ORIGINAL ARTICLE

Molecular Characterization of Staphylococcal Cassette Chromosome *mec*A and Concomitant Panton-valentine Leukocidine in Clinical Isolates of Community-acquired Methicillin-resistant *Staphylococcus aureus*

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Abstract:

Background: Staphylococcus aureus (*S. aureus*) remains, to date, one of the major causes of both Healthcare Associated (HA) and Community Associated (CA) infections. *S. aureus* causes a variety of infections, ranging from Skin and Soft Tissue Infections (SSTI) to life threatening endocarditis. Healthcare Associated Methicillin-resistant *Staphylococcus aureus* (HA-MRSA) and Community-associated Methicillin-resistant *Staphylococcus aureus* (CA-MRSA) have been increasingly reported from India. Efficacious dissemination of the complex and heterologous Staphylococcal Cassette Chromosome (SCC) *mec* elements necessitate to detect its SCC *mec* typing. Panton-Valentine Leucocidin (PVL) is a cytotoxin produced by *S. aureus* associated CA infections of *S. aureus. Aim and Objectives:* To investigate the prevalence of HA-MRSA and CA-MRSA in tertiary care hospital and to analyse the demographic and clinical characteristics of patients with Methicillin-resistant *Staphylococcus aureus* (MRSA) infections with *mec A* gene and PVL gene–positive strains and to compare with those for PVL gene–negative strains. *Material and Methods:* Isolation and identification has been done by standard conventional methods. *Results:* A total of 400 MRSA; 107 (26.75%) MRSA were from blood sample of Body Substance Isolation (BSI) and endocarditis 81(20.25%) were from osteomyelitis and septic arthritis, 97(24.25%) were from skin and soft tissue infections, 62(15.5%) were from pneumonia, 45(11.25%) were from Urinary Tract Infection (UTI). Of the total 400 MRSA strains; 183(45.75%) strains were isolated from paediatric and neonatal age group. All MRSA strians were susceptible to tigecycline and vancomycin. 95.5% strains were susceptible to linezolid. Of the total MRSA;40.5% strains were susceptible for clindamycin and all strains isolated from SSTIs were susceptible clindamycin. MRSA strains 248(62%) were resistant to tetracycline. 75.25% and 42.75% MRSA strains were defined as CA-MRSA and HA-MRSA respectively by clinical criteria: CDC epidemiologic definitions.76% (41/54) of patient isolates had presence of both the genes in the genome whereas 16% had only *mecA* gene. Small percent of isolates (5.5%) did not have both the genes on the genome. *Conclusion*: Our laboratory analysis of MRSA isolates indicated a high number of PVL-positive CA-MRSA isolates, carrying a novel *mecA* gene. The co-occurrence of multidrug-resistant MRSA and PVL-positive CA-MRSA highlights the risk for the emergence of a multidrug-resistant PVL-positive MRSA clone.

Keywords: Staphylococcal Cassette Chromosome *mecA*, Community Associated Methicillin-resistant *Staphylococcus aureus*, Healthcare Associated Methicillin-resistant *Staphylococcus aureus*, Skin and Soft Tissue Infection, Panton-Valentine Leucocidin

Introduction

Staphylococcus aureus (*S. aureus*) remains, to date, one of the major causes of both Healthcare Associated (HA) and Community Associated (CA) infections. *S. aureus* causes a variety of infections, ranging from Skin and Soft Tissue Infections [SSTI] to life threatening endocarditis. [1-2]. The prevalence of MRSA infections, especially bacteraemia, and neonatal septicaemia differs around the world. In 2014, the percentage of invasive MRSA isolates in Europe ranged from 0.9% in the Netherlands to 56% in Romania, with a population-weighted mean of 17.4%. MRSA prevalence exhibits a North–South variation in Europe, with a higher proportion of resistant isolates in southern countries as compared with northern countries [3].

MRSA is now endemic in India. The incidence of Methicillin-resistant *Staphylococcus aureus* (MRSA) varies from 25% in western part of India to 50% in South India. Community Associated-Methicillin-resistant *Staphylococcus aureus* (CA-MRSA) has been increasingly reported from India [2-4]. MRSA has spread and become established as major nosocomial pathogens and emerged as a major cause of community-acquired infections worldwide. MRSA strains have acquired and integrated into their genome a 21- to 67-kb mobile genetic element, termed the Staphylococcal Cassette Chromosome *mec* (SCC *mec*), which harbours the methicillin resistance (*mecA*) gene and other antibiotic resistance determinants [3-5]. These newly emerging community-acquired MRSA strains possess novel, small, mobile SCC *mec* type IV or V genetic elements which contain the *mecA* gene with or without additional antibiotic resistance genes and are more easily transferred to other strains of *S. aureus* than larger SCC *mec* (types I, II, and III) elements [4-6]. A systematic review of the molecular epidemiology and progression of MRSA in community as well as in hospital environment is vital. Efficacious dissemination of the complex and heterologous SCC *mec* elements necessitate to detect its SCC *mec* typing and concomitant subtyping of mec Types I to V which is an important molecular tool and playing substantial role for its detection in community clonal outbreaks globally. An alarming alert thing is that the frequency of single clone producing the Panton-Valentine Leucocidin (PVL) induced CA-MRSA infections has been increased worldwide.

