ORIGINAL RESEARCH PAPER
Volume - 12 | Issue - 01 | January - 2023 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

ANTIBIOTIC RESISTANCE PROFILE AND MOLECULAR CONFORMATION AND MRSA ISOLATE BY MULTIPLEX PCR FROM CLINICAL SAMPLE AT A TERTIARY CARE HOSPITAL IN INDIA

Medical Microbiology

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ABSTRACT

Methicillin Resistant Staphylococcus aureus (MRSA) is an important human pathogen. They are emerging as a serious public-health issue. It causes nosocomial and community acquired infections. MRSA is present throughout hospitals in many countries. Nowadays it is the most commonly isolated antimicrobial resistant pathogen worldwide. **Material Method –** The study included 450 isolates of Staphylococcus species from various clinical samples. The samples received from outpatient departments (OPDs), inpatient departments (IPDs) & various intensive care units (ICUs) included urine, blood, pus, sputum, vaginal swab, tissue, pleural fluid. All clinical samples were processed as per standard bacteriological techniques. Molecular identification of Antibiotic Resistance Gene-mecA gene was also performed. **Result–** Out of 450 Staphylococcus species, 150 isolates were Staphylococcus aureus & 300 isolates were Coagulase Negative Staphylococci (CONS). **Conclusion –** Methicillin resistance is caused by the presence of mec- A gene, which encodes a low affinity penicillin binding protein (PBP)-2a or PBP2' which has a low affinity for β-lactam antibiotics. Therefore, presence of mec-A gene indicates methicillin resistance in Staphylococci. Detecting mec-A gene by polymerase chain reaction is now considered the gold standard for identifying methicillin resistance in S. aureus.

KEYWORDS

Methicillin Resistant Staphylococcus aureus (MRSA), antimicrobial resistant pathogen, Coagulase Negative Staphylococci (CONS), mec- A gene.

INTRODUCTION:

Staphylococcus aureus (S.aureus) has been recognized as an important cause of human disease for more than 100 years.[1] Sir Alexander Ogston introduced the name Staphylococcus (from Greek staphylé, a bunch of grapes), first isolated S.aureus from a surgical abscess in 1880 and described the role of S.aureus in localized infection and septicemia.[2,3]

S. aureus is recognized as a cause of a wide range of infections, from minor skin infections and chronic bone infections to devastating septicemia and endocarditis.[4]

Significant events in the evolution of S. aureus have included the development of methicillin resistance, now a problem for many hospitals around the world, and the recent emergence of community strains of S. aureus that are methicillin resistant but also harbor genes associated with increased virulence.[5,6]

Methicillin Resistant Staphylococcus aureus (MRSA) is an important human pathogen. It causes nosocomial and community acquired infections. They are emerging as a serious public-health issue[7]. MRSA is present throughout hospitals in many countries. Nowadays it is the most commonly isolated antimicrobial resistant pathogen worldwide.

Resistance to penicillin by Staphylococcus aureus strains after the use of penicillin started emerging in 1940s (75-95%) - mainly the hospital strains[8]. Methicillin Resistant Staphylococcus aureus strains (MRSA) is associated with infection of skin, soft tissue, endovascular tissue, urinary tract infections, lung - pneumonia, heart - endocarditis and also causes septic shock. Internationally MRSA is spread throughout many hospitals and health care institutions. Worldwide it is the most commonly isolated antimicrobial resistant pathogen. In India, MRSA prevalence extends from 30 to 70 %[9].

Methicillin which was introduced in 1960s was effective against infections by Staphylococcus aureus which produce penicillinase enzyme. MRSA strains appeared in 1961 which had PBP - Pencillin Binding protein that was altered and spread worldwide. Resistance is because of the modified PBP2a. This has a low affinity to β Lactam antibiotics[10]. These strains will possess mecA gene. The mec A gene is detected by Polymerase Chain Reaction – PCR[12]. MRSA is detected by the presence of mecA gene, resulting in altered PBP2a or PBP2'. It is considered as the Gold Standard for detecting resistance in Staphylococcus to Methicillin[10,11]. The other gene fem (Factor essential for Methicillin resistance) like fem A/B/X along with mec A expresses Methicillin resistance [10].

