Original Article

Clonal Clusters and Virulence Factors of Methicillin-Resistant *Staphylococcus Aureus*: Evidence for Community-Acquired Methicillin-Resistant *Staphylococcus Aureus* Infiltration into Hospital Settings in Chennai, South India

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Abstract

Background and Objective: *Staphylococcus aureus* is one of the major pathogens of nosocomial infections as wells as community-acquired (CA) infections worldwide. So far, large-scale comprehensive molecular and epidemiological characterisation of *S. aureus* from very diverse settings has not been carried out in India. The objective of this study is to evaluate the molecular, epidemiological and virulence characteristics of *S. aureus* in both community and hospital settings in Chennai, southern India. **Methods:** *S. aureus* isolates were obtained from four different groups (a) healthy individuals from closed community settings, (b) inpatients from hospitals, (c) outpatients from hospitals, representing isolates of hospital–community interface and (d) HIV-infected patients to define isolates associated with the immunocompromised. Antibiotic susceptibility testing, multiplex polymerase chain reactions for detection of virulence and resistance determinants, molecular typing including Staphylococcal cassette chromosome *mec* (SCC*mec*) and *agr* typing, were carried out. Sequencing-based typing was done using *spa* and multilocus sequence typing (MLST) methods. Clonal complexes (CC) of hospital and CA methicillin-resistant *S. aureus* (MRSA) were identified and compared for virulence and resistance. **Results and Conclusion:** A total of 769 isolates of *S. aureus* isolates were studied. The prevalence of MRSA was found to be 7.17%, 81.67%, 58.33% and 22.85% for groups a, b, c and d, respectively. Of the four SCC*mec* types (I, III, IV and V) detected, SCC*mec* V was found to be predominant. Panton-Valentine leucocidin toxin genes were detected among MRSA isolates harbouring SCC*mec* IV and V. A total of 78 *spa* types were detected, t657 being the most prevalent. 13 MLST types belonging to 9 CC were detected. CC1 (ST-772, ST-1) and CC8 (ST238, ST368 and ST1208) were found to be predominant among MRSA. CA-MRSA isolates with SCC*mec* IV and V were isolated from all study groups including hospitalised patients and were found to be similar by molecular tools. This shows that CA MRSA has probably infiltrated into the hospital settings.

Keywords: Community-acquired methicillin-resistant *Staphylococcus aureus*, HIV, hospital-acquired methicillin-resistant *Staphylococcus aureus*, innate immune evasions, MLST, microbial surface component recognising adhesive matrix molecules, spa typing, ST 772

Introduction

The number of serious infections with *Staphylococcus aureus* is increasing worldwide; this is true for both community-acquired (CA) and hospital-acquired (HA) methicillin-resistant *S. aureus* (MRSA) infections. The first case of nosocomial MRSA infection in India was reported in 1988.[1] Subsequently, the Indian hospitals were identified to be endemic for MRSA

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with the attributed nosocomial infections ranging from 45% to 70%. Only 15 years later, in 2003, CA-MRSA was first reported from India.[2] Thereafter, only a few case reports and hospital-based studies have documented CA-MRSA in India with recent reports demonstrating CA-MRSA infections from hospitalised patients with limited diversity in India.[3,4]

Molecular characterisation and epidemiological typing of MRSA involving a large and diverse population has not been done in India. Such studies are urgently required to not only understand the current molecular epidemiology of the *S. aureus* in the country, but also to understand the impact of virulence and resistance determinants in hospital and community settings and in treatment outcomes. Hence, this study was aimed at performing a comprehensive phenotypic and molecular characterization of *S. aureus* obtained from various settings including community and hospital settings, their interface and from immunocompromised hosts in Chennai, South India. The results of this study showed unexpectedly high levels of CA-MRSA infiltration onto the hospitals, throwing light on the changing demographics of the *S. aureus* in India and the need for revision of screening and treatment strategies.

Methods

Study population

This is a cross-sectional study conducted between January 2010 and January 2014. *S. aureus* isolates from four different groups were included in this study: (a) healthy individuals from closed community settings to represent CA-MRSA (CA), (b) inpatients from hospitals that constitute HA-MRSA (HA), (c) outpatients from hospitals that represent CA–HA interface (CHI) and (d) HIV infected patients to represent isolates from immunocompromised individuals (HIV). Inpatient isolates were obtained from patients admitted in post-operative wards of hospitals for wound infection. Outpatient group represents patients visiting these tertiary hospitals for skin and soft tissue infections. *S. aureus* isolates were obtained from HIV patients visiting Government hospital for Thoracic Medicine for treatment of HIV and secondary infections. *S. aureus* isolates from individuals with prior treatment history for MRSA infections or history of long stay in hospital or association with hospital by any means (employed, patient attendee) were excluded from the study Groups a and c. The study was approved by Institutional ETHICAL committees of Dr ALM PG Institute of Basic Medical Sciences, Madras Medical College and Government Hospital for Thoracic Medicine, Tambaram. Informed consent was obtained from all the study participants.

Sample collection

Nasal swabs were obtained from individuals of various community settings including old age homes, orphanages and sports teams and were included in this group. Pus and pus swabs from various pyogenic infections were collected by standard procedures. The patient details including name, age, sex, date and hours of hospital admission, underlying clinical condition, previous medical history and antibiotic treatment if any were noted using a sample request form cum questionnaire.