PVL is a cytotoxin produced by *S. aureus*. PVL exhibits highly specific lytic activity against polymorphonuclear cells, monocytes, and macrophages in humans. PVL is a bicomponent toxin that consists of the polypeptides *luk S-PV* and *luk F-PV*, and genes for these PVL components have been found in the genomes of various temperate phages [5-7]. PVL gene is detected in <5% of *S. aureus* strains of clinical origin, and it is mainly associated with necrotic lesions of the skin and subcutaneous tissues, such as furuncles, and also with community-acquired, severe, necrotizing pneumonia [6-8]. Recent findings suggest that severe pneumonia caused by PVL-producing *S. aureus* can occur in healthy children and young adults. Prevalence of MRSA with high vancomycin Minimum Inhibitory Concentrations (MICs) have been increased and usually associated with prolonged bacteraemia and increased mortality. Recent consensus guidelines recommend that clinicians consider using alternative agents for MRSA infection when the vancomycin MIC is greater than 1 mcg/ml. Recent

reports comparing daptomycin and vancomycin in treatment of Body Substance Isolation (BSI) by MRSA with high vancomycin MIC demonstrated that daptomycin presented improved outcome, both in terms of rates of clinical success and mortality, compared to vancomycin. Therapeutic use of daptomycin in treating BSIs caused by MRSA remained under investigation.

We investigated the prevalence of HA-MRSA and CA-MRSA in tertiary care hospital with special reference to their antimicrobial susceptibility pattern and molecular characterization of *mecA* gene and PVL gene among isolates recovered from various clinical samples. Also, we analysed the demographic and clinical characteristics of patients with MRSA infections with *mec A* gene and PVL gene–positive strains, and compared these findings with those for PVL gene–negative strains.

Material and Methods:

The present study was conducted in the Department of Microbiology of Dr. D. Y. Patil Medical College, Hospital and Research Centre Pimpri Pune which is tertiary care teaching hospital, India. The study was conducted from 1st January 2015 to 31st December 2017. The study was approved by the Institutional Ethics Committee of the Vidyapeeth.

Samples:

All clinical samples received from various clinical departments for culture and sensitivity laboratory diagnosis.

Inclusion Criteria:

Isolates of MRSA from the clinical samples received in the microbiology department.

Clinical Samples:

Blood, pus, wound swab, urine, CSF, and body fluids.

Sample Processing:

S. aureus isolates were identified by the standard conventional methods from various clinical specimens. Antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion method for co-trimoxazole (25 µg), gentamycin (30 µg), erythromycin (15 µg), linezolid (30 µg), tetracycline (30 µg), and vancomycin (30 µg) as per guidelines from Clinical and Laboratory Standards Institute (CLSI). Screening for oxacillin resistance using oxacillin (1 µg) on (Muller-Hinton) M-H agar supplemented with 2% NaCl followed by overnight incubation at 35°C [9-10].

Phenotypic Detection of Inducible Resistance to Clindamycin by D-zone Test:

The inducible Clindamycin resistance was performed by D-zone test using erythromycin (15 µg) and clindamycin (2 µg) discs as per CLSI guidelines. Three different phenotypes were interpreted as MS phenotype, Inducible MLSB phenotype and Constitutive MLSB phenotype [11].

Quality Control:

S. aureus ATCC 25923 strain was used as the quality control strain. Medical records for the source patients were reviewed for the demographic information, history of prior hospitalization, presence of major comorbid conditions (e.g. Diabetes mellitus, renal dysfunction, post-surgical status, malignancy, solid organ or stem cell transplantation, neutropenia, trauma or burn injury) and antibiotic exposure within the preceding year.

MRSA isolates were designated as HA-MRSA if the source patient had any of the following risk factors: a history of hospitalization, residence in a

long-term care facility (e.g. nursing home), dialysis, or surgery within one year to the date of specimen collection; growth of MRSA within 48 h or more after admission to a hospital, presence of permanent indwelling catheter or percutaneous device at the time of culture; or prior positive MRSA culture report. If none of the above risk factors were present, the isolates were considered CA-MRSA [12-13].