Several molecular markers have been identified that would be useful for epidemiological and diagnostic purpose, among them is the Staphylococcal Cassette chromosome (SCC mec).

Traditionally, many strains of MRSA got isolated from hospitalized patients. Internationally MRSA have started appearing in the community in patients with or without risk factor for MRSA infections suggesting a changing epidemiology. The importance of MRSA as a nosocomial as well as community acquired pathogen is well documented.

Rapid and accurate detection of MRSA and their antimicrobial susceptibility pattern is important for treatment with sensitive antibiotics. Hygienic precautions are important to reduce the spread of such strains like hand washing and screening for carrier in the hospital.

MATERIALS & METHODS

The study included 450 isolates of Staphylococcus species from various clinical samples that were received in the Clinical Microbiology Laboratory of Department of Microbiology. The samples received from outpatient departments (OPDs), inpatient departments (IPDs) & various intensive care units (ICUs) included urine, blood, pus, sputum, vaginal swab, tissue, pleural fluid. All clinical samples were processed as per standard bacteriological techniques.[12] The isolates were then identified by colony morphology and biochemical reactions such as catalase, slide coagulase, tube coagulase & mannitol as per standard guidelines & identified as S. aureus & CONS. Out of 450 Staphylococcus species, 150 isolates were Staphylococcus aureus & 300 isolates were Coagulase Negative Staphylococci (CONS). The culture media, reagents and chemicals used in the study were purchased from HiMedia Laboratories Private Limited, Mumbai, India. Staphylococcus aureus ATCC 43300-mecA positive (zone ≤21 mm), Staphylococcus aureus ATCC 25923-mecA negative (zone 23-29mm), were used as controls.

Methicillin resistance in S.aureus detected by phenotypic method namely Cefoxitin disc diffusion method. Testing for Methicillin resistance was done by using 30µg Cefoxitin disc by Kirby Bauer disc

diffusion method. 0.5 McFarland standard suspension of the isolate was made and lawn culture was done on MHA plates, and incubating at 37°C for 18-24 hours and zone diameter was measured[13].

The results were interpreted as per CLSI standards with appropriate ATCC controls.

	SUSCEPTIBLE	RESISTANT
Cefoxitin (30µg)	>22mm	<21mm

Antibiotic susceptibility testing was done for all the isolates by the Kirby Bauer disk diffusion method using the drugs recommended for Staphylococcus aureus by CLSI.[14]

- Cefoxitin
- Contrimoxazole
- Erythromycin
- Ciprofloxacin
- Gentamicin
- Amikacin
- Chloramphenicol
- Vancomycin
- Linezolid

The diameters of the zones of complete inhibition, including the diameter of the disk was measured to the nearest whole millimeter, using a ruler on the back of the inverted Petri plate.

The zone diameters measured around each disc were interpreted as per CLSI guidelines.

Molecular identification of Antibiotic Resistance Gene-mecA gene

HiPurA™ Bacterial Genomic DNA Purification Kit from HiMedia was used for DNA extraction. Kit contained Gram Positive Lysis Solution (GPLA), Lysis Solution, Prewash Solution, Wash Solution Concentrate, Elution Buffer, Proteinase K, RNase A Solution, HiElute Miniprep Spin Column (Capped, Uncapped). 2X PCR Master Mix, MRSA Primer Mix, 6X Loading Dye, 100 bp DNA Ladder, Positive control DNA were the constituents of Methicillin Resistant Staphylococcus aureus (MRSA) Detection Kit (Multiplex) from HiMedia. The procedure elaborated in kit literature was followed for DNA extraction, purification and PCR.

Agarose gel electrophoresis

1.8% of low EEO agarose gel, 1X TAE buffer, 6X DNA loading dye, Ready to use MRSA Primer Mix- 16 µl/ pcr reaction (Primer concentration provided: 16S rRNA – 0.6 pmol, femA – 0.8 pmol, mecA – 1.0 pmol, PVL – 0.6 pmol) were used for gel electrophoresis bands were observed in UV transilluminator for analysis.