Microbiological methods

All reference strains of *S. aureus* were obtained from Institute of Hygiene and Microbiology, University of Wuerzburg. The cultures were maintained as glycerol stocks at −80°C. Specimens were processed using standard microbiological methods.[5]

Culture and biochemical tests

The clinical specimens were processed for bacteria culturing in blood agar followed by Gram's staining, catalase, slide and tube coagulase test and growth on mannitol salt agar.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out by Kirby Bauer disc diffusion method and interpreted using CLSI guidelines. Inducible clindamycin resistance was detected by disc approximation test.[6] *S. aureus* ATCC 43300 (MRSA) and *S. aureus* ATCC 25923 methicillin sensitive *S. aureus* (MSSA) were employed as positive control and negative control, respectively.

Molecular methods

S. aureus cultures were grown overnight in 45 mL of LB broth at 37°C in shaker incubator. The cells were pelleted by centrifugation at 8,000 rpm for 15 min. The cells were lysed using lysostaphin (15 mg/mL) (Sigma) by incubation at 37°C for 15 min. DNA was extracted from lysed cells using the QiagenDNAeasy Extraction kit following manufacturer's protocol and was stored at 4°C until use.

Staphylococcal cassette chromosome *mec* typing and detection of virulence factors

Multiplex polymerase chain reactions (PCRs) were used for the specific detection of MRSA, panton valentine leucocidin (*pvl)* genes[7] and Staphylococcal cassette chromosome *mec* (SCC*mec*) typing[8,9] and virulence genes such as enterotoxins, exfoliative toxins, hemolysins and leucocidins.[10-13] *S. aureus* COL, *S. aureus* BK2464, *S. aureus* ANS 46, *S. aureus* MW2 and *S. aureus* WIS were used as reference strains for SCC*mec* typing, *ccr* typing and *mec* gene complex detection. *S. aureus* MW2, *S. aureus* N315, *S. aureus* COL, *S. aureus* RN 6607, *S. aureus* ATCC 27664 and *S. aureus* USA300 FPR3757 were used as reference strains for the detection of virulence factors. All the PCR methods were carried out using Eppendorf Mastercycler PCR and Taq DNA polymerase (NEB).

Genotyping

Genotyping of *S. aureus* was done by *agr* typing, *spa* typing and MLST using standard protocols.[14-16]

Statistical methods

The data were stored in MS excel and analysed using Minitab software v-15. ANOVA and Mann–Whitney tests were done for comparison of various groups included in the study.

Table 1: Demographic Characteristics of study groups

Demographic Characteristics	Group 1 Hospital Associated (*n*)	Group 2 Hospital Outpatients (*n*)	Group 3 HIV positive patients (*n*)	Group 4 Community associated (*n*)	
# of *S. aureus*	251	225	70	223	
				128 (old age)	95 (Young adults)
Males	148	128	48	63	68
Females	103	97	22	65	27
M: F Ratio	3:2	4:3	2:1	1:1	6:3
Median age (range) in years	48 (11-91)	25 (8-61)	38 (32-52)	71 (63-93)	18 (17-22)

Results

Staphylococcus aureus in study groups

A total of 769 non-duplicate isolates of *S. aureus* were collected from four different study populations: Hospital-associated *S. aureus* isolates (HA; *n* = 251) included isolates from inpatients of septic wards (*n* = 99), dermatology wards (*n* = 80), orthopaedic wards (*n* = 37) and ENT wards (*n* = 35). Community-associated clinical isolates (CA) of *S. aureus* were from outpatients attending the dermatology (*n* = 156), general surgery (*n* = 23) and ENT (*n* = 46) departments. *S. aureus* isolates (HIV) from HIV-infected patients were collected from the inpatient and outpatient settings. Carrier isolates of *S. aureus* included isolates from healthy individuals (CHI) and were obtained from three different closed communities at risk namely orphanages128/356 (35.95%), sportspersons 67/225 (29.77%) and old age homes 28/251 (11.15%). Of 852 healthy individuals from various communities, 223 (26.17%) were found to be carriers of *S. aureus*. The demographic characteristics of the groups are presented in Table. 1.

Prevalence of methicillin resistant *Staphylococcus aureus* and staphylococcal cassette chromosome *mec* types among various groups

The overall prevalence of MRSA as identified by PCR among the *S. aureus* isolates included in this study was found to be 48% (368/769). The prevalence of MRSA was the highest among HA infections (82%; 205/251) followed by CA infections (58%; 131/225). Among HIV patients, 23% (16/70) were MRSA, which was at least three times higher than what is observed in the community (CA-7%; 16/223).

