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Original Article

Microbiology Section

Molecular Typing of Methicillin Resistant *Staphylococcus aureus* using *coa* Gene Polymerase Chain Reaction-Restriction Fragment Length Polymorphism: A Cross-sectional Study

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ABSTRACT

Introduction: Methicillin Resistant *Staphylococcus aureus* (MRSA) are the most important multidrug resistant pathogen of humans causing a wide array of infections. Polymorphic Coagulase gene (*coa*) could be targeted for specific typing of *Staphylococcus aureus* isolates. 'Possible source' can be identified and discriminated rapidly for control and prevention of *Staphylococcus aureus* infections especially in case of suspected outbreaks. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) have high discriminatory power and enhanced reproducibility. Mutation and variation in number of short sequence repeats lead to distinct amplification of this region of *coa* gene by PCR. Deoxyribonucleic Acid (DNA) fragments of different sizes obtained by digestion with specific restriction enzymes like *Arthrobacter luteus I (AluI)* help to discriminate various types based on the patterns produced as a result of PCR-RFLP.

Aim: To determine molecular typing of MRSA by *coa* gene PCR-RFLP.

Materials and Methods: A cross-sectional study was done in the Department of Microbiology involving 150 clinical isolates of *Staphylococcus aureus* obtained from various clinical specimens. Genotypic identification of MRSA was done by detecting *coa* gene and *mecA* gene by PCR. Molecular typing of MRSA was done by *coa* gene PCR-RFLP.

Results: *Coa* gene PCR identified six Genotypic codes (Code I to Code VI) ranging from 300 bp-800 bp size. Restriction digestion of the amplicons of the *coa* gene by PCR-RFLP using the enzyme *AluI* provided 17 unique restriction patterns among MRSA isolates in toto. The predominant *coa* gene PCR Genotype code was "Type IV" which was 600 bp size and predominant RFLP fragment for Genotype code "Type IV" was RFLP Pattern 'b' (500 bp, 240 bp and 140 bp fragments) among the study population.

Conclusion: Genotype code 'IV' and RFLP pattern 'b' was found to be predominant *coa* gene type among MRSA in this study.

Keywords: *mecA*, Outbreak, Panton-valentine leukocidin, Typing

INTRODUCTION

Bacterial taxonomy plays a major role in epidemiology, prevention, and effective management of infectious diseases. Recently, both phenotypic and genotypic methods were employed using a polyphasic taxonomic approach to define and identify specific genera, species and subspecies of taxonomical relevance and importance, based on inherently polymorphic loci present in the genomes of all bacterial species by using data sets generated by such methods [1-3]. Primary structure and number of copies per chromosome of conserved and variable nucleotide sequence motifs could be established by direct or indirect DNA typing methods [2], culminating in subspecies level identification. Pulsed field Gel Electrophoresis (PFGE) is considered as the gold standard for molecular typing of MRSA detection and characterisation [4].

The MRSA is a widespread commensal and opportunistic pathogen causing infections both in the community and hospital set-up. Approximately 30% of human populations are nasal carriers of *Staphylococcus aureus* [5]. Increasing incidence of hospital acquired and community acquired MRSA and its carrier state among healthcare workers pose difficulty in treatment and result in poor clinical outcome. MRSA is detected by identification of *mecA* gene by PCR. This method is considered to be the gold standard when compared to other phenotypic methods (cefoxitin susceptibility test, oxacillin disc diffusion test, oxacillin screening agar, CHROMagar, and latex agglutination test) [3,5].

'Possible source' could be rapidly and rightly identified and discriminated for enhanced control and prevention of *Staphylococcus aureus* infections, especially in case of suspected outbreaks in hospital environment by various molecular typing methods like PCR based molecular techniques, and typing methods like PFGE and Multilocus Sequence Typing (MLST) [6,7]. These methods are not widely available, time consuming, costly and not feasible despite its discriminatory power to detect the potential pathogens like *Staphylococcus aureus* [8].

The PCR-RFLP and Random Amplified Polymorphic DNA (RAPD)-PCR have high discriminatory power and enhanced reproducibility compared to the above-mentioned methods and is suitable for all molecular biology laboratories worldwide because of its ease and the reduced cost [9,10]. Plasma coagulation is the result of coagulase, an extracellular virulence factor, that is produced by pathogenic staphylococci. Coagulase not only acts as a cofactor but also induces a conformational change in prothrombin, by converting serum fibrinogen to fibrin. Polymorphic Coagulase gene (*coa*) could be targeted for specific typing of *Staphylococcus aureus* isolates [11]. Coagulase enzyme consists of repeat regions in the 3′ end sequence and RFLP analysis of *coa* gene encoding this enzyme could be very useful for *Staphylococcus aureus* strain typing and would serve as an excellent epidemiological marker [12]. About 81 bp tandem Short Sequence Repeats (SSRs) encoding repeats of 27 amino acids in the C-terminal region together comprise the