FIGURE

Identification OF Staphylococcus aureus

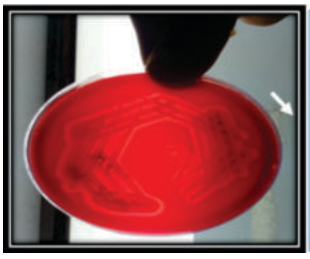

Fig1: Blood Agar showing β-hemolysis

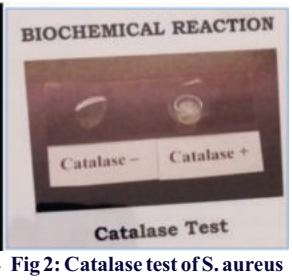

Fig 2: Catalase test of S. aureus

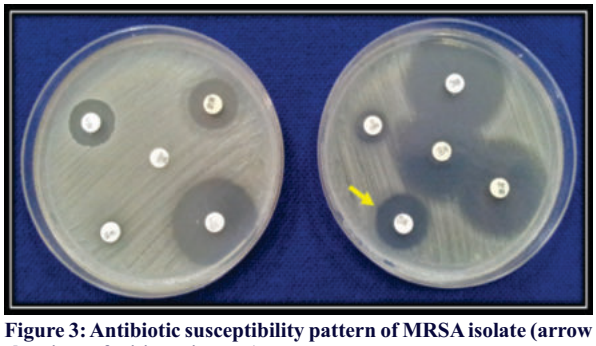

Figure 3: Antibiotic susceptibility pattern of MRSA isolate (arrow showing cefoxitin resistance)

RESULT:

The present study included 450 isolates of Staphylococci from various clinical samples like pus, urine, blood, sputum, vaginal swab, tissue, pleural fluid etc that were received in the Clinical Microbiology Laboratory. Out of 450 Staphylococcus species, 150 isolates were Staphylococcus aureus & 300 isolates were Coagulase Negative Staphylococci (CONS). The isolated 150 Staphylococcus aureus were further analysed as below:

Table 1: Sex wise distribution of S. aureus isolates patients (N= 150)

SEX	PATIENT	PERCENTAGE
Male	94	62.67
Female	56	37.33
TOTAL	150	100

Out of 150 S. aureus isolates, 62.67% (94) were obtained from males while 37.33% (56) were obtained from female.

Table 2: Overall Pattern of methicillin resistance in S.aureus isolates using cefoxitin disc (N= 150)

PATTERN OF METHICILLIN RESISTANCE	No. of CASES	PERCENTAGE
Susceptible	102	68
Resistant	48	32
TOTAL	150	100

Out of 150 S. aureus isolates, 32% (48) were methicillin resistant while 68% (102) isolates were sensitive to methicillin.

Table 3: Sex wise Distribution of MSSA & MRSA isolates in patients (N= 150)

SEX	MSSA	MRSA	TOTAL
Male	60 (58.82%)	34 (70.83%)	94
Female	42 (41.17%)	14 (29.16%)	56
TOTAL	102	48	150
Chi-square=2.01 p = 0.15*			

Not significant at p<0.05*

58.82% (60) MSSA isolates were obtained from males while 41.17% (42) MSSA isolates were obtained from females. Similarly, 70.83% (34) MRSA isolates were obtained from males while 29.16% (14) MRSA isolates were obtained from females.

Table 4: Age wise Distribution of S. aureus isolates in patients (N= 150)

AGE (yr.)	FREQUENCY	PERCENTAGE
<10	14	9.33
11 to 20	15	10.00
21 to 30	19	12.67
31 to 40	30	20.00
41 to 50	41	27.33
51 to 60	26	17.33
61 to 70	5	3.33
TOTAL	150	100
Mean =36.04; Std.Deviation=16.287; p<.001		

Out of 150 S. aureus isilates maximum number of S. aureus isolates (27.33%) (41) were obtained from the patients in age group 41 – 50 years.