Presence of SCC*mec* is the defining characteristic of MRSA, which encodes resistance for β-lactam antibiotics. Multiplex PCR of study MRSA isolates identified four types of SCC*mec* types: SCC*mec* type I, III, IV and V. Other SCC*mec* types such as SCC*mec*II, VI, VII–XIII were not detected in any of the MRSA isolates included in the study. SCC*mec* types V dominated in all the settings with 51%, 72%, 63% and 81% distribution among HA, CHI, HIV and CA-MRSA isolates. Next to SCC*mec* types V, SCC*mec* type IV was present in all study settings (41% – HA, 28% – CHI, 31% – HIV and 13% – CA). Four isolates from HA-MRSA and one isolate from CA-MRSA harboured SCC*mec* type I. Only one isolate among all the study isolates, present in HIV-MRSA group carried SCC*mec* type III, making it a rare type to be found among South Indian MRSA isolates. Comparison of study groups showed that there was a significant difference in the prevalence of SCC*mec* type V and SCC*mec* type IV between groups ($f = 5.55$, $P = 0.001$ and $f = 12.59$, $P = 0.000$).

Table 2: Antibiotic resistance among MRSA and MSSA

Antibiotics	MRSA *n*=368 (%)*	MSSA *n*=401 (%)
Amikacin	186 (50.5)	0 (0)
Ciprofloxacin	267 (72.55)	51 (12.71)
TMP-SMX	224 (60.86)	78 (19.45)
Clindamycin	10 (2.71)	0 (0)
Erythromycin	368 (100)	82 (20.44)
Fusidic acid#	0 (0)	4 (1)
Gentamicin	250 (67.93)	24 (5.98)
Netilmicin	144 (39.13)	0 (0)
Ofloxacin	239 (64.94)	23 (5.73)
Rifampicin	64 (17.39)	41 (10.22)
Tetracycline	176 (47.82)	26 (6.48)
Mupirocin	56 (15.21)	13 (3.24)

*Antibiotic resistance was significantly higher among MRSA than MSSA, #Significantly high among MSSA ($P<0.005$)

Antibiotic resistance among *Staphylococcus aureus* isolates from various groups

All the tested *S. aureus* isolates from the four different settings were found to be susceptible to linezolid and vancomycin. MRSA isolates showed higher resistance compared to MSSA irrespective of the settings [Table 2].

A total of 70 (19.23%) isolates including 51 from HA, 14 from CHI, 2 from group HIV and 3 from CA-MRSA showed inducible clindamycin resistance (iMLSB). Comparison of inducible clindamycin resistance among MRSA isolates from different group by ANOVA showed that there was a significant difference between inpatient and outpatient isolates ($f = 5.16$, $P = 0.002$). Both constitutive and inducible clindamycin-resistant isolates were found to harbour the *ermA* gene by PCR. All MRSA isolates included in this study were susceptible to fusidic acid. Four MSSA isolates showed resistance to fusidic acid and were found to be positive for *fusC* gene by PCR. Forty-three (23.71%) of 205 MRSA isolates from hospital setting showed high-level mupirocin resistance by disc diffusion method (mupirocin 200 µg). All the isolates which showed mupirocin resistance were found to be positive for the *mupA* gene by PCR.

Table 3: Virulence determinants among various groups

Virulence determinants	Group I *n*=251(%)	Group II *n*=225 (%)	Group III *n*=70 (%)	Group IV *n*=223 (%)
Enterotoxins	*n* (%)	*n* (%)	*n* (%)	*n* (%)
SEA[#]	160 (63.7)	88 (38.9)	28 (40)	19 (8.5)
SEB[@]	11 (4.0)	38 (16.8)	12 (17.1)	(5.3)
SEC[#]	104 (41.4)	38 (16.8)	13 (18.6)	14 (6.3)
*SED**	3 (1.2)	5 (2.2)	0	3 (1.3)
SEE	0	5 (2.2)	0	0
SEG[#]	141 (56.2)	134 (56.2)	41 (58.6)	88 (39.4)
SEH[@]	3 (1.2)	5 (2.1)	5 (7.1)	7 (3.2)
*SEI**	141 (56.2)	134 (56.2	41 (58.6)	145 (65.0)
SEJ	3 (1.2)	5 (2.2)	1 (1.4)	3 (1.3)
SEK[@]	15 (6.0)	45 (20.0)	12 (17.4)	12 (5.4)
SEL	109 (43.4)	45 (20.0)	15 (21.4)	52 (23.3)
SEM[#]	141 (56.1)	128 (56.1)	26 (37.1)	95 (42.6)
SEN	152 (60.6)	88 (39.1)	28 (40.0)	119 (53.4)
SEO	141 (56.2)	132 (58.7)	26 (31.7)	100 (44.8)
SEP[#]	15 (6.0)	9 (4.0)	2 (2.9)	3 (1.3)
Exfoliatin (et) a[@]	3 (1.2)	25 (11.1)	2 (2.9)	15 (6.7)
etb[@]	0	19 (8.4)	2 (2.9)	12 (5.4)
etd	0	0	0	0
*Toxic shock syndrome toxin (tsst-1)**	3 (1.2)	2 (0.9)	1 (1.4)	3 (1.3)
Panton - Valentine leucocidin (pvl)[#]	129 (52.5)	84 (37.3)	26 (37.1)	54 (24.5)
Leucocidin (Luk) D[#]	157 (62.5)	130 (57.6)	23 (32.9)	59 (26.4)
Luk E[#]	157 (62.5)	130 (57.6)	23 (32.9)	64 (28.7)
Luk M	0	0	0	0
*Hemolysin (hl) a**	246 (98.0)	225 (100)	70 (100)	223 (100)
hlb$	3 (1.2)	7 (3.1)	1 (1.4)	16 (7.2)
*hld**	251 (100)	225 (100)	70 (100)	223 (100)
Hlg[#]	233 (88.8)	171 (76)	31 (57.4)	67 (30.4)
hlg2[#]	219 (87.3)	178 (79.1)	22 (40.7)	103 (36.2)
*Staphylokinase (sak) **	248 (98.8)	219 (97.3)	69 (98.6)	207 (92.8)
*Staphylococcal complement inhibitor (scn)**	248 (98.8)	219 (97.3)	69 (98.6)	207 (92.8)
Chemotaxis inhibitory protein (chp)[#]	171 (68.1)	145 (64.4)	16 (22.9)	54 (24.2)
*Clumpiong factor (clf) A**	236 (94.0)	216 (96.0)	67 (95.7)	213 (95.5)
clfB[#]	104 (41.4)	94 (41.8)	31 (44.3)	52 (23.3)
Fibrinogen binding protein (fib)[$]	83 (33.1)	82 (36.4)	22 (31.4)	139 (62.3)
Fibronectin binding protein (fnb) A[#]	6 (2.4)	12 (5.3)	3 (4.3)	5 (2.2)
*fnbB**	12 (4.8)	8 (3.6)	2 (2.9)	12 (5.4)
*Enolase (eno)**	251 (100)	225 (100)	70 (100)	223 (100)
Elastin binding proteins (ebps)[#]	190 (75.7)	151 (67.1)	44 (62.9)	52 (23.3)
Collagen binding protein (cna)[$]	110 (43.8)	96 (42.6)	31 (44.3)	152 (68.2)
Bone sialoprotein binding protein (bbp)[#]	85 (33.9)	52 (23.1)	16 (22.9)	21 (9.4)