variable region of *coa* gene in *Staphylococcus aureus*. Mutation and variation in number of SSRs lead to distinct amplification of this region of *coa* gene by PCR. DNA fragments of different sizes obtained by digestion with specific restriction enzymes like *AluI, Hae* III etc. helps to discriminate various types based on the patterns produced as a result of PCR-RFLP [1]. Amplification of 3' region of *coa* gene produces five classes of bands expressing heterogeneity ranging from 300 bp to 800 bp. Digestion of PCR amplicons by *AluI* results in eight different RFLP patterns designated as *coa* 1-8. Most commonly encountered RFLP pattern in clinical specimens and carriers is Class 3 and *coa* 3 [13,14].

Early detection and characterisation of MRSA is crucial to prevent disease transmission, appropriate management and its containment, outbreak investigation. As an epidemiological tool to understand geographic distribution, clonal relationship, disease transmission, and also its recurrence.

Only a very few studies have been done to evaluate specific genotypes by molecular characterisation of MRSA using PCR-RFLP in view of Indian context. Brakstad OG et al., [15] reported that PCR-RFLP typing methodology could trace the source of infection and routes of transmission and help to control infections and outbreaks. This technique cannot be used to distinguish MRSA and MSSA. The discriminatory index of *AluI* is much greater than *HaeIII* and it was able to discriminate unrelated strains well. PCR-RFLP was found useful, technically simple, reproducible, rapid and efficient for typing *Staphylococcus aureus* strains isolated from clinical specimens. Hence, the present study was undertaken to understand the utility of PCR-RFLP in molecular typing of MRSA and also to evaluate the genotypic variations among *S. aureus* strains.

MATERIALS AND METHODS

A cross-sectional study was conducted in the Department of Microbiology, from December 2018 to June 2020. Institutional Human Ethics committee clearance (Reference Number: PSG/IHEC/2019/APPR/fb/003) was obtained for this project (No. 18/338).

Inclusion criteria: A total of 150 clinical non duplicate *Staphylococcus aureus* isolates obtained from various clinical samples comprised the study population, which was conveniently selected from 1209 culture-positive *Staphylococcus aureus* isolates isolated in the Microbiology laboratory during the study period.

Exclusion criteria: *Staphylococcus aureus* isolates isolated from healthcare providers, environmental surveillance, patients below 18 years and those with Pulmonary tuberculosis, were excluded from the study.

Sample size calculation: Proportion, $N=4PQ/d^2$. Where 'P' is the Expected prevalence; Q=100-P; d=Precision of the estimate. P=20%; Q=100-20=80%; d=5%.

Proportion, $N=4\times20\times80/25=256$.

The MRSA was identified using the routine conventional phenotypic methods namely positive catalase, tube coagulase tests and resistance to cefoxitin (30 µg). *Staphylococcus aureus* was detected and genotyped by *coa* gene PCR and *coa* gene PCR-RFLP, respectively. Identification of MRSA was confirmed by *mecA* gene PCR. *PVL* gene PCR was performed to detect CA-MRSA from HA-MRSA.

Pure DNA was extracted [15,16] from 10 µL loopful of 16-24 hours young bacterial culture and was inoculated into 100 µL of Tris 10X lysis buffer which contains 2 µL of lysostaphin. The suspension was incubated at 37°C for 10 minutes and then boiled for 10 minutes and was then cooled to room temperature for 5 minutes. Finally centrifuged at 16000 X g for one minute. The aliquot of supernatant containing extracted DNA were used for amplification. Amplification of genes [16-19] done by Veriti 96 well Conventional Thermal cycler (Applied biosystem- Thermo Fisher Scientific) PCR was employed under appropriate thermal conditions for each gene of interest (*coa* gene, mecA gene and *PVL* gene) as per standard reference methods discussed below. Primers were procured from Yaazh genomics, India. Amplification of *coa* gene [14] was done by adding 1 µL of Forward primer 5'-ATAGAGATGCTGGTACAGG-3', 1 µL of Reverse primer 5'-GCTTCCGATTGTTCGATGC-3', 5 µL of extracted DNA template 12.5 µL of Master mix (dNTPs, PCR buffer 10X, MgCl2, Taq DNA polymerase) and 5.5 µL of distilled water making a final reaction volume of 25 µL was used for the reaction. Specific thermal conditions for *coa* gene amplification was performed with the following thermocyclic conditions: a. denaturation at 94°C for 45 seconds, b. followed by 30 cycles at 94°C for 1 minute, c. annealing at 57°C for 15 seconds, d. elongation at 72°C for 15 seconds and e. final elongation at 72°C for 2 minutes. Amplification of mecA gene [15-17] was done with 1 µL of forward primer 5'-GTAGAAATGACTGAACGTCCGATAA-3', 1 µL of reverse primer 5'-CCAATTCCACATTGTTTCGGTCTAA-3', 12.5 µL of Master mix, 5 µL of extracted DNA template and 5.5 µL of distilled water making a final reaction volume of 25 µL was used for the reaction. Specific thermal conditions for *mecA* gene PCR consists of 40 cycles of a. denaturation step at 94°C for 30 seconds, b. annealing step at 55°C for 30 seconds, c. elongation step at 72°C for 1 minute and d. final elongation step at 72°C for 5 minutes. Amplification of PVL [15-17] was done by adding 1 µL of forward primer 5'-ATCATTAGGTAAAATGTCTGCACATGATCCA -3', 1 µL of reverse primer 5'- GCATCAASTGTATTGGATAGCCAAAAGC -3', 5 µL of extracted DNA template, 12.5µl of master mix and 5.5 µL of distilled water making a final reaction volume of 25 µL was used for the reaction. Specific thermal conditions for PVL gene detection are: a. denaturation step at 97°C for 6 minutes b. followed by 35 cycles at 92°C for 30 seconds; c. annealing step at 55°C for 30 seconds, d. elongation step at 72°C for 45 seconds and e. final elongation step carried out at 72°C for 10 minutes.