Dual MRSA Detection EZY MIC Strip EM063 [Hi - media] for Detection of Oxacillin and Vancomycin by E-test:

It is a unique MIC determination paper strip which is coated with two different antibiotics on a single strip in a concentration gradient manner [14]. The upper half has oxacillin with a highest concentration tapering downwards and capable of showing MIC in the range of 0.064 – 8.0 mcg/ml, whereas lower half is similarly coated with vancomycin concentration gradient in reverse direction to give MIC in the range of 0.19 – 16.0 mcg/ml.

CLSI Recommendation for Vancomycin Sensitivity Test:

High molecular weight antibiotics such as vancomycin do not diffuse in concentration gradient manner while diffusing through the agar medium when the disc susceptibility test is employed. The antimicrobial susceptibility testing using disc diffusion test does not differentiate vancomycin-susceptible isolates of *S. aureus* from vancomycin intermediate isolates, nor does the test differentiate among vancomycin–susceptible, intermediate, and resistant isolates of coagulase-negative staphylococci, all of which may give similar size zones of inhibition. CLSI therefore recommends that MIC test should be performed to determine the susceptibility of all isolates of staphylococci to vancomycin.

MIC Reading:

Reading the plates only when sufficient growth was seen. Reading the MIC where the ellipse intersects the MIC scale on the strip. For bactericidal drugs such oxacillin, vancomycin, Gentamicin, and other members of β-lactams class of drugs, always read the MIC at the point of completion inhibition of all growth, including hazes, microcolonies and isolated colonies. If necessary, use magnifying glass. Isolated colonies, microcolonies and hazes appearing in the zone of inhibition are indicative of hetero nature of the culture having resistant subpopulation in it. In such cases, consider reading for MIC determination at a point on the scale above which no resistant colonies are observed close to MIC strip (within 1-3 mm distance from the strip). Since Ezy MIC™ strip has continuous gradient, MIC values "in-between" two-fold dilutions can be obtained. If the ellipse intersects the strip in between 2 dilutions, read the MIC as the value which is nearest to the intersection. When growth occurs along the entire strip, report the MIC as > the highest values on the MIC strip. When the inhibition ellipse is below the strip (does not intersect the strip),report the MIC < the lowest value on the MIC scale.

Interpretation:

Use following interpretive criteria for susceptibility categorization of oxacillin and vancomycin.

Test organism	Incubation	Interpretative criteria		
***Staphylococcus spp.* for Oxacillin**	35-37^{0}C for 18-24 hrs	**< S**	**I**	**>R**
		2	-	4
***Staphylococcus spp.* for Vancomycin**	35-37^{0}C for 18-24 hrs	2	4-8	16

Quality Control: Quality control of Ezy MIC™ Strips was carried out by testing the strips with standard ATCC cultures recommended by CLSI on suitable medium incubated appropriately.

Daptomycin E test; Daptomycin Ezy MIC™ Strip (DAP) (0.016-256 mcg/ml) EM088 [Hi-Media]:

Antimicrobial Susceptibility Testing by E-test: It is a unique MIC determination paper strip which is coated with Daptomycin in a concentration gradient manner, capable of showing MICs in the range of 0.016 mcg/ml to 256 mcg/ml, on testing against the test organism [15-16]. Ezy MIC™ strip is useful for quantitative determination of susceptibility of bacteria to antibacterial agents. The system comprises of a predefined quantitative gradient which is used to determine the MIC in mcg/ml of different antimicrobial agents against microorganisms as tested on appropriate agar media, following overnight incubation.

Preparation of inoculum-From the pure colony of MRSA,2-3 pure isolated colonies were inoculated in the 5 ml tryptone soya broth and were incubated at 35-37^{0}C for 2-4 hrs until moderate turbidity developed. The inoculum turbidity was matched with the turbidity of 0.5 McFarland. Daptomycin MIC strips are supplemented with calcium ions therefore it can be tested on regular Muller Hinton Agar (MHA). Dip a sterile nontoxic cotton swab into the standardized inoculum and rotate the soaked swab firmly against the upper inside wall of the tube to express the excess fluid. Streak the entire agar surface of the entire plate with the swab by rotating the plate. Place the strip at the desired position on the plate pre-lawned with test organism aseptically. Incubate the plates in the incubator at 35-37^{0}C for 18-24 hrs.

MIC Reading:

Reading the MIC where the ellipse intersects the MIC scale on the strip. Quality control- Quality control of Ezy MIC strips were carried out by testing the strips with standard *S. aureus* ATCC 29213 as per CLSI guidelines.