*No significant difference was obtained; [#]Significantly high among clinical isolates ($P<0.005$);[$]significantly high among carrier isolates ($P<0.005$); [@]Significantly high among community associated isolates ($P<0.005$)

Virulence determinant genes

A total of40 virulence determinant genes including 15 enterotoxins, 3 exfoliative toxins, 5 hemolysins, 3 leucocidins, *tsst-1*, *pvl*, 3innate immune evasions and 9 microbial surface component recognising adhesive matrix molecules (MSCRAMMs) were detected. All toxin genes except leukocidin M (*lukM*) and exfoliative toxinD (*etd)* were present [Table 3]. No single virulence factor was found to be exclusive for any group of isolates included in this study. However, varying prevalence was detected for some of the virulence genes tested (*pvl, sea, sec* and *sel*). Toxigenic virulence factors such as *pvl*, *sea* and *sec* were highest for HA infections and MSCRAMMS such as *clf, fib* and *fnbA* were slightly high for CA infections. *S. aureus* isolates from asymptomatic carriers of community settings and infected HIV patients showed lowest prevalence of toxigenic virulence factors.

Genotyping

All four types of accessory gene regulator subtypes were detected among the *S. aureus* isolates included in the study. The predominant *agr* type was *agr II* (n = 427), followed by *agr I* (n = 208), *agr III* (n = 105) and *ag rIV* (n = 16). 13

Table 4: Characteristics of the MLST - Clonal complexes (CC) obtained in this study