Gel electrophoresis of amplified PCR [13,19] products of *coa* gene, *mecA* gene and *PVL* gene were analysed individually in 1.5% agarose gel prepared with 1X TBE (Tris Borate EDTA) buffer and 0.5 µg/mL ethidium bromide. The melted agarose gel was allowed to cool and solidify in the cast with comb for to create the samples well and disperse them. 10 µL of PCR product along with loading dye 2 µL of bromophenol blue were dispersed into the well create. A DNA ladder of 100 base pairs was used to measure the amplicons size and amplified product of ATCC 25923 *Staphylococcus aureus* control was used as positive control for each run. Electrophoresis of PCR amplicons was done for 1 hour at 100 volts and were examined under Gel doc EZ imager documentation system, Biorad. PCR-RFLP of *coa* gene [13] was done for *coa* gene in MRSA isolates. RFLP of *coa* gene was carried out using the *AluI* restriction enzyme (Sisco Research Laboratories Pvt. Ltd., India) according to the manufacturer's recommendations. 10 µL of *coa* amplicons was digested with 10U of *AluI* enzyme along with 7.5 µL deionised water and 2 µL 10X buffer incubated at 37°C for 1 hour in a water bath. The restricted fragments were electrophoresed in 1.5% agarose gel and were analysed under automated gel documentation system.

STATISTICAL ANALYSIS

Data entry was made in Microsoft excel and Statistical analysis was done using International Business Machines (IBM) Statistical Package for the Social Sciences (SPSS) statistical software version 17.0.

RESULTS

A total of 77,768 specimens were received in the Microbiology laboratory for aerobic bacterial culture and sensitivity during the study period. Culture positivity was found to be 15,129 (19.5%). Among them *Staphylococcus aureus* accounted for 1207 (8%) of culture positive isolates isolated from various clinical specimens received during the study period. Incidence of MRSA was found to be 388 (32.15%) among the *Staphylococcus aureus* isolates. Out of these, 150 *Staphylococcus aureus* clinical isolates isolated were

conveniently selected for further study. Common sources of isolation of *Staphylococcus aureus* were found to be blood 50 (33.33%), wound swab 46 (30.67%), pus 34 (22.67%), tissue, tracheal aspirate, cerebrospinal fluid and others being 20 (13.3%). Total 91 (60.7%) were MSSA and 59 (39.3%) were MRSA. Total of 50 (84.7%) were from inpatients and 9 (15.3%) were outpatients out of 59 MRSA isolates. Male:Female ratio was 1.36. Age-wise distribution showed a higher incidence in 19-65 years age group as depicted [Table/Fig-1]. Among the inpatients, 32 (54.2%) were admitted in the ward and remaining 18 (31%) were admitted in ICU. High incidence of MRSA isolation from clinical specimens represented Surgery department 20 (33.89%) followed by Medicine 10 (16.94%). Co-morbid conditions commonly associated with MRSA infections were diabetes mellitus (28,47.5%), systemic hypertension (20,33.9%), abscess (8,13.56%), as depicted in [Table/Fig-1].