Table 5: Specimen wise Distribution of MSSA & MRSA isolates in patients (N= 150)

SPECIMEN	MSSA	MSSA (%)	MRSA	MRSA (%)	TOTAL
Pus	45	44.12	11	22.92	56
Urine	16	15.69	8	16.67	24
Blood Culture	11	10.78	3	6.25	14
Sputum	9	8.82	6	12.5	15
Vaginal Swab	7	6.86	7	14.58	14
Tissue Culture	8	7.84	11	22.92	19
Pleural Fluid	6	5.88	2	4.17	8
TOTAL	102	100 %	48	100 %	150

Maximum number of S. aureus isolates 56 were obtained from pus samples, out of which 11 were MRSA and 45 were MSSA.Out of 24 S. aureus isolates from urine, 8 were MRSA while 16 were MSSA.

Similarly out of 14 blood culture isolates 3 were MRSA while 11 were MSSA.

Likewise, MRSA isolates obtained from sputum 6, vaginal swab 7, tissue culture 8 and pleural fluid 6.

Table 6: Antibiotic Pattern of MRSA Isolates in patients (N=48)

Antibiotics	Susceptible		Resistant	
	No. of isolates	%	No. of isolates	%
Cefoxitin	0	0 %	48	100 %
Penicillin	NA	NA	NA	NA
Cotrimoxazole	6	12.5%	42	87.5%
Erythromycin	18	37.5%	30	62.5%
Clindamycin	20	41.66%	28	58.33%
Ciprofloxacin	11	22.9%	37	77.08%
Gentamicin	14	29.16	34	70.84%
Amikacin	23	47.9%	25	52.1%
Chloramphenicol	21	43.75%	27	56.5%
Vancomycin	48	100%	0	0%
Linezolid	48	100%	0	0%

The MRSA isolates were associated with a high degree of co-resistance to other groups of antimicrobial agents. In our study, 87.5% were resistant to cotrimoxazole, 62.5% were resistant to erythromycin, 58.33% were resistant to clindamycin, 77.08% isolates were resistant to ciprofloxacin, 70.84% isolates were resistant to gentamicin, 52.1% were resistant to amikacin & 56.5% isolates were resistant to chloramphenicol. All the isolates, however, were sensitive to vancomycin & linezolid.

Table 7: Genotypic characterization of MRSA Isolates (N=48)

GENE	16SrRNA gene (Staphylococcus genus)	mecA gene (methicillin-resistant staphylococci)	lukPVL gene (Panton Valentine Leucocidin toxin)	femA gene
Positive	48	48	34	48
Negative	0	0	14	0
Total	48	48	48	48

Table 8: Comparison of cefoxitin disc diffusion test with PCR for detection of MRSA

Method	No. of False negatives	No. of False positives	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Concordance with PCR (%)
Cefoxitin disc diffusion	0	0	100	100	100	100	100

PPV- Positive Predictive Value, NPV- Negative Predictive Value

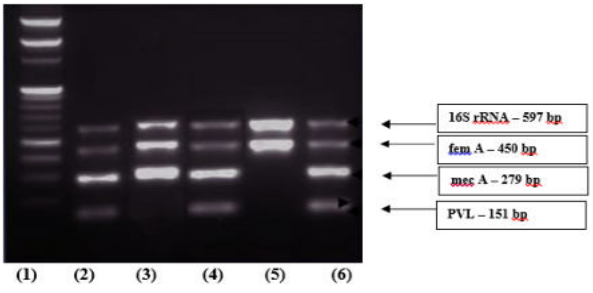

Fig4: Gel image representing amplification of MRSA clinical samples

Lane	1	2	3	4	5	6
Identification	100 bp Ladder	MRSA PVL+	MRSA PVL-	MRSA PVL+	MSSA	MRSA PVL+

DISCUSSION:

S.aureus is one of the most important pathogens, causing severe morbidity and fatal infections. The rapid evolution of antibiotic resistance in S.aureus is of considerable concern. Methicillin was indicated for treatment of staphylococcal infections due to penicillinase producing staphylococci. Methicillin resistant strains gradually evolved during last three decades which accounted for less than 0.1% of S.aureus in 1960s. Since then MRSA have become well established as hospital acquired pathogen.[15]

Resistance to this antibiotic implies resistance to all β-lactam antibiotics including cephalosporins and monobactams, the most important group of antibiotics to treat staphylococcal infection.