Interpretation:

Test organism	Incubation	Interpretative criteria		
Staphylococcus spp.	35-37^{0}C for 18-24 hrs	**< S**	**I**	**>R**
		1	-	-
Enterococcus spp	35-37^{0}C for 18-24 hrs	4	-	-

Fig. 1: Zone of Inhibition of Oxacillin-Vancomycin Ezy MIC Strip EM063: showing MIC of 2 mcg/ml for Vancomycin and MIC of >8 mcg/ml for Oxacillin

According to the CLSI, daptomycin MICs of 1 mcg/ml or lower in *S. aureus* isolates were considered to be susceptible.

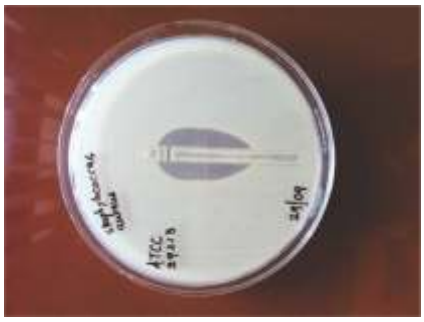

Fig. 2: Zone of Inhibition of Daptomycin Ezy MIC Strip (EM088) for Standard *S. aureus* ATCC29213 Culture MIC: 10 mcg/ml

Fig. 3: Zone of Inhibition of Daptomycin Ezy MIC Strip (EM088) for *S. aureus* Culture MIC: 10 mcg/ml

Detection of *mecA/PVL* gene in the selected MRSA isolates

Fifty-four representative isolates with special reference to isolates from SSTI infections were selected for detection of *mecA/PVL gene*. For molecular detection, MRSA and MSSA reference cultures were also processed in the same manner as the isolates obtained from the patient samples

Genomic DNA Extraction:

Genomic DNA of reference MRSA, MSSA and isolates obtained from various samples was extracted following standard extraction procedures with some modifications. Cells from overnight grown pure culture were harvested by centrifugation and were resuspended in TE buffer pH-8. Cells were treated by lysozyme and were mechanically disrupted with glass bead lysis (8 cycles of vortexing). Cell lysate was centrifuged at 10000 rpm for 10min and the supernatant was separated and further treated with 1% SDS and 100 µg Proteinase K (incubation at 50^0C for 30 minutes). Post incubation, equal volume Phenol: chloroform: Isoamyl alcohol (25: 24:1) was added to remove the denatured protein. After centrifugation, the aqueous layer was collected and 50 µg of *RNAse* A was added and the sample was incubated at 37^0C for 30min. Additional extraction step of Chloroform: Isoamyl alcohol (24:1) was given and the aqueous layer was separated post centrifugation. Genomic DNA in aqueous layer was precipitated with 0.15M NaCl and 2 volume chilled absolute ethanol. Pellet obtained after centrifugation was subjected to 70% ethanol wash. Final pellet of genomic DNA post centrifugation was dissolved in sterile distilled water and stored at 4^0C till further use. The quality of the DNA was checked on 0.8% Agarose gel [16].

PCR Amplification:

Primers for both the genes (*mecA and PVL*) were synthesized from Sigma. Primer sequences were adapted from Zhang *et al.*, 2005 for *mecA* and Lina *et al.*, 1999 for *PVL gene* [17-18].

***mecA*:**

FP 5′ GTG AAG ATA TAC CAA GTG ATT 3′and
RP 5′ ATG CGC TAT AGA TTG AAA GGA T 3′where as

***PVL*:**

FP 5′ ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A 3′and
RP 5′ GCA TCA AST GTA TTG GAT AGC AAA AGC 3′

Each 25ul reaction for either gene, had 1x PCR Buffer with $MgCl_2$, 0.25mM each dNTP, 15nM primers of respective gene, 1U *Taq* polymerase and1ng of genomic DNA. Negative control was set up with same components except the Template DNA was not added. Genomic DNA of the reference MRSA/MSSA served as positive control for each PCR reaction with patient isolates. Amplification conditions (Agilent Tech, Sure Cycler 8800) were, initial denaturation for both genes at 94^{0}C for 5 min, followed by 35 cycles for *mecA* as 94^{0}C for 30 sec, 43^{0}C for 30 sec, 72^{0}C for 30 sec; For PVL as 94^{0}C for 30 sec, 55^{0}C for 30 sec, 72^{0}C for 30 sec. Final extension for both genes was at 72^{0}C for 7 min. The PCR amplicons were visualized on 1.8% agarose gel. Expected amplicon size of *mecA* and *PVL* was 147bp and 433bp, respectively.