Results of *coa* gene PCR for coagulase enzyme was positive for all 150 (100%) *Staphylococcus aureus* isolates. *MecA* gene (310 bp) was detected in all 59 (100%) isolates of MRSA. *PVL* gene (433 bp) was identified among 9 (15.25%) MRSA isolates and 31 (34.06%) MSSA isolates. In this study, the typing method namely *coa* gene

A: Age and sex-wise distribution of study population (N=150)

Sex	Age (years)			
	<18	19-65	>65	Total
Male (N-87)	10 (6.6%)	58 (38.7%)	19 (12.7%)	87 (58%)
Female (63)	6 (4%)	48 (32%)	9 (6%)	63 (42%)
Total (N-150)	16 (10.6%)	106 (70.7%)	28 (18.7%)	150 (100%)

B: Age and sex-wise distribution of MRSA clinical isolates (N=59)

Sex	Age (years)			
	<18	19-65	>65	Total
Male (N-34)	3 (8.8%)	28 (82.35%)	3 (8.8%)	34 (57.6%)
Female (N-25)	3 (12%)	19 (76%)	3 (12%)	25 (42.4%)
Total (N-59)	6 (10.16%)	47 (79.66%)	6 (10.16%)	59 (100%)

C: Age and sex-wise distribution of MSSA clinical isolates (N=91)

Sex	Age (years)			
	<18	19-65	>65	Total
Male (N-53)	4 (7.5%)	39 (73.58%)	10 (18.87%)	53 (58.2%)
Female (N-38)	6 (15.79%)	19 (50%)	13 (34.21%)	38 (41.8%)
Total (N-91)	10 (10.99%)	58 (63.74%)	23 (25.27%)	91 (100%)

D: Distribution of patients with *Staphylococcus aureus* infection (N=150)

Isolates	Outpatients	In-patients		Total
		ICU	Ward	
MRSA (N-59)	9 (15.36%)	18 (31%)	32 (54.2%)	59 (39.33%)
MSSA (N-91)	13 (14.2%)	32 (35.2%)	46 (50.6%)	91 (60.67%)
Total (N-150)	22	50	78	150 (100%)

E: Age-wise mortality among study population (N=150)

Isolates	Age (years)			
	<18	19-65	>65	Total
MRSA (N-59)	0	2 (3.38%)	2 (3.38%)	4 (6.78%)
MSSA (N-91)	0	0	1 (1.09%)	1 (1.09%)
Total (N-150)	0	2 (1.33%)	3 (2%)	5 (3.33%)

F. Source of isolation of *Staphylococcus aureus*

Isolates	Blood	Wound swab	Pus	Tissue	Tracheal aspirate	CSF	Others	Total
MRSA (N-59)	8 (13.56%)	21 (35.59%)	21 (35.59%)	3 (5.08%)	2 (3.39%)	1 (1.69%)	3 (5.08%)	59 (39.33%)
MSSA (N-91)	42 (46.15%)	25 (27.47%)	13 (14.29%)	3 (3.3%)	3 (3.3%)	2 (2.2%)	3 (3.3%)	91 (60.67%)
Total (N-150)	50 (33.33%)	46 (30.6%)	34 (22.67%)	6 (4%)	5 (3.33%)	3 (2%)	6 (4%)	150 (100%)

G. *Co-morbid conditions among study population

Isolates	DM	SHT	Abscess	PSI	Sepsis	Cellulitis	CKD	CHD	Others
MRSA (N-59)	28 (47.4%)	20 (33.%)	8 (13.5%)	7 (11.8%)	6 (10.1%)	6 (10.1%)	4 (6.7%)	3 (5%)	5 (8.47%)
MSSA (N-91)	39 (42.8%)	46 (50.5%)	16 (17.6%)	11 (12%)	13 (14.2)	9 (9.9%)	9 (9.9%)	8 (8.8%)	8 (8.8%)
Total (N-150)	67 (44.67%)	66 (44%)	24 (16%)	18 (12%)	19 (12.67%)	15 (10%)	13 (8.67%)	11 (7.33%)	13 (8.67%)

H. Clinical outcome (N=150)

Isolates	Cured	Improved	Not improved	Died	Discharged		Total
					At request	Against medical advice	
MRSA (N-59)	4 (6.78%)	40 (67.79%)	6 (10.17%)	4 (6.67%)	1 (1.14%)	4 (6.67%)	59 (39.33%)
MSSA (N-91)	12 (13.19%)	54 (59.34%)	16 (17.58%)	1 (1.1%)	2 (2.2%)	6 (6.59%)	91 (60.67%)
Total (N-150)	16 (10.67%)	94 (62.67%)	22 (14.67%)	5 (3.33%)	3 (2%)	10 (6.67%)	150 (100%)

[Table/Fig-1]: Demography and epidemiology of the study population (N-150).
*DM: Diabetes mellitus; SHT: Systemic hypertension; PSI: Post-surgical infections; CKD: Chronic kidney disease; CHD: Coronary heart disease