Hospital infection by MRSA strains has not only caused therapeutic problems, but also put a tremendous pressure on resources controlling their spread. Therefore it is important that clinical microbiology laboratories identify the organism accurately. This will help in determining the appropriate antimicrobial therapy.

Consequent benefits will be shorter hospital stay, lower hospital cost (by preventing unnecessary use of Vancomycin-glycopeptides and isolation precautions), limiting the emergence and cross transmission of antimicrobial resistant bacteria and in turn decreased morbidity and mortality.

Polymerase chain reaction (PCR) for mecA gene is presently considered as the standard method for detecting methicillin resistance in S.aureus. Inspite of growing consensus in the literature for this method, it is not available in all clinical laboratories due to financial and technical constraints, Therefore phenotypic methods, although dependent on many environmental and conditional factors still remain a method of choice in resource constraint laboratories.

In our study, the prevalence of S.aureus among clinical isolates is 33.33% which is similar to the study done by Bhatt, et al. in 2014[16] which showed the prevalence of 30.4%. But, our S.aureus isolation rate is higher than the study conducted by Shahi, et al. in 2018[17] which shows the prevalence of 14.4% and Dilnessa T et. Al[18] who also reported lower S.aureus isolation rates at 14.3%.

Our prospective study revealed that overall rate of prevalence of Methicillin resistance S.aureus was 32% (table-2) (figure.3) in a tertiary care hospital at Indore. Similarly ,Tiwari et al. in their study conducted in BHU, Varanasi have also reported 38.44% MRSA isolates.[19] Slightly higher MRSA isolation rates have been reported by Shanthi et al. at 45.5% in their study.[20] Perwaiz et al. have also reported that 43% isolates of S.aureus were MRSA.[21] Parveen et al. have observed isolation of 48% MRSA in their study from Andhra Pradesh.[22] Still higher isolation of MRSA was observed in a tertiary referral hospital in eastern Uttar Pradesh by Anupurba et al.who have reported isolation of 54.85% of MRSA which is much higher than our study.[23] Likewise , Behera et al. have reported a higher isolation of MRSA(75%) in their study from All India Institute Of Medical Sciences, New Delhi.[24] On the contrary, Pai et al. in their study have observed that only 69/ 237 (29.1%) of S.aureus isolates were found to be MRSA which is lower than our study.[25] Similarly, Mehta et al. from Chandigarh have reported a low (24%) isolation of MRSA in their study.[26]

In a comparative sex wise study of MRSA prevalence, males (were more affected than females (prevalence ratio of 34:14). Likewise Rao BN et al. in 2012 in their study conducted in Andhra Pradesh also reported a higher male to female prevalence ratio (60:40)[27]. On the contrary a different prevalence ratio (38:62) of male and female was reported by Ankur Goyal[28] in Agra and (39:61) by Sharma & Mall in Himachal Pradesh[29]

In the present prospective study, out of 150 isolates of S.aureus, 56(37.33%) isolates were obtained from pus samples which formed the largest group of samples followed by tissue culture, urine, blood, sputum, vaginal fluid & pleural fluid. Similar type of pattern was also observed by Shanthi et al. in their study who have also reported that maximum number of Staphylococcus aureus (67.5%) were obtained from samples like pus & wound swabs.[20] The study conducted in Mangalore by Pai et al. also showed that pus & wound swabs accounted for majority (76.3%) of isolates followed by urine, respiratory specimens, blood and body fluids.[25]Similarly, Perwaiz et al. have also reported that majority of S.aureus isolates were from pus(59.47%) followed by endotracheal aspirates, blood, urine & body fluids.[21] On the contrary, Ahmad et al. in their study isolated the maximum number of S. aureus from blood (40%) followed by wound swabs(12%),respiratory specimens(6%) & urine(2.5%).[30]