Results:

Total of 400 MRSA were collected from various clinical samples received from various wards and ICUs. 107 (26.75%) MRSA were from blood sample of BSI and endocarditis 81(20.25%) were from osteomyelitis and septic arthritis, 97(24.25%) were from skin and soft tissue infections, 62(15.5%) were from pneumonia and 45(11.25%) were from UTI. Of the total 400 MRSA strains; 183(45.75%) strains were isolated from paediatric and neonatal age group

52.72% MRSA infections were detected in female and predominance were also detected in clinical syndrome like SSTI and osteomyelitis and UTI whereas 47.25% MRSA infections were from male predominantly detected in bacteraemia, endocarditis, and pneumonia. 75.25% and 42.75% MRSA strains were defined as CA-MRSA and HA-MRSA respectively by clinical criteria: CDC epidemiologic definitions (Table 1). All MRSA strians were susceptible to tigecycline and vancomycin. 95.5% strains were susceptible to linezolid. Of the total MRSA; 40.5% strains were susceptible for clindamycin and all strains isolated from SSTIs were susceptible clindamycin. 248(62%) MRSA strains were resistant to tetracycline. Of the total 81 MRSA strains isolated from osteomyelitis ; 44 strains were resistant to tetracyline. 69.5% MRSA strains showed susceptibility for gentamycine, 305(76.25 %) strains were susceptible for TMP-SMX (Table 2).

Of the total 400 MRSA strains; 183(45.75%) strains were isolated from paediatric and neonatal age group. About 41% MRSA strains were isolated from various intensive care units. Of the total MRSA isolated from various ICUs; maximum strains were from MICU (35.35%) and NICU 28.04%) (Table 3).

Table 1: Demographic and Clinical Characteristics of Patients with MRSA Infections (N=400)

Clinical syndromes	
Bacteremia, endocarditis or sepsis	107 (26.75%)
Osteomyelitis or septic arthritis	81 (20.25%)
Pneumonia	62 (15.5%)
Skin and soft tissue infection [SSTI]	97 (24.25%)
Urinary tract infection	45 (11.25%)
Other	08 (2%)
Age group	
Paediatric	183 (45.75%)
Adult	217 (54.25%)
Gender	
Male	189 (47.25%)
Female	211 (52.75%)
Presence of risk factors for HA MRSA(n=229)	50.5%
Inpatient culture obtained >48 hr after admission	84 (21%)
Hospital stay, past year	27 (6.75%)
Surgery, Past 6 months	21 (5.25%)
Haemodialysis, past year	19 (4.75%)
Indwelling catheter	63 (15.75%)
Stay in long care facility, past year	15 (3.75%)
Location of care	
Intensive care units	164 (41%)
Various wards	231 (57.75%)
Emergency Department	4 (1%)
Outpatient Department	23 (5.75%)
CDC criteria for infection type	
CA-MRSA	229 (57.25%)
HA-MRSA	171 (42.75%)

Table 2: Distribution of the Antibiotic Resistance Pattern among Isolates

Antibiotics	n=400	
	Sensitive	Resistant
Oxacillin	00(0%)	400(100%)
Gentamycin	278(69.5%)	122(30.5%)
Tetracycline	152(31.25%)	248(62%)
TMP-SMX	305(76.25%)	97(24.25%)
Linezolid	398(99.5%)	2 (0.5%)
Erythromycin	139(34.75%)	261(65.25%)
Clindamycin	162(40.5%)	238(59.5%)
Vancomycin	400 (100%)	00(0%)
Cefotaxime	189(47.25%)	211(52.75%)
Tigecycline	400(100%)	00(0%)
Ciprofloxacin	232(58%)	168(42%)

Table 3: Distribution of MRSA among Different Wards and ICUs

Ward	No. of MRSA isolation (n=400)
NICU [Neonatal ICU]	46 (11.5%)
PICU [Paediatric ICU]	26 (6.5%)
SICU [Surgical ICU]	34 (8.5%)
MICU [Medicine ICU]	58 (14.5%)
OBGY [Obstetrics and Gynaecology]	26 (6.5%)
Surgery	62 (15.5%)
Medicine	44 (11.5%)
Orthopaedic	35 (8.75%)
Paediatrics	26 (6.55)
Skin and VD	12 (3%)
Ophthalmology	04 (1%)
Emergency department	04 (1%)
OPD	23 (5.75%)
Total	400