PCR identified six Genotypic codes (Code I to Code VI) ranging from 300 bp-800 bp size. Restriction digestion of the amplicons of the *coa* gene by PCR-RFLP using the enzyme *AluI* provided 17 unique restriction patterns among MRSA isolates in total as depicted in [Table/Fig-2]. Moreover 300 bp (Genotype code VIa), 600 bp (Genotype code IVa), 700 bp (Genotype code IIa) and 800 bp (Genotype code Ia) resulted in undigested single fragments appearing as band patterns. Double fragment patterns were observed in 800 bp (Genotype code Ib and Ic), 700 bp (Genotype code IIc and IId), 600 bp (Genotype code IVc, IVd and IVe) and 400 bp (Genotype code Va) and 300 bp (Genotype code VIb). Triple fragment patterns were seen in 700 bp (Genotype code IIb), 650 bp (Genotype code IIIa and IIIb) and 600 bp (Genotype code IVb), respectively showing enormous heterogeneity in the *coa* gene of *S. aureus*. The predominant *coa* gene PCR Genotype code was "Type IV" which was 600 bp size and predominant RFLP fragment for Genotype code "Type IV" was RFLP Pattern 'b' (500 bp, 240 bp and 140 bp fragments) [Table/Fig-3-6].

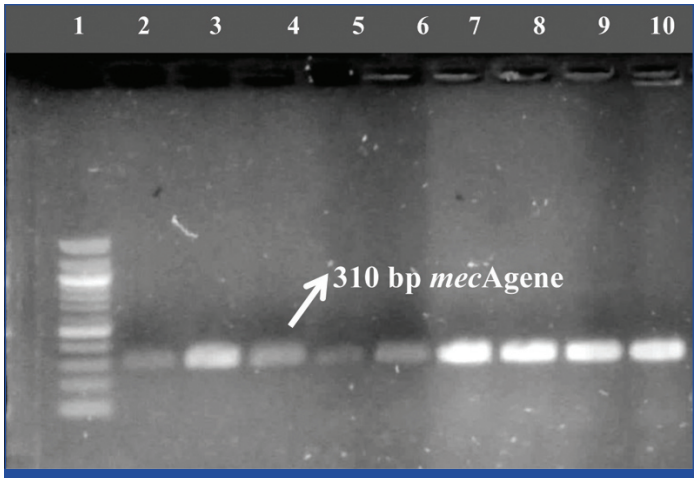
S. No.	*coa* gene PCR products (bp)	Genotype code	No. of isolates	*coa* gene PCR-RFLP pattern/s	MRSA isolates N (%)
1	800	I	06	a. Non digested	03 (50%)
				b. 400 bp and 250 bp fragments	02 (33.33%)
				c. 400 bp, 200 bp fragments	01 (16.66%)
2	700	II	11	a. Non digested	03 (27.27%)
				b. 400 bp, 210 bp and 100 bp fragments	03 (27.27%)
				c. 400 bp and 250 bp fragments	02 (18.18%)
				d. 400 bp and 200 bp fragments	03 (27.27%)
3	650	III	06	a. 540 bp, 240 bp and 100 bp fragments	05 (83.33%)
				b. 400 bp, 210 bp and 100 bp fragments	01 (16.67%)
4	600	IV	29	a. Non digested	04 (13.79%)
				b. 500 bp, 240 bp and 140 bp fragments	17 (58.62%)
				c. 500 bp and 280 bp fragments	04 (13.79%)
				d. 500 bp and 180 bp fragments	02 (6.89%)
				e. 300 bp and 180 bp fragments	02 (6.89%)
5	400	V	02	a. 240 bp and 100 bp fragments	02 (100%)
6	300	VI	05	a. Non digested	02 (40%)
				b. 200 bp and 100 bp fragments	03 (60%)
Total isolates			59	Total isolates	59

[Table/Fig-2]: *coa* gene PCR and PCR-RFLP patterns of MRSA.

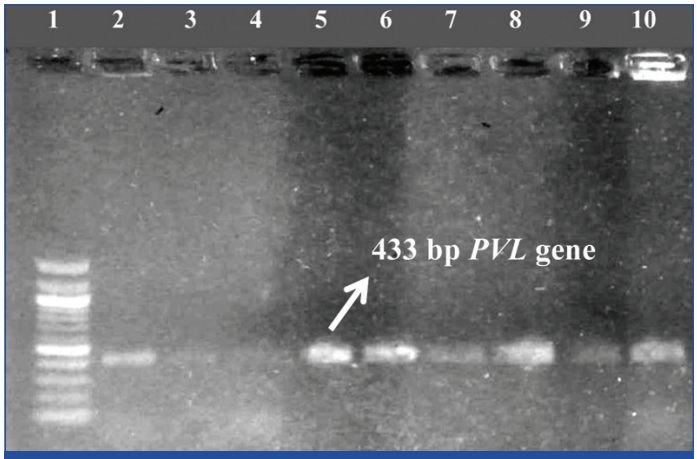
All the 59 isolates (100%) of MRSA and 91 isolates (100%) of MSSA were sensitive to vancomycin and linezolid. Quality control for susceptibility to antibiotics, performed using ATCC *Staphylococcus aureus* 25923 strain and was found to be satisfactory. Inducible clindamycin resistance (D test positive) was observed in 14% of MRSA and 6.7% MSSA isolates. Co-resistance among MRSA and MSSA isolates of different drugs is depicted in [Table/Fig-7].

