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RESEARCH ARTICLE

STAPHYLOCOCCAL CASSETTE CHROMOSOME *mec* TYPING IN CLINICAL ISOLATES OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS IN A TERTIARY CARE HOSPITAL IN MUMBAI

Raut S, Nataraj G*, Mehta P

Seth G.S.M.C. & K.E.EM.H., Parel, Mumbai

Corresponding Author: Dr. Gita Nataraj, Professor, Department of Microbiology, 5th floor, MSB, KEM Hospital, Acharya Donde Marg, Parel, Mumbai 400012.

Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA), considered for long as being healthcare associated, is increasingly being reported in community settings also. In India, the few studies published so far have shown that a large majority of the isolates were SCC*mec* type III while the prevalence of other SCC*mec* types was found to be low. The burden of MRSA in outpatient settings may be relevant to the choice of empiric therapy given for common staphylococcal infections. This study was undertaken to characterize Staphylococcal Cassette Chromosome in genotypically proven clinical isolates of MRSA (*mec*A gene positive) and determine the susceptibility profile of these isolates. The goal of the present study was to provide additional data on the epidemiology of MRSA. **Method**: 100 consecutive, non-duplicate, clinical isolates of MRSA showing resistance to cefoxitin 30µg disc and presence of *mecA* gene were included in a prospective cross-sectional study. These were further subjected to antimicrobial susceptibility testing and molecular characterization of *SCCmec* gene. **Results:** Prevalence of MRSA was found to be 31.85% with a significantly higher prevalence in indoor patients(43.88%)as compared to outdoor(16.95%). 92% belonged to SCC*mec* type III and 8% belonged to SCC*mec* type IV and V. **Conclusion:** The results of the present study suggest that MRSA most likely remains a hospital-acquired infection, but a small proportion of cases may be community acquired. Efforts to reduce MRSA should include strengthening infection control practices especially hand hygiene and prudent antimicrobial usage in terms of dosage, drugs and duration not only in the hospital but also in the community setting.
Key words: MRSA, *mecA*, SCC*mec* typing.

INTRODUCTION

Staphylococcus aureus is an important pathogen causing skin, soft tissue and bone infections. It is one of the most common causes of healthcare-associated bacteraemia and surgical site infection.[1] The mainstay of treatment for staphylococcal infections remains β-lactam antibiotics and surgical drainage.[1] The emergence of methicillin resistant strains both in health care as well as community settings however has compromised efficacy of therapy.[1]

In methicillin-resistant *S.aureus* (MRSA), resistance to β-lactam antibiotics is mediated by the *mecA* gene which encodes an additional methicillin-resistant penicillin-binding protein (PBP2a), having a very low affinity for β-lactam antibiotics.[2]It is situated on a mobile genetic element, the Staphylococcal Cassette Chromosome *mec* (SCC*mec*).[3] Numerous SCC*mec* types

(I–XI) have been identified till date, and several variants of these SCC*mec* types have been described.

MRSA infections, long considered as being healthcare associated, are being increasingly reported from community settings as well.[3,5,6,7] To minimize the spread of MRSA, newer strategies have to be devised which necessitate thorough knowledge of dissemination and epidemiology of MRSA strains. Various molecular typing techniques have been developed for this purpose include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), SCC*mec* typing and typing of the variable tandem repeat region of protein A (*spa* typing)[3].

Very few studies in India have dealt with molecular characterization of the SCC*mec* element of MRSA. The studies published so far have shown that a large majority of the isolates were SCC*mec* type III while the prevalence of other SCC*mec* types was low[6, 7, 8.] This study was undertaken to characterize Staphylococcal Cassette Chromosome in genotypically proven clinical isolates of MRSA (*mec*A gene positive) and determine the susceptibility profile of these isolates. The goal of the present study was to provide additional data on the risk factors and epidemiology of MRSA.

MATERIALS AND METHOD:

Consecutive, non-duplicate, clinical isolates of MRSA were identified using standard microbiological procedures. The isolates demonstrating resistance to cefoxitin 30µg disc by Kirby Bauer Disk Diffusion Method performed and interpreted as per CLSI standards 2014 were included [9,10]. The strains were tested for *mec*A gene by Polymerase Chain Reaction (PCR). 100 such *mecA* positive strains were included in a prospective cross-sectional study after obtaining Ethics Committee approval. These were further subjected to antimicrobial susceptibility testing and molecular characterization of *SCCmec* gene.[11] Clinical diagnoses, along with risk factors for acquiring MRSA were documented with specific reference to previous antimicrobial exposure and hospitalization, time from admission to culture of more than 48 hrs, invasive procedures carried out and immunocompromised status.[9,12,13,14] Individual patient identifiers were not recorded.

