacterial DNA was extracted by Qiagen DNA KIT by following manufactures guidelines. S. aureus previously extracted DNA was used for the amplification of mec A gene. A volume of 20 µl PCR reaction mixture consisting of 10 µl master mix, 1µl of each forward and reverse primers gene specific for the target gene, 3 µl of DNA template, and the volume was made up by adding nuclease free water. A 336-bp fragment of the mec A gene was obtained.

Table No 1: Primers for MECA Gene Polymorphism

Gene	Primer sequence	Length (bp)
MEC A	Forward- 5-GTTGTAGTTGTCGGGTTTGG -3	336bp
	Reverse 5-CTTCCACATACCATCTTCTTTAAC 3	

The mixture was briefly centrifuged and the tubes were transferred into PCR machine which has been programmed with the following conditions. The initial denaturation step for 7 minutes at 94°C followed by 94°C for 30 sec, 47°C for 40 sec, 72°C for 45 sec, 72°C for 7 min. The total cycles were 37. The PCR programming was very similar as followed by Jonas et al., 2002. The PCR products were electrophoresed, stained with 10 µM ethidium bromide, bromophenol blue visualized by using UV trans illuminator [17].

Results

A total of 965 clinical samples were studied in the present study. Out of 210 S.aureus isolates 58 isolates were identified as the MRSA isolates by both the phenotypic and genotypic method. The gold standard method was chosen to be the genotypic method as well as the Cexofitin disc diffusion method for the phenotypic detection. The MRSA isolates were highly susceptible to teicoplanin, vancomycin and linezolid.

Table No 1: Total number of Isolates

Type of Isolates	No. of Isolates
Clinical Isolates	965
MSSA	210
MRSA	58

Table 2: Phenotypic Identification with the use of different test

Microscopic observation	Gram's test	Catalase test	Coagulase test	Urease test	Cefoxitin (cx) and Oxacillin (ox)
Cocci form (For all 58 cases)	+	+	+	+	+

ssIt was observed that the maximum number of cases of MRSA reported was that of Males being affected with 35 (60.3%) followed by Females with 23 (39.6%) [Table No. 3].

Table No 3: Gender wise distribution of the isolates

Type of isolates	Gender	No. of isolates	Percentage
MRSA (N=58)	Male	35	60.3%
	Female	23	39.6%
Total		58	

Table No 4: Age wise distribution of the isolates

S.No.	Age (in years)	No. of Cases	Percentage
1	0- 10	1	1.70%
2	20-Nov	8	13.70%
3	21-30	15	25.80%
4	31-40	19	32.70%
5	41-50	11	18.90%
6	51-60	3	5.70%
7	≥61	1	1.70%

Genotypic Method Mec A Gene

The DNA was extracted using the Qiagen DNA Extraction kit as per the manufactures guidelines, and the DNA was run on the Electrophoresis which was visualized using the DNA gel Documentation System

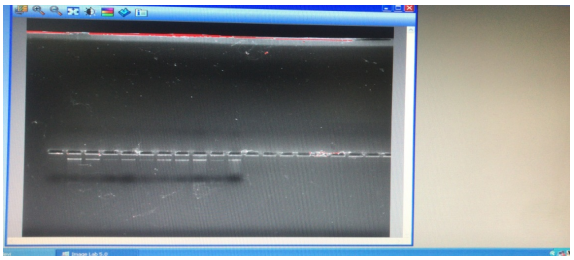

Figure No1: Isolated DNA from Bacterial culture of S. aureus

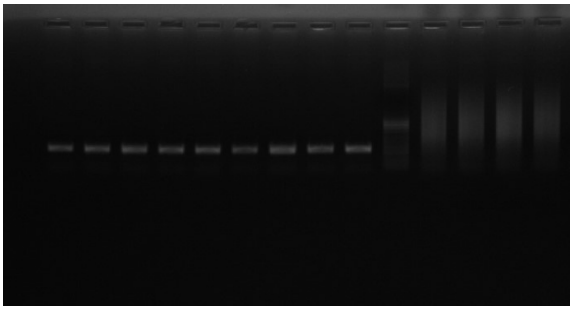

Figure No. 2: Amplified DNA with PCR for Mec A gene of S. aureus. Lane -1-9 is Positive control of Mec A gene; Lane 10 is the DNA Ladder

Table No 5: Phenotypic and Genotypic detection of MRSA

In the present study it was observed both the phenotypic and genotypic method gives 100% accuracy results. The Gold standard test for detecting MRSA is identification of the mecA gene using polymerase chain reaction (PCR) but in phenotypic methods, cefoxitin disc diffusion (CDD) test can also be recommended for detection of methicillin resistance in constraint setups that cannot afford PCR testing for mecA as a confirmatory test

Discussion

Methicillin-resistant Staphylococcus aureus (MRSA) is a pathogen with a worldwide distribution. Given the

increasing rate of MRSA infections, implementing of reliable, accurate and rapid testing for diagnosis of MRSA is necessary.