DISCUSSION

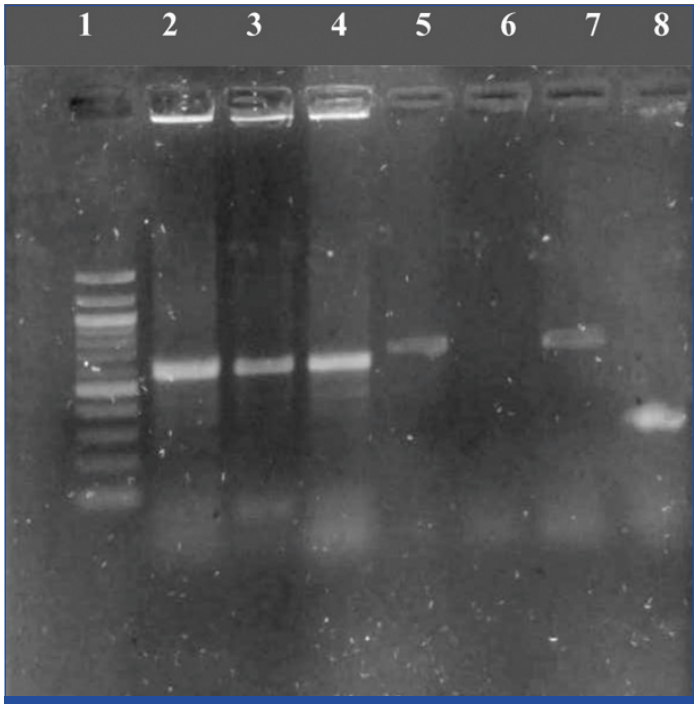
Hospital-Acquired MRSA (HA-MRSA) and Community-Acquired MRSA (CA-MRSA) are important public health concern causing localised skin infections to life threatening fulminant infections. Establishing the source and detection of HA-MRSA infections



[Table/Fig-3]: Molecular detection of *mecA* gene in MRSA.
Lane 1-100 bp ladder; Lane 2 to 9-*mecA* gene positive; Lane 10- In-house MRSA strain- Positive for *mecA* gene

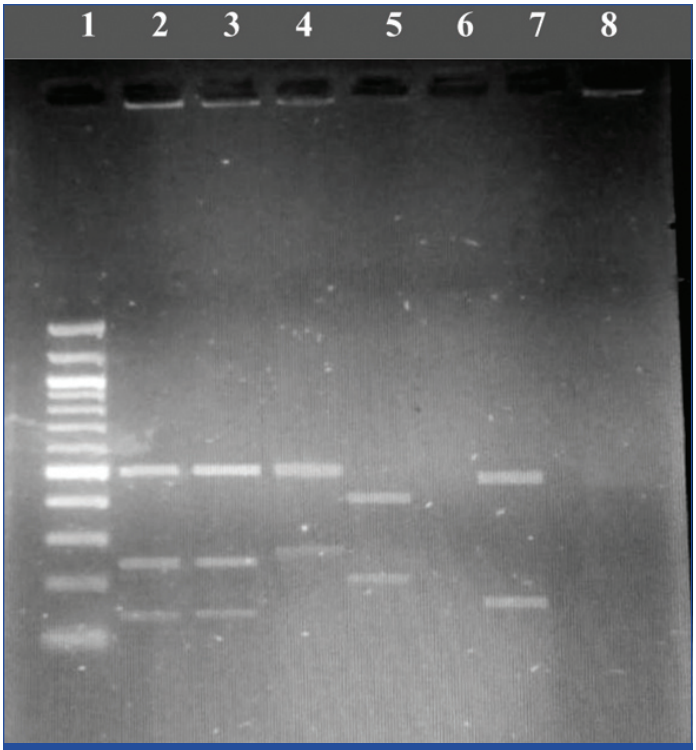


[Table/Fig-4]: Molecular detection of *PVL* gene in *MRSA*.
Lane 1-100 bp ladder; Lane 2, 5, 6, 7, 8, 9 and 10-PVL gene positive; Lane 3 and 4-PVL gene negative



[Table/Fig-5]: Molecular detection of *coa* gene in MRSA.
Lane 1-100 bp ladder; Lane 2 to 4-600 bp; Lane 5 and 7-700 bp; Lane 6-no band and Lane 8-300 bp

in healthcare facility is a challenge for both physicians and microbiologists. Methicillin resistance in MRSA is due to the presence of mutated *mecA* gene which encodes for penicillin binding protein 2a (PBP2a). PBP2a blocks the binding site of β-lactam antibiotics resulting in multidrug resistance. The incidence of MRSA in this study was 32.15%. In India, prevalence of MRSA varied between