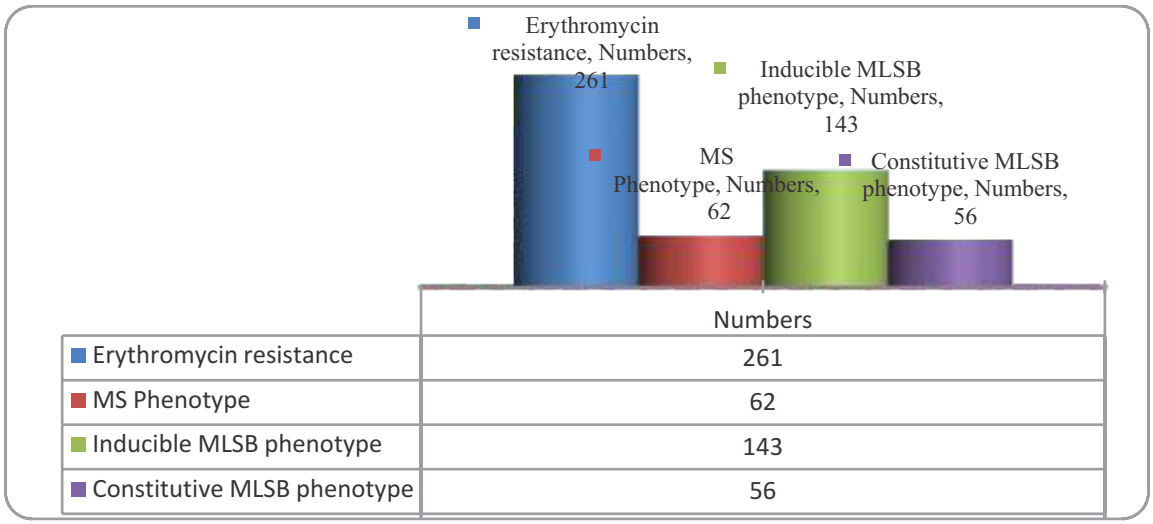

Fig. 4: Detection of Inducible MLSB Phenotype

Of the total 400 MRSA strains 261(65.25%) strains were erythromycin resistance and further detected for production of inducible clindamycin. About 143(35.75%) strains were positive for D-zone test i.e. Inducible MLSB phenotypes of which 44(11%) strains were isolated from various ICUs. About 56 (14%) strains were constitutive MLSB phenotype (Fig. 4) Two linezolid resistant strains were positive for Inducible clindamycin resistant and were isolated from MICU and SICU (Fig. 4).

Distribution of *mecA/PVL* gene in MRSA isolates from patient samples

Based on the microbiological analysis of 400 patient isolates, 54 MRSA positive isolates were further selected for the molecular characterization to evaluate the presence of *mecA* and *PVL gene* in their genome. All the isolates including reference MRSA and MSSA were subjected to genomic DNA extraction and the quality of the genomic DNA was checked on 0.8% agarose gel (Fig. 5). This DNA was used as a template for amplification of *mecA* and *PVL genes*.

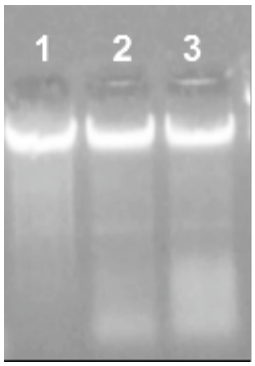

Fig. 5: Extraction of Genomic DNA from Reference Culture and Pure Culture Isolated from Patient Sample. Lane 1: Methicillin sensitive *S. aureus* (MSSA) genomic DNA, lane 2: Methicillin resistant *S. aureus* (MRSA) genomic DNA, lane 3: genomic DNA of *S. aureus* isolated from clinical sample.

Amplification of *mecA/PVL* genes by PCR:

PCR parameter optimization for *mecA/PVL* amplification was carried out using genomic DNA of reference strains (MSSA and MRSA. As seen in Fig. 6, the amplicons were detectable on 1.8% agarose gel. *mecA* gene could be amplified only from genomic DNA of reference MRSA whereas

reference MSSA could not give *mecA* amplicon. *PVL* gene could be amplified from both reference cultures. The amplicon size for both the genes was as expected. Since the MRSA reference culture could detect both the genes, it was used as a positive control in subsequent PCR reactions with patient isolates.

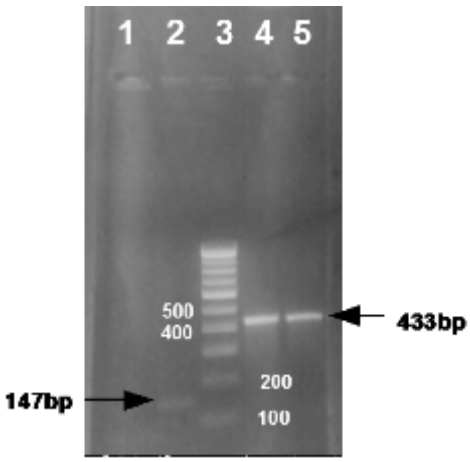

Fig. 6: PCR for the Amplification of *mecA* and *PVL* Gene from the Genomic DNA of Reference Strains Loaded on 1.8% Agarose Gel.