In the present study 965 clinical samples were studied in the present study. Out of 210 S.aureus isolates 58 isolates were identified as the MRSA isolates. This study was in support with the study performed by the other author where a high prevalence of MRSA (35% in ward and 43% in ICU) was observed from blood culture specimens in a study in Delhi [18]. The prevalence of MRSA in the present study was found to be 27.6%. This study was similar to the study in South India where the incidence of MRSA varies from 25 per cent in western part of India to 50 per cent [19]. It was observed that the maximum number of cases of MRSA reported was that of Males being affected with 35 (60.3%) followed by Females with 23 (39.6%).

Due to high prevalence of MRSA infections among hospitalized patients, rapid and accurate identification of MRSA is needed to initiate appropriate antimicrobial therapy and prevent the spread of MRSA infections. Usually, molecular methods such as detection of the mecA gene are preferred for this task because of high sensitivity and specificity. The results of molecular methods are also usually available faster than that of phenotypic methods [20].

In recent years, detection of mecA by PCR is considered as the gold standard for identification of MRSA. In this study, we evaluated other methods as alternatives to PCR [21], where phenotypic method of Cefoxitin was equally accurate for the detection of MRSA. Cefoxitin disc diffusion test was perceived to be the most sensitive method for detection of mecA-mediated resistance. CLSI has also recently substituted the oxacillin disc with cefoxitin disc for detection of MRSA [22]. Numerous studies including the current one have informed that the results of the cefoxitin disc diffusion test correlates better with the presence of mecA compared with those of the oxacillin disc diffusion test

The results about cefoxitin disc diffusion method are consistent with previous report [23]. However, Broekeme et al., reported the sensitivity and specificity of this method 97.3% and 100%, respectively among 1,611 S. aureus isolates [24].

In current study, MIC strip test showed the sensitivity and specificity about 91.6% and 100%, respectively. In the study of Rahbaret al., sensitivity and specificity were both 100% [25]

Cefoxitin is taken into consideration as it is a more potent inducer of mec-A gene expression than oxacillin or methicillin and the results obtained are comparable with detection of mec-A gene using PCR and also can be used in the constraint setups that cannot afford PCR testing for mecA as a confirmatory test [14, 15].

Conclusion

The present study showed that cefoxitin disc diffusion has both high sensitivity and specificity as compared with mecA gene by PCR. Therefore, it can be a good alternative to molecular methods due to its low cost for clinical laboratories.