[Table/Fig-6]: Molecular detection of *coa* gene in MRSA by PCR-RFLP.
Lane 1-100 bp ladder; Lane 2 and 3-600 bp-500, 240, 140; Lane 4-600 bp-500, 280; Lane 5-700 bp-400, 200; Lane 6-no band-no band; Lane 7-700 bp-500, 180; Lane 8 -300 bp-undigested

Drugs	MRSA (N-59)	MSSA (N-91)	p-value
Fluoroquinolones (Ciprofloxacin)	48 (81.35%)	68 (74.72%)	0.176
Macrolides (Erythromycin)	38 (64.40%)	24 (26.37%)	<0.05
Lincosamide (Clindamycin)	36 (61.01%)	16 (17.58%)	<0.05
Cotrimoxazole	16 (27.11%)	24 (26.37%)	0.458
Aminoglycosides (Gentamicin)	17 (28.81%)	7 (7.69%)	<0.05

[Table/Fig-7]: Co-resistance among MRSA and MSSA isolates.

23.3-59.3% [20-24]. MRSA was more commonly isolated in 19-65 years age group, predominantly in males. The mortality due to MRSA among study population was 6.67% which was higher than MSSA (1.09%). It was similar to those studies done by Godwin PG et al., and Goh SH et al., [25,26]. The mortality rate of MRSA ranges from 5-60% [26-28] and it depends on patient population and site of infection namely infective endocarditis, skin and subcutaneous tissues, followed by invasive infections like osteomyelitis, meningitis, pneumonia, lung abscess, and empyema.

Conventional methods to identify *Staphylococcus aureus*, like, tube coagulase test has some pitfalls though it is routinely practiced in most of the microbiology laboratories. Phenotypic assays like Phage typing, biotyping, SDS-PAGE are available but laborious, time consuming and is available only in few reference laboratories. Whereas, molecular-based typing is much rapid, easier, specific, descriptive with high discriminative power, reproducibility and stability to establish timely diagnosis, and to initiate appropriate therapy so as to contain the epidemic. Molecular detection of *coa* gene confirms the diagnosis of *Staphylococcus aureus* with high specificity, reproducibility and rapid detection of pathogen. Typing of *Staphylococcus aureus* helps in characterisation and discrimination of different strains from different sources especially those from hospitals and community.

Genotypic methods like Monoplex, duplex and Multiplex PCR, Molecular beacon, DNA probe, DNA hybridisation, Amplified Restriction Fragment Polymorphism (AFLP), PCR based DNA fingerprinting technique, Plasmid profile analysis, Ribotyping, Restriction Fragment Length Polymorphism (RFLP), Real time PCR, RAPD, PFGE, SCCmec typing, MLST, Hypervariable region typing and spa typing are available for molecular detection and characterisation of MRSA. Methods like PCR-RFLP typing seems to be an alternative technique to high expensive, laborious and time consuming PFGE [2,27]. *coa* gene encoding coagulase enzyme is highly polymorphic as this shows difference in the amino acid sequence and number of 81 bp tandem repeats of 3' variable end. This heterogeneity in *coa* gene among MRSA and MSSA isolates were analysed by PCR-RFLP. Novel *coa* gene PCR-RFLP for *Staphylococcus aureus* first introduced by Goh SH et al., in 1992 [26]. In India, Tiwari HK et al., [27] were the first to attempt *coa* gene PCR-RFLP in the year 2008. *coa* gene PCR-RFLP would serve as an excellent molecular epidemiological tool as it is cheaper, simple, inexpensive and reproducible and also is helpful in analysing large number of MRSA isolates especially during outbreak investigation.

Panton-Valentine leukocidin (PVL) toxin gene (433 bp) was identified among 9 (15.25%) MRSA isolates and 31 (34.06%) MSSA in present study which was low compared on a global scale. Global prevalence of PVL toxin producing CA-MRSA strains range from 77-100% [29-31]. Reports from many countries show increasing prevalence of PVL positive MRSA isolates [32,33]. Studies from India, have reported 62.85% of PVL positivity among MRSA. Another study from Mumbai by Souza ND et al., reported a prevalence of 64 % PVL positive MRSA isolates. These studies reported a higher prevalence compared to this study [34,35]. Studies done elsewhere have reported a lower prevalence [32] of PVL positive MRSA strains viz. 14.3 % in Bangladesh, 8.1 % in Saudi Arabia, 5% in France, and 4.9 % in UK [36-39]. PVL toxin production in CA-MRSA strains could be confirmed by lukS/F-PV genes co-amplification method [33]. Adults or children infected with strains of harnessing PVL genes as a constant and stable genetic markers is primarily known to cause skin and soft tissue infections and rarely nectorising pneumonia with poor prognosis [40-42]. PVL toxin serves as a genetic marker for CA-MRSA [43] strains and few studies reports that PVL is a major virulent determinant in pathogenesis of Staphylococcal infections [34,35]. PVL gene positivity in MRSA is one of the criteria to differentiate HA-MRSA from CA-MRSA where it is highly prevalent in CA-MRSA [44,45]. By identifying Sequence Type (ST), agr type, SCCmec type, and toxin gene profile these PVL positive MRSA strains could be further be typed into various clones. Most isolates harboured SCCmec type IV, a supposed marker for CA-MRSA and some HA-MRSA which is easily transmissible and commonly found [46].