Clonal complex (CC)	Sequence type (ST)/Allelic profile	Spa type	MRSA-/ MSSA SCCmec (*n*)	pvl	Enterotoxin	Hemolysin	Leukocidins tsst-1/ exfoliatins	MSCRAMMs	Innate Immune evasions	Antibiotic resistance	Age type	Infections	Hospital/ Community
CC1	ST1 1-1-1-1-1-1-1	t1931	MSSA (10)	-	sea, sel, sem, sen & seo	α, δ & γv	-	Eno, clfA, ebpS & cn-a	sak, scn	Ery, Co	II	skin & soft tissue	CA-infection
	ST772 1-1-1-1-22-1-1	t657	MRSA-V (169)	+	sea, (sec), seg, sei, sel, sem, sen, seo	α, δ, γ , γv	-	Eno, clfA, ebpS, clfB & cn-a	sak, scn, chps	Ak, Cip, Co, Ery, G, Of, (Tet)	II	Pyogenic abscesses, cellulitis, postoperative wound	Carriage & CA/HA-infection
		t345	MSSA (15)	+	sec, sek, sel, sem, sen, seo	α, δ, γ , γv	lukD, lukE	Eno, clfA, ebpS, clfB & cn-a	sak, scn	-	II	-	Carriage
CC5	ST5 1-4-1-4-12-1-10	t1154	MRSA-V (16)	-	Seb, seg, sei, sek	α, δ & γv	lukD, lukE	Eno, clfA, ebpS, clfB & cn-a	sak, scn	Cip, Co, Ery, G & Of	I	cellulitis & wound infections	CA-infection
		t442	MSSA (10)	-	Sed, seg, sei, sej, sem, sen, seo	α, δ & γv	lukD, lukE	Eno, clfA, cn-a, fib	Sak, scn	Ery, Cip	III	Ear infections, Skin & soft tissue infections	
CC6	ST6 12-4-1-4-12-1-3	t4615	MSSA (19)	-	-	α, δ	lukD, lukE	Eno, clfA, cn-a, fib	sak, scn	-	III	-	Carriage
CC8	ST239 2-3-1-1-4-4-3	t037	MRSA-III (49)	-	sea, (sek)	α, δ, γ & γv	lukD, lukE	Eno, clfA, ebpS, clfB, fib	sak, scn, chps	Ak, Cip, Co, Ery, G, Mu, Nt, Of & Tet	I	Post-operative wound	HA -infection
	ST368 2-3-1-1-65-4-3	t425	MRSA-III (15)	-	Sea	α, δ, γ & γv	lukD, lukE	Eno, clfA, ebpS, clfB, fib	sak, scn, chps	Ak, Cip, Co, Ery, G, Nt, Of & Tet	I	diabetic foot ulcers	
	ST1208 3-3-1-1-142-4-3	t1223	MRSA-III (23)	-	sea, (sek)	α, δ	lukD, lukE	Eno, clfA, ebpS, clfB, fib	sak, scn	Ak, Cip, Co, Ery, G & Of	II	post operative wound	
		t064	MRSA-V (40)	-	seb, seg, sei, (sek), sem, sen, seo	α, δ, γ & γv	lukD, lukE	Eno, clfA, ebpS, clfB & cn-a	sak, scn	Cip, Co, Ery, G & Of	II	Middle ear, skin & soft tissue	CA-infection
Clonal complex (CC)	Sequence type (ST)/Allelic profile	Spa type	MRSA/ MSSA-SCCmec (n)	pvl	Enterotoxin	Hemolysin	Leukocidin tsst-1/ exfoliatin	MSCRAMMS	Innate Immune evasions	Antibiotic resistance	Age type	Infections	HA/CA -infections
CC9	ST109 3-27-1-1-1-1-10	t209	MSSA (13)	-	-	α, δ & γ	Etb	Eno, clfA, cn-a, fib	sak, scn	-	IV	-	Carriage
CC18	ST18 13-15-1-1-12-11-13	t9037	MSSA (7)	-	sem, sen	α, δ	-	Eno, clfA, cn-a, fib	sak, scn	-	III	-	Carriage
CC20	ST20 4-9-1-8-1-10-8	t164	MSSA (10)	-	Sel	α, δ	-	Eno, clfA	sak, scn	-	III	-	Carriage
CC22	ST22 7-6-1-5-8-8-6	t005	MSSA-I* (20)	+	Seg, sei, sem, sen,& seo	α, δ	lukD, lukE	Eno, clfA, cn-a, fib	sak, scn, chps	-	I	skin & soft tissue	CA/ HA-Infection
		t852	MRSA-IV (48)	+	sea, (seh), sel, sem, sen & seo	α, δ, γ & γv	luD, lukE	Eno, clfA	sak, scn	Cip, Co, Ery, G & Of	II	Cellulitis, wound infections	

Contd...

Table 4: Contd...

Clonal complex (CC)	Sequence type (ST)/Allelic profile	Spa type	MRSA-/ MSSA SCCmec (*n*)	pvl	Enterotoxin	Hemolysin	Leukocidins tsst-1/ exfoliatins	MSCRAMMs	Innate Immune evasions	Antibiotic resistance	Age type	Infections	Hospital/ Community
CC30	ST30 2-2-2-2-6-3-2	t021	MSSA (19)	+	seg, sei, sen, seo	α, δ & γv	lukD, lukE	Eno, clfA, fib	sak, scn	Cip, Ery	III	skin & soft tissue	CA-infection
		t021	MRSA-IV (1)	+	sec, seg, sei, sen, seo, sep	α, δ, γ & γv	lukD, lukE	Eno, clfA, fib	sak, scn	Cip, Co, Ery, G & Of	III	Pyogenic Abscesses	
CC45	ST45 10-14-8-6-10-3-2	t015	MSSA (17)	-	sec, seg, sei, sel, sem, sen, seo	α, δ, γ & γv	lukD, lukE	clfA, eno	sak, scn	-	I	wound infection	Carriage, CA-infection
CC121	ST120	t159	MSSA (12)	-	Sea	α, δ & γ	eta, etb	Eno, clfA, cn-a, fib	sak, scn	-	IV	-	Carriage
	6-5-6-2-7-14-2	t272	MSSA (15)	-	sea, sen, seo	α, δ & γ	-	Eno, clfA, cn-a, fib	sak, scn	-	III	-	
		t3204	MSSA (20)	-	sem, seo	α, δ & γ	-	Eno, clfA, cn-a, fib	sak, scn	-	III	-	
	ST672	t3841	MSSA (29)	-	(seb), seg, sei	α, δ & γ	lukD, lukE	Eno, clfA, ebpS, clfB, fib	sak, scn	-	I	-	Carriage
	4-3-1-1-11-72-11	t3841	MRSA-I (5)	-	(sea), seg, sei	α, δ, γ & γv	lukD, lukE	Eno, clfA, ebpS, clfB, fib	sak, scn	Ery, G, Tet	III	skin & soft tissue	HA-infection