Antimicrobial Susceptibility Testing (AMST) :

AMST and interpretation were carried out by Kirby Bauer disc diffusion/ E test method as per CLSI standards[9] for Amikacin(30µg), Cefoxitin(30µg), Chloramphenicol(30µg), Ciprofloxacin(5µg), Clindamycin(2µg), Co-trimoxazole(25µg), Doxycycline(30µg), Erythromycin(15µg), Fusidic acid(10µg), Gentamicin(10µg), Linezolid(30µg), Moxifloxacin(5µg), Mupirocin (20µg), Netilmycin(30µg), Ofloxacin(5µg), Penicillin(10U), Rifampicin(5µg), Teicoplanin(30µg), Tetracycline (30µg) and Vancomycin.

Mupirocin and fusidic acid were interpreted as per British Society for Antimicrobial Chemotherapy (BSAC) standards, 2014[15] since CLSI standards were not available. For vancomycin, E test (Ezy MIC™ Strip) was used to determine MIC. Inducible clindamycin resistance (ICR) was detected by the D-test in erythromycin-resistant but clindamycin-susceptible strains as described by Sutliffe, et al. [10,16,17] Quality control strains used for AMST

were *Staphylococcus aureus* ATCC 25923 and Methicillin resistant *Staphylococcus aureus* ATCC 33591.

mecA detection by Nuclei Acid Amplification Test (NAAT)

All strains phenotypically characterized as MRSA were tested for presence of *mec*A by PCR as described by Zhang et al.[11]

DNA extraction (Heat Extraction):

Frozen bacteria were sub-cultured twice onto 5% sheep blood Columbia agar plates 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 min, 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 (table 1), provided by Genetix Biotech, Eurofins Genomics India Pvt Ltd.

Table 1: Primers used for _mecA_ PCR

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), Forward and Reverse Primer (10 pmol/ µl) 1 µl each and water. The amplification was performed in a thermal cycler (Eppendorf Master cycler 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; 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 followed by a hold at 4°C.The cycle parameters were confirmed using known positive and negative controls.

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.

Molecular characterization of _SCCmec_:

All strains genotypically characterized as *mec*A positive were further evaluated for their *SCCmec* type as per procedure described by Zhang et al[11] with modifications. For each *SCCmec* type individual amplification run was carried out instead of a multiplex format using known positive

controls for Type III, Type IV and Type V. Controls were provided by Benjamin M Pulimood Laboratories for Infection, Immunity and Inflammation (BMPLIII), Department of Medicine Unit I and Infectious Diseases, Christian Medical College, Vellore, India and the Infectious Disease Unit, St. John's National Academy of Health Sciences at Bangalore Controls for Type I and Type II were not available. *mec*A primers were used as internal amplification control.

DNA extraction (Lysostaphin)[18]:
A suspension of bacteria with saline solution (at 5 McFarland level) was prepared and centrifuged at 8000 rpm for 5 min. After discarding supernatant, the sediment was resuspended in 50µl of lysostaphin (100µl/ml) and incubated at 37°C for 10 min. 50 µl proteinase K (100 µl/ml) and 150 µl buffer (0.1 M Tris 7.5 pH) were added, followed by incubation at 37°C for 10 min, heating for 5 min at 100°C and centrifugation at 8000 rpm for 3 min. Lysostaphin (cat no L7386) required for extraction was obtained from Sigma and Proteinase K and Tris buffer were obtained from Fermentas, Mumbai.

PCR amplification:
Optimization of the PCR protocol was performed by following the general principles as described by Henegariu et al.[19] Each pair of primers was first tested for amplification specificity using annealing temperatures between 47°C to 57°C. For the best result it was necessary to decrease the annealing temperature, increase the extension time and adjust primer amounts. These alterations were tested in small steps. Reliable amplification of two bands was obtained for all strains tested when the final concentration of the different primers were adjusted to 20 pmol/µl with 30 reaction cycles, increasing the extension time to 2 min and decreasing the annealing temperature to 50°C.
Amplification was performed using thermal cycler by following set of primers (Table 2) provided by Genetix Biotech, Eurofins Genomics India Pvt Ltd.

All PCR assays were performed directly on extracted DNA obtained after the lysostaphin extraction method. An aliquot of 2 µl of extracted DNA was added to 23 µl of PCR mixture containing 12.5 µl of PCR Master Mix (Fermentas), primers for *mec*A and each individual *SCCmec* type (20 pmol/ µl) of 2 µl and molecular grade 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 60 seconds, 50°C for 60 seconds, and 72°C for 2 min, ending with a final extension step at 72°C for 5 min followed by a hold at 4°C. The cycle parameters were confirmed using known positive and negative controls.