In this study, six Genotype codes of *coa* gene were detected by PCR and 17 *coa* PCR-RFLP patterns using *AluI* enzyme among MRSA clinical isolates. Six genotypes were designated as Genotype code I to VI with *coa* gene bands 800 bp, 700 bp, 650 bp, 600 bp, 400 bp and 300 bp, respectively by gel electrophoresis. *AluI* restriction enzyme digested *coa* PCR products demonstrated about 17 unique restriction fragment patterns among the MRSA isolates. The predominant Coa gene PCR Genotype code was "Type IV" (600 bp) and predominant RFLP fragment for Genotype code IV is RFLP Pattern 'b' (500 bp, 240 bp and 140 bp fragments) was observed in the present study population. Studies done by Mahumoudi H et al., observed five different bands (300 bp, 500 bp, 600 bp, 700 bp and 800 bp). The 600-bp amplicon was observed in 150 out of 200 (75%) which was identified as *coa* 3 genotype predominated in *S. aureus* isolated from clinical and carrier specimens in by PCR-RFLP of MRSA [13]. Mohajeri P et al., [46] observed five types of unique bands (810 bp, 770 bp, 740 bp, 650 bp and 490 bp) in their study. Babu NR et al., [17] studied 12 isolates of *Staphylococcus aureus* and reported only two types of bands (600 bp and 700 bp) after coa gene PCR-RFLP. By using *coa* gene PCR-RFLP, authors could very well understand the heterogeneity, pathogenicity and clinical spectrum of various CA-MRSA, HA-MRSA and MSSA infections [47]. The present study also identified which particular genotype and RFLP pattern is common in specific locations especially in cases of suspected outbreaks. PCR-RFLP

was performed using restriction endonuclease *AluI* (endonuclease enzyme obtained from strain of *Arthrobacter luteus*). Himabindu M et al., from southern India observed that *AluI* enzyme is better for genotyping of *Staphylococcus aureus* compared to *HaeIII* enzyme obtained from *Haemophilus aegypticus* [48]. In developing countries like India with resource poor setting, we require a highly economical but more powerful and precise molecular assay like *coa* gene PCR-RFLP which is a simple, feasible, and precise *coa* typing method that would be very useful for understanding the heterogeneity and epidemiology of MRSA obtained from relevant clinical specimens.

The MRSA strains were 100% resistant to beta lactam antibiotics like penicillins and cephems. Co-resistance in MRSA was observed in ciprofloxacin 48 (81.3%), erythromycin 38 (64.40%), clindamycin 36 (61.01%), gentamicin 17 (28.81%), and cotrimoxazole 16 (27.11%) apart from resistance to penicillins, I, II and III generation cephems. Also, co-resistance with other groups of antibiotics were commonly observed in MRSA and was found to be high when compared to MSSA which was similar to studies done in other countries [49,50]. Clindamycin is considered as a useful alternative to vancomycin and other costlier antibiotics to treat both MSSA and MRSA infections. However, inducible clindamycin resistance (iMLSB phenotype) is very common and can be detected in the laboratory by 'D test'. Inducible clindamycin resistance in MRSA was 14% and in MSSA was 6.7% in this study. Ciraj AM et al., from Manipal reported very high inducible clindamycin resistance in MRSA and MSSA (38.4%, 12.9% respectively) [50]. Similarly, Vidhya R et al., have reported the incidence of inducible clindamycin resistance as 23.07% for MRSA and 3.52% for MSSA isolates [51]. Current therapeutic options for MRSA are vancomycin, linezolid, teicoplanin. Alternative options are daptomycin, quinupristine-dalfopristine, cefiderocol, ceftabiprole, ceftaroline, levonadiflxacin etc.

Limitation(s)

The MRSA carriers among front line workers (staff nurses, doctors and other paramedics) were not included in the study. Evaluation of a larger sample size would help us understand the exact epidemiology, genotypic variations, drug resistance, pathogenesis and molecular characteristics of CA-MRSA and HA-MRSA. More specific and gold standard typing method like MLST was not done for *coa* typing due to unavailability and high cost.

CONCLUSION(S)

Early and accurate identification and characterisation of MRSA both by phenotypic and genotypic typing assays are of paramount importance to identify the source and to prevent further spread. The predominantly observed MRSA coa genotype was "Genotype code IV" and RFLP pattern 'b' in present study.

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