ST- sequence type; CC-clonal complex; Allelic profile - arcc-aroe-glpf-gmk-pta-tpi-yqil; spa-staphylococcal protein A; pvl-Panton-Valentine leukocidin & SCCmec -Staphylococcal cassette chromosome mec; se-staphylococcal enterotoxins; eta&b-exfoliative toxins a & b; α, δ, γ & γv -alpha, delta, gamma and variant gamma-hemolysins; lukDE-leukocidin DE; MSCRAMMs-Microbial surface components recognizing adhesive macromolecules; ebpS -elastin binding protein of S. aureus; eno -enolase; clfA & B-clumping factors; fib- fibrinogen binding protein; cn-a- collagen binding protein; sak- staphylokinase; scn- staphylococcal complement inhibitor; chp- chemotaxis inhibitory protein; (x) - variable; agr-accessory gene regulator; Ak -amikacin, Cip -ciprofloxacin, tmp-smz-trimethoprim-sulfamethoxazole, Ery-erythromycin, G-Gentamicin, Mu-mupirocin, Nt-netilmicin, Of-ofloxacin, Tet - tetracycline; CA-Community Associated; HA-Hospital Associated; Carriage- nasal carriage among healthy individuals from communities at risk; + -positive; - -negative; *-MSSA with mecA negative SCC-MEC elements

isolates were found to be negative for the *agr*. *Agr* types I and III were predominant among MSSA isolates, while *agr* type II was predominant among MRSA isolates. The major *agr* subtype in this study was found to be *agr* II. Agr typing showed good discriminatory power in relation to the virulence determinants.

A total of 78 different *spa* types were obtained. The major *spa* types ($n \geq 10$) obtained in this study were typed for sequence type using MLST. The BURP analysis of obtained *spa* sequence types (ST) i shown in Figure 1.

The representative ST and their clonal complexes (CC) were ST1; 1-1-1-1-1-1-1 (CC1), ST772 (CC1), ST5 (CC5), ST6 (CC6), ST239 (CC8), ST 368 (CC8), ST1208 (CC8), ST20 (CC20), ST22 (CC22), ST30 (CC30), ST45 (CC45), ST109, ST672 and ST120 (CC121). The characteristics of major CC of *S. aureus* identified in this study were compared [Table 3].

Discussion

In recent years, the prevalence of MRSA in Indian hospitals is continuously increasing.[17] The current study observed that the prevalence of MRSA among hospitalised patients which hits a new high to 82% and is so far the highest compared to all reports of HA-MRSA in India and other parts of the world including developed countries.[17-19] One would expect a concomitant increase in CA-MRSA as, 1990 onwards, increasing CA-MRSA infections have been reported across the world. In this study, the prevalence of MRSA among community-associated skin and soft tissue infections was found to be 58%, which is lower compared to previous and recent reports from South India studying superficial and deep-seated infections[18,20] and is significantly lower compared to HA-MRSA. This observed prevalence of CA-MRSA is lower than the current prevalence in the USA and other developed countries.[21] Recently, it has been reported that HIV-infected patients have increased rates of *S. aureus* colonisation and skin and soft tissue infections.[22] Interestingly, the prevalence of MRSA infections among HIV-infected patients included in this study was detected as 22.85%, which is again, very low when compared to reports from developed countries.[23,24] The prevalence of MRSA among HIV-infected patients was found to be significantly lower compared to both hospital- and community associated infections ($P < 0.005$).

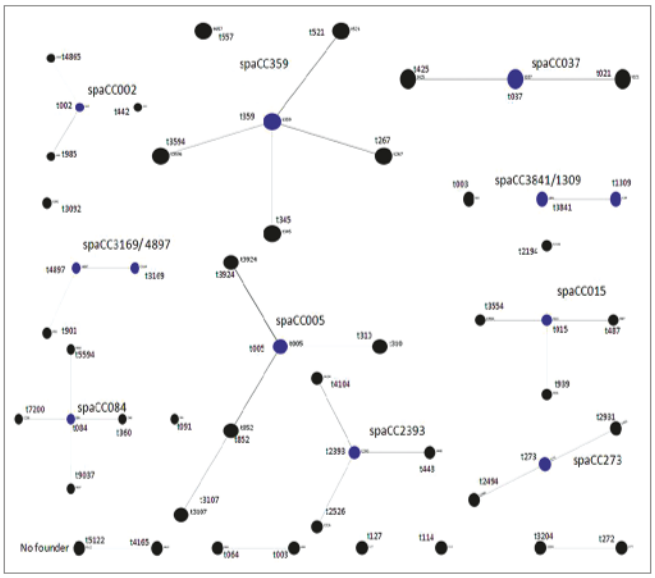

Figure 1: Snapshot of BURP showing the spa clusters obtained in this study; Blue dot – represents the founders of the clonal complex; black dot – members of the clonal complexes; Size of the cluster corresponds to the number of isolates in the cluster. The major clonal complexes in this study were spaCC359, spaCC005 and spaCC037

Endogenous infections are common in healthcare settings and similar circumstances may occur in communities like sports, orphanages, day care centres and prisoners, where there is crowding of people. Recent studies report nasal carriage of MRSA among healthy individuals from various communities at risk indicating it as a source of CA-MRSA infections. The overall carriage rate of *S. aureus* among healthy individuals included in this study was found to be 26.17%, which is in the reported range of 20%–30% by different studies.[25] The prevalence of MRSA among carrier isolates of *S. aureus* in the current study was found to be 7.17%, which is comparatively lower than the recent reports from India.[20] Overall, our study shows that while HA-MRSA is increasing at an alarming rate in Chennai, the CA-MRSA and MRSA infections observed in immunosuppressed individuals are much lower than expected/reported levels, indicating a need for public health efforts to focus on hygiene practices in hospitals.