Lanes 1 & 2: Amplicon of *mecA gene* (147bp) from genomic DNA of reference cultures of MSSA and MRSA, lane 3: 100bp ladder, lane 4 & 5: Amplicon of *PVL* gene (433bp) from genomic DNA of reference cultures of MSSA and MRSA

The optimized PCR parameters were further used for the amplification of *mecA/PVL* genes from genomic DNA of MRSA isolates from 54 patients. Fig. 6 is a representation of amplicons of both the genes from patient isolates.

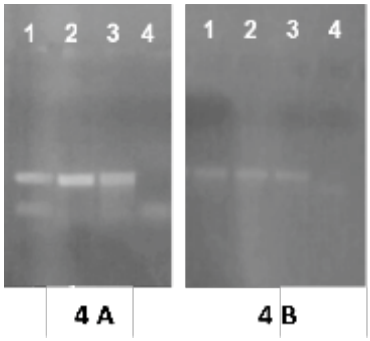

Fig. 7: Amplification of *PVL* and *mecA* gene from genomic DNA of MRSA isolated from patient samples:4A: Amplicon of *PVL* Gene Loaded on 1% Agarose Gel.

Lane 1: amplicon from reference MRSA, lanes 2 & 3: clinical samples (genomic DNA as template), lane 3: non-template (negative) control. 4B: Amplicon of *mecA* gene loaded on 1.8% agarose gel. Lane 1: amplicon from reference MRSA, lanes 2 & 3: clinical samples (genomic DNA as template), lane 3: non-template (negative) control. As is depicted in the Table 4, 76% (41/54) of patient isolates had presence of both the genes in the genome whereas 16% had only *mecA* gene. Interestingly, a small percent of isolates (5.5%) did not have both the genes on the genome.

Table 4: Presence of *mecA* and *PVL* Gene in Genomic DNA of MRSA Isolated from Clinical Samples of 54 Patients

***mecA/PVL* presence**	**Total number of isolates**
***mecA* (+ve), *PVL* (-ve)**	9
***mecA* (-ve), *PVL* (+ve)**	1
***mecA* (+ve), *PVL* (+ve)**	41
***mecA* (-ve), *PVL* (-ve)**	3
Total	54

Discussion:

MRSA carrying the important virulence determinant PVL, is an emerging infectious pathogen associated with SSTI as well as life threatening invasive diseases. In the present study of the total 400 MRSA; 107(26.75%) MRSA were from blood sample of BSI and endocarditis, 81(20.25%) were from osteomyelitis and septic arthritis, 97(24.25%) were from SSTI, 62(15.5%) were from pneumonia, 45(11.25%) were from UTI. Of the total 400 MRSA strains; 183(45.75%) strains were isolated from paediatric and neonatal age group. Significant morbidity and mortality in neonatal age group were associated with sepsis and SSTI. In a recent Europe-wide survey, the most common organism in SSTIs was *S. aureus* (71% cases) with 22.5% being MRSA [4]. The prevalence of MRSA varies between regions and between hospitals in the same region as reported by Wattal *et al*. (2010) in a study from Delhi [5]. 52.72% MRSA infections were detected in female predominantly in clinical syndrome like SSTI and osteomyelitis and UTI whereas 47.25% MRSA infections were detected in male predominantly in bacteraemia, endocarditis, and pneumonia. In the present study, 75.25% CA-MRSA and 42.75% HA-MRSA strains were defined by clinical criteria: CDC epidemiologic definitions (Table1). CA-MRSA were mainly isolated from SSTI and osteomyelitis. CA-MRSA isolates are now being increasingly reported from India. D'souza *et al.* [6] studied 412 confirmed cases of MRSA and found that 54% were true CA-MRSA possessing the SCC *mecIV* and SCC *mecV* genes.

In the present study, all MRSA strians were susceptible to tigecycline and vancomycin while 95.5% strains were susceptible to linezolid. Of the total MRSA, 40.5% strains were susceptible to clindamycin and all strains isolated from SSTIs were susceptible to clindamycin. 248(62%) MRSA strains were resistant to tetracycline. Of the total 81 MRSA strains isolated from osteomyelitis; 44 strains were resistant to tetracyline. About 69.5% MRSA strains showed susceptibility to gentamycine, 305(76.25 %) strains were susceptible to TMP-SMX (Table 2). In a study reported by Arora *et al*. (2010) from North India [8], the prevalence of MRSA was 46% were found to be more resistant to other antibiotics such as erythromycin, ciprofloxacin, gentamicin and amikacin.