The MRSA isolates were associated with a high degree of co-resistance to other groups of antimicrobial agents. In our study, 87.5% were resistant to cotrimoxazole, 62.5% were resistant to erythromycin, 58.33% were resistant to clindamycin, 77.08% isolates were resistant

to ciprofloxacin, 70.84% isolates were resistant to gentamicin, 52.1% were resistant to amikacin & 56.5% isolates were resistant to chloramphenicol. All the isolates, however, were sensitive to vancomycin & linezolid. Similar high level of co resistance to other group of antibiotics has been observed by other workers too. Pai et al. observed in their study that about 40-50% of MRSA isolates were resistant to erythromycin, gentamicin & chloramphenicol.[25] Similarly, Anupurba et al. have also reported that MRSA isolates were found to have multi drug resistance. More than 80% of MRSA were resistant to cotrimoxazole, ciprofloxacin, gentamicin, erythromycin, tetracycline.[23] Likewise, Perwaiz et al. have reported 90- 95% resistance to erythromycin & clindamycin, 93% resistance to gentamicin & 54% resistance to amikacin in MRSA isolates.[21] In their study, Tiwari et al observed that almost all MRSA strains were resistant to penicillin, 95.68% were resistant to cotrimoxazole, 92.36% were resistant to chloramphenicol, 90.7% were resistant to norfloxacin, 76.1% were resistant to tetracycline, and 75.75% were resistant to ciprofloxacin.[19]

All the isolates were sensitive to vancomycin and linezolid in our study. Similar study done by Loveena Oberoi et al[31] who also found 100% sensitivity for these drugs in their study in Punjab and Haryana.

All the 48 MRSA isolates were positive for 16S rRNA, mecA gene and femA gene. 34 (70.8%) isolates were positive for lukPVL gene (Panton Valentine Leucocidin toxin) and 14 (29.1%) isolates were negative.

In our study, the result of cefoxitin disk diffusion with 100% sensitivity and specificity was the same as PCR method for detection mecA gene. Considering PCR as the gold standard, concordance with PCR was 100%.

Similar findings were observed by Koupahi H et al.[32] in their study in 2016. All 105 mec A gene positive samples by PCR were also detected positive by cefoxitin disc diffusion test with 100% sensitivity and specificity.

Panda RK et al.[33] in their study in 2016 observed the sensitivity of cefoxitin disc in detecting MRSA was 96.7% and specificity was 100%. Out of 62 mec A confirmed samples by PCR only 60 were detected by cefoxitin disc diffusion test. Considering PCR as the gold standard, concordance with PCR was 99%.

In their study in 2020, Madhavan A et al.[34], out of 72 mec A gene confirmed MRSA samples by PCR, 70 were identified as MRSA by cefoxitin disc diffusion test with 97.2% sensitivity, 100% specificity and 98% concordance of cefoxitin disc diffusion test with PCR.

CONCLUSION

Methicillin resistance is caused by the presence of mec- A gene, which encodes a low affinity penicillin binding protein (PBP)-2a or PBP2' which has a low affinity for β-lactam antibiotics[35]. Therefore, presence of mec-A gene indicates methicillin resistance in Staphylococci. Detecting mec-A gene by polymerase chain reaction is now considered the gold standard for identifying methicillin resistance in S. aureus[36]. In spite of the growing consensus in the literatures for this method, it is not yet available in all clinical laboratories, therefore phenotypic methods still remain as the methods of choice in the resource constraint settings.

Cefoxitin disc diffusion test has shown promising results in detection of MRSA & is recommended by CLSI for detection of methicillin resistance. The advantage of using cefoxitin is that the test conditions are similar to those used for other antibiotics. Studies have shown Cefoxitin is a better inducer of the expression of the mecA gene, so the heterogeneous population that variably express the mecA gene is better detected by disc diffusion with cefoxitin than with oxacillin, which is a weak inducer of PBP2 a production.

Results of cefoxitin disc diffusion were in concordance with results of PCR in our study. PCR is too costly to be routinely implemented in most of the clinical laboratories. The cefoxitin disc diffusion method was found to be a reliable, cost effective method for detection of MRSA & the result was concordant with PCR mecA gene detection method.

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