The prevalence of SCC*mec* types among MRSA from both hospital and community settings were reported to vary with geographical location. In this study, 4 different SCC*mec* types, *namely* SCC*mec* type I, type III, type IV and type V were detected. SCC*mec* type V was found to be the predominant SCC*mec* type irrespective of the source of the MRSA. Among MRSA isolates from hospitalised patients, SCC*mec* type I and III were exclusively associated, whereas only SCC*mec*t ype IV and V were present in CA-MRSA.

The study found that only 41% of HA-MRSA isolates harboured SCC*mec* type III, the predominant SCC*mec* type among HA-MRSA isolates endemic in hospitals of South Asian, European and American countries.[17] The presence of SCC*mec* V and SCC*mec* IV among HA-MRSA isolates indicates that the CA-MRSA strains are gradually infiltrating into the Indian hospitals. The results of this study were in agreement with the results from recent reports of MRSA among hospitalised patients.[4,23]

An interesting finding of this study was that among CA-MRSA, SCC*mec* type V was predominant unlike reports from developed countries such as USA where SCC*mec* type IV was reported as the predominant type among CA-MRSA infections.[10,24] Our results show that the SCC*mec* type V is predominant in CA-MRSA indicating changing virulence patterns.[3,20]

In the present study, results of SCC*mec* typing of MRSA isolates from HIV-infected patients showed that SCC*mec* type V (81.25%) to be the predominant type followed by SCC*mec* type IV (12.5%). MRSA from healthy individuals from various communities at risk, showed three SCC*mec* types (I, IV and V). SCC*mec* type V was predominant and was detected in 62.5% of MRSA, followed by SCC*mec* type IV (31.25%). The above findings were in agreement with a recent study from India,[20] reporting 52.63% of CA-MRSA with SCC*mec* V. Interestingly, for the first time in India, we identified SCC*mec* type I from healthy individuals of community settings. Insights into all study groups showed that SCC*mec* type V was significantly higher among MRSA isolates from carriers, non-hospitalised patients and HIV-infected patients when compared to MRSA from hospitalised patients ($P = 0.001$), revealing that in this geographical location, SCC*mec* V type is predominant.

MRSA gained much importance mainly because of its potential multi-drug resistance to antibiotics. Recent reports show that the CA-MRSA strains entered into hospitals and were found to be multi-drug resistant. Among tested antibiotics, MRSA isolates showed highest resistance to erythromycin (100%), followed by ciprofloxacin (72.55%), gentamicin (67.93%), ofloxacin (64.94%), TMP-SMX (60.86%), amikacin (50.5%), tetracycline (47.82%) and netilmicin (39.13%). Comparatively, low level resistance was observed for rifampicin (17.39%) and mupirocin (15.21%). Resistance to aminoglycosides, fluoroquinolones, tetracycline and erythromycin were found to be high in MRSA isolates, when compared to previous reports. A community-based study[25] on the nasal carriage of *S. aureus* from North India has reported about 13% of MSSA to be erythromycin resistant, which is in concordance with the erythromycin resistance of carrier isolates of MSSA from healthy community in our study. Furthermore, there was a significant difference in the prevalence of erythromycin resistance of MSSA between the carrier isolates and the clinical isolates from hospitalised and community patients ($P < 0.005$).

Our study reports 3% of MRSA causing clinical infections to be positive for constitutive clindamycin resistance. Deotale *et al*.[26] from North India has shown 7.3% clinical isolates of MRSA to be clindamycin resistant, which is higher than our study. Studies from developed countries report a high percentage of HA-MRSA to be constitutively resistant to clindamycin and also a significant difference in constitutive clindamycin resistance between HA-MRSA and CA-MRSA. Our study reports 19% of MRSA and 12.21% of MSSA isolates to show inducible clindamycin resistance, which is comparable with the 2011 study[27] from south India, which reports 14.67% and 4.92% of MRSA and MSSA, respectively. There was a significant difference in iMLSB phenotype between MRSA isolates from hospitalised patients (24.67%) and community patients (10.68%) ($P < 0.005$). In this study, second only to erythromycin, highest resistance was observed for ciprofloxacin among MRSA isolates causing infections of hospitalised patients (93.17%), HIV-infected patients (75%) and among MRSA isolates from healthy carriers (93.75%). In a study from Mumbai,[3] about 98% HA-MRSA and 87% CA-MRSA isolates causing infections were found to be ciprofloxacin resistant which is higher than the present study. The prevalence of rifampicin resistance is significantly higher among MRSA from HIV-infected patients compared to the MRSA from community patients and hospitalised patients ($P < 0.005$). The highest rifampicin resistance among MRSA from HIV-infected patients may be due to the extensive use of rifampicin in HIV/TB co-infected patients to treat the tuberculosis infection. No significant difference on rifampicin resistance was observed between HA-MRSA and CA-MRSA isolates.