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. Results were interpreted as per amplicon sizes mentioned in the Table 2.

Statistical analysis:

Odds ratio was calculated to measure the strength of association between the risk factors and infection. Odds ratio value of >1 was considered statistically significant.

Table 2: Primers used for SCCmec by PCR

Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Specificity
Type I-F Type I-R	GCTTTAAAGAGTGTCGTTACAGG GTTCTCTCATAGTATGACGTCC	613	*SCCmec*I
Type II-F Type II-R	CGTTGAAGATGATGAAGCG CGAAATCAATGGTTAATGGACC	398	*SCCmec*II
Type III-F Type III-R	CCATATTGTGTACGATGCG CCTTAGTTGTCGTAACAGATCG	280	*SCCmec*III
Type IVa-F Type IVa-R	GCCTTATTCGAAGAAACCG CTACTCTTCTGAAAAGCGTCG	776	*SCCmec*IVa
Type IVb-F Type IVb-R	TCTGGAATTACTTCAGCTGC AAACAATATTGCTCTCCCTC	493	*SCCmec*IVb
Type IVc-F Type IVc-R	ACAATATTTGTATTATCGGAGAGC TTGGTATGAGGTATTGCTGG	200	*SCCmec*IVc
Type IVd-F5 Type IVd-R6	CTCAAAATACGGACCCCAATACA TGCTCCAGTAATTGCTAAAG	881	*SCCmec*IVd
Type V-F Type V-R	GAACATTGTTACTTAAATGAGCG TGAAAGTTGTACCCTTGACACC	325	*SCCmec*V

RESULTS:

The first 100 non-duplicate MRSA (*mecA* positive) isolates were detected from 314 *S.aureus* isolates. The MRSA prevalence was 43.88% in indoor setting and 16.95% in outdoor settings, resulting in an overall prevalence of 31.85%. Of 100 MRSA strains isolated, 79 were from indoor patients and 21 from outdoor patients. Of the 79 isolates, 9 came from ICU setting and 70 came from general wards. Majority of the isolates (82%) were from patients from surgical branches and were recovered from pus (91%).

SCC*mec* typing:
SCC*mec* typing revealed 92 strains as SCC*mec* III, 5 as SCC*mec* IV, and 3 as SCC*mec* V while types I and II were not detected. All three types were found in both indoor patients and outdoor patients.
A specimen-wise distribution of SCC*mec* types (Table 3) revealed that types IV and V were present in only pus specimens.

Antibiotic susceptibility testing:
Antibiotic susceptibility testing (Table 4) demonstrated 100 % susceptibility to chloramphenicol, mupirocin, vancomycin and linezolid irrespective of the SCC*mec* type. Susceptibility of the eight SCC*mec* type IV and V strains ranged from 0% (β-lactams) to 100% (chloramphenicol, mupirocin, vancomycin and linezolid). Susceptibility of SCC*mec* Type III strains to the same antimicrobials was lower and ranged from 10.86% (ciprofloxacin) to 98.91% (rifampicin). All 92 SCC*mec* Type III strains of MRSA were resistant to erythromycin and 76 of these were found to be sensitive to clindamycin. ICR in these strains was found to be 75%.

Risk factors
An analysis of the associated risk factors (Table 5) showed that all patients with SCC*mec* III strains had risk factors for MRSA infections. Of the patients with SCC*mec* IV and V strains, 37.5% had risk factors while 62.5% had no documented risk factors. The most common risk factors were previous antibiotic exposure (95%) followed by time elapsed >48 hrs from admission to culture (79%).

Table3: Specimen wise distribution of SCC*mec* types

Specimen	**Scc*mec* Types (n)**						**MRSA**	
	Type III		**Type IV**		**Type V**			
Pus	83		5		3		91	
Mini broncho alveolar lavage	4		0		0		4	
Blood culture	2		0		0		2	
Conjuctival swab	1		0		0		1	
Cerebrospinal shunt fluid	1		0		0		1	
Sputum	1		0		0		1	
Total	**92**		**5**		**3**		**100**	
Setting	IPD 76	OPD 16	IPD 2	OPD 3	IPD 1	OPD 2	IPD 79	OPD 21

Table 4: Antimicrobial susceptibility pattern (% sensitive)