Therapy for staphylococcal infections may be complicated by the possibility of inducible Macrolide-Lincosamide-Streptogramin B resistance (MLSBi). Of the total 400 MRSA strains 261(65.25%) strains were erythromycin resistance and further detected for production of inducible clindamycin *i.e.* MLSBi. Of which 143(35.75%) strains were positive for D-zone test *i.e.* Inducible MLSBi phenotypes. A total of 143 MLSBi phenotype; 44(11%) strains were isolated from various ICUs and NICU. About 56 (14%) strains were constitutive MLSB phenotype (Fig. 4).We have detected more prevalence of MLSBi in CA-MRSA as compared to HA-MRSA. Two linezolid resistant strains showed inducible clindamycin resistant and were isolated from MICU and SICU (Fig. 4). Lina *et al*. (2014) [18] Pune India reported higher prevalence of MLSBi in health care-associated than community associated *S. aureus* (86.5% versus 13.4% respectively).

Patel *et al*.(2006) [19] from Division of Infectious Diseases, Department of Medicine, University of Alabama at Birmingham, reported overall

prevalence of MLSBi was 52%, with 50% of MRSA and 60% of methicillin-susceptible *S. aureus* isolates exhibiting MLSBi. CA-MRSA represented 14% of all isolates and had a lower prevalence of MLSBi than hospital-associated MRSA (33% versus 55%).

The presence of skin or soft-tissue infection was predictive for CA-MRSA, and the presence of a comorbidity was predictive for MLSBi. Preservation of glycopeptides and linezolid use for treatment of MRSA cases should be encouraged. In our previous finding (2011) reported MLSBi phenotype 25% in CA-MRSA while 75% in HA-MRSA [11]. Study by Bouchiat *et al*. (2015) Bangalore India reported 52.2% were MRSA, isolated from CA infections in 60.4% and HA infections in 39.6%. MRSA were found to be significantly more resistant to gentamicin, cotrimoxazole and ciprofloxacin than MSSA, but no significant difference was observed between CA- and HA-MRSA [19-23].

In the present study molecular characterization has been done to evaluate the presence of *mecA* and *PVL* gene in their genome. Present study showed; 76% (41/54) of patient isolates had presence of both the genes in the genome whereas 16% had only *mecA* gene. Expression of *PVL* gene is enhanced by sub-MIC values of *β*-lactam antibiotics.

Interestingly, a small percent of isolates (5.5%) did not have both the genes on the genome (Table 4). It is possibly for the reason that resistance determinants be carried on other mobile elements, such as plasmids, transposons, and phages; so, their elimination from bacterial cell would result in the absence of *mecA* gene and consequently no association with *PVL* gene [20-24].

Okon *et al.* [23] (2009) Nigeria reported only 30% of isolates identified as MRSA were positive for *mecA* gene. This explains that it maybe *mecA* positive. MRSA isolates have different mechanism for methicillin resistance than *mecA*. These results are in agreement with other reports such as that by Bagdonas *et al.* in Lithuania (23.4%) [21].

In the present study only one isolate out of 54 expressed *PVL* gene but is not expressed *mecA* gene. Moreover Xiadiang *et al.* [24] (2014) Cambridge, UK mentioned specific alterations in different amino acids present in protein binding proteins cascade (PBPs 1, 2, and 3) which may be the basis of resistance. These alterations were found to include three amino acid substitutes which were identical and were present in PBPs 1, 2, and 3. Besides, the same amino acid was found to have two other different substitutes in PBP1. Both the identical and different amino acid substitutes were observed in isolates from different multilocus types. These findings provided clear evidence that there are mechanisms other than the presence of *mecA* gene responsible for beta-lactam resistance of MRSA and that molecular methods alone are not enough for confirmed characterization of MRSA isolates, a point that should be under consideration by regional and reference laboratories. The majority of researches in this field suggested that *mecA* gene that is present in all MRSA strains and is known to encode penicillin binding protein 2a (PBP2a), which has a low tropism to all *β*-lactam antibiotics, is the corner stone responsible for producing MRSA phenomenon [2-3, 25-26]. Beta-lactam resistance is attributed mostly to mutations in the *mecA* gene, but other genetic elements may also be considered for the explanation of the mechanism of resistance [4, 23-26]. However, the

absence of *mecA* gene in a considerable percentage of MRSA isolates requires investigating the alternative genetic possibilities related to the resistance phenomena.

Conclusion:

This study demonstrates that treatment outcome for infections caused by CA-MRSA is a challenge due to changing epidemiology in India. More number of MRSA isolates were multidrug resistant. Our laboratory analysis of MRSA isolates indicated a high number of PVL-positive CA-MRSA isolates, carrying a novel *mecA* gene. The co-occurrence of multidrug-resistant MRSA and PVL-positive CA-MRSA highlights the risk for the emergence of a multidrug-resistant PVL-positive MRSA clone. This point further underlines the need for surveillance studies in India and the implementation of antibiotic stewardship and infection control to prevent further dissemination of epidemic clones.

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