References

1. Pavillard R, Harvey K, Douglas D, Hewstone A, Andrew J,Collopy B, et al. Epidemic of hospital-acquired infection due to methicillin-resistant Staphylococcus aureus in major Victoria hospitals. Med J Aust. 1982; 1:451-4.
2. Lucet JC, Paoletti X, Demontpion C, Degrave M, Vanjak D, Vincent C, et al.; Staphylococcus aureus Resistant a la Meticilline en Hospitalisation A Domicile (SARM HAD) Study Group. Carriage of methicillin-resistant Staphylococcus aureus in home care settings: Prevalence, duration, and transmission to household members. Arch Intern Med. 2009; 169:1372-8.
3. Sekiguchi JI, Fujino T, Saruta K, Konosaki H, Nishimura H, Kawana A, et al. Prevalence of erythromycin-, tetracycline-, and aminoglycoside-resistance genes in methicillin-resistant Staphylococcus aureus in hospitals in Tokyo and Kumamoto. Jpn J Infect Dis. 2004; 57:74–7.
4. Qureshi AH, Rafi S, Qureshi SM, Ali AM. The current susceptibility patterns of methicillin resistant Staphylococcus aureus to conventional anti Staphylococcus antimicrobials at Rawalpindi. Pak J Med Sci. 2004; 20:361–4.
5. Batabyal B, Kundu GK, Biswas S. Methicillin-resistant Staphylococcus aureus: A brief review. Int Res J Biol Sci. 2012; 1:65–71.
6. Kumar S, Budhani D, Sayal P, Sindwani P, Jain PK. Emphasis on antibiotic optimisation in difficult to treat methicillin resistant staphylococcus aureus infection. J Evolution Med Dent Sci. 2017; 6:5055–8.
7. Kulkarni S, Khare A, Charan Kaur D. Prevalence of methicillin resistant staphylococcus aureus-A study in a tertiary care rural hospital. Indian J Basic Appl Med Res. 2014; 3:414–21.
8. Mehta AP, Rodrigues C, Sheth K, Jani S, Hakimiyan A, Fazalbhoy N. Control of methicillin resistant Staphylococcus aureus in a tertiary care centre: A five year study. Indian J Med Microbiol. 1998; 16:31.
9. David MZ, Daum RS. Community-associated methicillin-resistant Staphylococcus aureus: Epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010; 23:616–87.
10. Ko JH, Moon SM. Evaluation of Methicillin-resistance rates among community-associated Staphylococcus aureus infections in Korean military personnel. J Korean Med Sci. 2018; 33:e250.
11. Kim ES, Kim HB, Kim G, Kim K-H, Park K-H, Lee S, et al. Clinical and epidemiological factors associated with methicillin resistance in community-onset invasive Staphylococcus aureus infections: Prospective multicenter cross-sectional study in Korea. PLoS One. 2014; 9:e114127.
12. Kali A, Stephen S, Umadevi S. Laboratory evaluation of phenotypic detection methods of methicillin-resistant Staphylococcus aureus. Biomed J. 2014; 37:411–4.
13. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, 15th Informational Supplement, M100-S15. Wayne, PA: Clinical and Laboratory Standards Institute; 2021.
14. Anand KB, Agrawal P, Kumar S, Kapila K. Comparison of cefoxitin disc diffusion test, oxacillin screen agar, and

PCR for mecA gene for detection of MRSA. Indian J Med Microbiol. 2009;27:27–9.
15. K B Anand , P Agrawal, S Kumar, K Kapila. Comparison of cefoxitin disc diffusion test, oxacillin screen agar, and PCR for mecA gene for detection of MRSA. Indian J Med Microbiology. 2009; 27(1):27-9.
16. Alam SMS, Kalam MA, Munna MS, Munshi SK, Noor R. Isolation of pathogenic microorganisms from burn patients admitted in Dhaka Medical Collage and Hospital and demonastration of their drug-resistant traits. Asian Pac J Trop Dis. 2014; 4:402-7.
17. Jonas D, Speck M, Daschner FD, Grundmann H. Rabid PCR-based identification of methicillin resistant Staphylococcus aureus from screening swabs. J Clin Microbiol 2002; 40(5):1821-23.
18. Wattal C, Goel N, Oberoi JK, Raveendran R, Datta S, Prasad KJ. Surveillance of multidrug resistant organisms in tertiary care hospital in Delhi, India. J Assoc Physicians India. 2010; 58(Suppl):32–6.
19. Gopalakrishnan R, Sureshkumar D. Changing trends in antimicrobial susceptibility and hospital acquired infections over an 8 year period in a tertiary care hospital in relation to introduction of an infection control programme. J Assoc Physicians India. 2010; 58(Suppl):25–31.
20. Barski P, Piechowicz L, Galinski J, Kur J. Rapid assay for detection of methicillin-resistant Staphylococcus aureus using multiplex PCR. Mol Cell Probes. 1996; 10:471–475.
21. Bhutia KO, Singh TS, Biswas S, Adhikari L. Evaluation of phenotypic with genotypic methods for species identification and detection of methicillin resistant in Staphylococcus aureus. Int J Appl Basic Med Res. 2012; 2:84–91
22. Adaleti R, Nakipoglu Y, Karahan ZC, Tasdemir C, Kaya F. Comparison of polymerase chain reaction and conventional methods in detecting methicillin-resistant Staphylococcus aureus. J Infect Dev Ctries. 2008; 2:46–50.
23. Ananl K, Agrawal P, Kumar S, Kapila K. Comparison of cefoxitin disc diffusion test, oxacillin screen agar, and PCR for mecA gene for detection of MRSA. Indian J Med Microbiol. 2009; 27:27–29.
24. Broekema NM, Van TT, Monson TA, Marshal SA, Warshauer DM. Comparison of cefoxitin and oxacillin disk diffusion methods for detection of mecA-mediated resistance in Staphylococcus aureus in a large-scale study. J Clin Microbiol. 2009; 47:217–219.
25. Rahbar M, Yaghoobi M, Fattahi A. Comparison of different laboratory methods for detection of methicillin resistant Staphylococcus aureus. Pak J Med Sci. 2006; 22:442–445.