Antibiotics	**Type III**	**Type IV**	**Type V**	**Total**
	N=92	N=5	N=3	N=100
Amikacin	60(65.21%)	5(100%)	3(100%)	68
Cefoxitin	0	0	0	0
Chloramphenicol	**92(100%)**	**5(100%)**	**3(100%)**	**100**
Ciprofloxacin	10(10.86%)	1(20%)	1(33.33%)	12
Clindamycin	68(73.9%)	5(100%)	3(100%)	76
Co-trimoxazole	1(1.08%)	0	1(33.33%)	2
Doxycycline	67(72.82%)	5(100%)	3(100%)	75
Erythromycin	0	5(100%)	3(100%)	8
Fusidic acid	87(94.56%)	5(100%)	3(100%)	95
Gentamicin	23(25%)	2(40%)	1(33.33%)	26
Linezolid	**92(100%)**	**5(100%)**	**3(100%)**	**100**
Moxifloxacin	24(26.08%)	3(60%)	2(66.67%)	29
Mupirocin	**92(100%)**	**5(100%)**	**3(100%)**	**100**
Netilmycin	67(72.83%)	5(100%)	3(100%)	75
Ofloxacin	13(14.13%)	2(40%)	1(33.33%)	16
Penicillin	0	0	0	0
Rifampicin	91(98.91%)	5(100%)	3(100%)	99
Teicoplanin	70(76.08%)	5(100%)	3(100%)	78
Tetracycline	71(77.17%)	5 (100%)	3(100%)	79
Vancomycin	**92(100%)**	**5(100%)**	**3(100%)**	**100**

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Risk factors	Type III	Type IV	Type V	Total	Odds ratio
	N=92	N=5	N=3	N=100	
Antimicrobial exposure	92	2	1	95	--
Time from admission to culture more than 48 h	76	2	1	79	7.91

Table 5: Risk factors associated with acquisition of MRSA

Catheterization	62	0	0	62	--
Previous hospitalization	59	2	0	61	5.36
Surgery	55	0	1	56	10.40
Intubation	30	0	0	30	--
Patients with loss of skin integrity	22	1	1	24	0.94
ICU stay	22	0	0	22	--
Central venous line	20	0	0	20	--
Immunocompromised status (Diabetes, steroids)	18	0	1	19	1.70

DISCUSSION:

Molecular typing is considered an important tool in understanding the epidemiology of MRSA. The present study using SCC*mec* typing to characterize 100 consecutive clinical isolates of MRSA (*mec*A positive) showed that 92% belonged to SCC*mec* type III.

A majority of the published literature on SCC*mec* from India upto the year 2010 indicates a preponderance of type III.[6, 20, 21] Studies published after 2010 however demonstrate an increase in the prevalence of SCC*mec* types IV and V.[7, 22] The higher prevalence of SCC*mec* Type III in the present study may be due to the patient population selected as majority of the isolates were recovered from either admitted patients or those with a history of hospitalization/ surgery in the immediate past.

An overlap of epidemiologic characteristics in the SCC*mec* types was observed. SCC*mec* type III was found in 76 isolates from indoor patients(96.20%) and 16 isolates from outdoor patients(76.19%) Three of the eight outpatients however had history of hospitalization. As such, there was no clear demarcation as to the SCC*mec* types found in the in-patient and the out-patient populations. Such overlap has been noted in other studies from India as well.[7, 22]

The results of the present study suggest that in tertiary care settings such as ours, MRSA most likely remains a hospital- acquired pathogen, but a small proportion of cases may be seen in outpatient settings as well.

A comparison of the susceptibility profile of different SCC*mec* types revealed that SCC*mec* type III strains were multidrug resistant as compared to SCC*mec* type IV and type V strains which were multidrug sensitive. These findings have also been reported by D'Souza, *et al.*[7] from Mumbai (2010) in which all SCC*mec* type III strains were multidrug resistant.[7]

In the current study 57% of the MRSA isolates demonstrated ICR and all of these belonged to SCC*mec* type III. Similar results have been reported in other studies with ICR ranging from

38.4% from Karnataka, India[27] to 50% from USA.[28] High prevalence of ICR emphasises the need to detect the same before clinical use.

In the present study the most common risk factor identified was previous antimicrobial use, probably because this study was conducted at a tertiary care centre where a large proportion of patient population has previous exposure to health care system. Other significant risk factors for acquisition of MRSA SCC*mec* Type III documented in this study include admission for more than 48 hrs, a history of surgery and previous hospitalization. Surgical intervention is associated with loss of normal host defences and administration of antibiotics for prophylaxis; both proven risk factors for MRSA.

Though the small sample size may not allow for tangible conclusions, it appears that in tertiary care settings SCC*mec* type III is still the predominant *mec* type. Epidemiologic criteria overlap between the SCC*mec* types. The widespread occurrence and dissemination of resistant strains of *S.aureus* renders multiple antibiotics ineffective and increases the cost of health care. This needs to be tackled by judicious use of existing antibiotics and enhanced infection control practice.

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