CA-MRSA isolates containing *pvl* genes have been epidemiologically linked to recurrent and often severe skin and soft tissue infections. The prevalence of *pvl*-positive CA-MRSA varies considerably from one continent to another. European countries have the least prevalence of approximately 1%–3%, the USA has up to 50% of clinical isolates causing community associated skin and soft tissue infections. 100% *pvl* prevalence among CA-MRSA has been reported from developed countries including France, Switzerland, USA, Oceania, South West pacific and Australia. We observed *pvl* gene among MRSA and MSSA isolates from all the four study groups. Our study reports 57% of MRSA from hospital-associated infections, 58% of MRSA from community-associated infections, 94% of MRSA from HIV-infected patients and 75% of MRSA from anterior nares of healthy individuals to be positive for *pvl* genes. A hospital based study from Mumbai[3] reports 56.7% of MRSA to be *pvl* positive which is in concordance with the prevalence of *pvl* among MRSA from hospital inpatients of our study. A community based study from several cities in India,[20] reports about 72% of clinical isolates of MRSA from community to be positive for *pvl*, which is comparatively higher than our study (58%). The same study also reports 50% of carrier isolates of MRSA to be positive for *pvl* gene, which is comparatively lower than our study. No significant difference on prevalence of *pvl* was observed between MRSA from community and hospital-associated infections. However, significant difference in the prevalence of *pvl* was seen between carrier isolates of MRSA and MRSA from HIV, community and hospital-associated infections.

Of the other virulence factors studied, among clinical isolates of *S. aureus, lukD* was always present with *lukE*. 62% of clinical isolates from hospitalised patients, 57% of isolates from community patients and 32.8% of isolates from HIV-infected patients were found to be positive for *lukD-E* genes. The prevalence of *lukD-E* was low, when compared to studies from USA, Japan and European countries. Among carrier isolates, the presence of *lukD* and *lukE* varied and was found to be 26% and 28% respectively. This was in agreement with the previous reports, which also have shown varying prevalence rates of *lukD* and *lukE*.[18]

Of the enterotoxins studied, *sea* gene for staphylococcal enterotoxin A was frequently detected in 64% from

hospital-associated infections, 39% from community-associated infections, 40% from infections of HIV-infected patients and 8% from healthy carriers. The prevalence of *sea* (63.6%) among *S. aureus* from hospitalised patients of our study is high, when compared to a nationwide hospital-based study from Japan, which reports 9.6% of MRSA to be *sea* positive. The prevalence of *sea* among carrier isolates of *S. aureus* in this study was found to be lower when compared to studies from developed countries.[18] In a worldwide study of CA-MRSA, Vandenesch *et al*.[28] reported about 79% of CA-MRSA isolates from USA to be positive for *sea* gene. No significant difference on prevalence of *sea* was observed between *S. aureus* from community and HIV-associated infections; while, the prevalence of *sea* was significantly high among clinical isolates of *S. aureus* from hospitalised patients than the clinical isolates from community and HIV-infected patients ($P < 0.005$).

The type of *agr* polymorphism has been reported to be associated with the clinical significance, specific virulence factors (pathogenicity) and resistance. Our study reports *agr* I and *agr* II in 74% of the isolates, which shows their high prevalence in this geographical location. In this study, 31% of MRSA from hospital associated infections, 12% of MRSA from community-associated infections and 6% isolates from HIV-infected patients belonged to the *agr*-I and 67% of MRSA causing hospital-associated infections, 87% of isolates causing community associated infections, 94% of isolates from HIV-infected patients and 62% of isolates from healthy carriers belonged to the *agr*-II subtype. This shows that in this geographical location, the antibiotic resistance is more common among the *agr* I and II. All the isolates with *agr*-IV harboured the exfoliative toxins ETA and/ETB, which is in agreement with the previous reports.[12] 90% of the isolates belonging to *agr*-III were found to be MSSA.

Genotyping

Spa typing

A total of 78 *spa* types were detected in this study. Among *S. aureus* isolates from hospitalised patients, 26 different *spa* types were found, which constituted 33% of the total *spa* types detected. Majority of the *S. aureus* isolates from hospital-associated infections, belonged to *spa* types t657, t037, t1223 and t852 and were also found to be MRSA. *S. aureus* causing community-associated infections belonged to 23 different *spa* types constituting 29% of total *spa* types. The majority of isolates from community-associated infections belonged to *spa* types t657, t064, t1154 and t852 which were also found to be MRSA. The major *spa* types of MSSA isolates from community-associated infections were found to be t3841 and 3204. The results were comparable with the previous report from India.[20]

S. aureus isolates causing infections among HIV-infected patients belonged to 19 different *spa* types, which constituted 24% of the total *spa* types obtained in this study.

Carrier isolates of *S. aureus* included in this study showed high diversity belonging to 58 different *spa* types, which constituted 75% of total *spa* types obtained in this study. Spa types t4615, t272, t159, t021, t005, t015, t3841, t701, t937 and t9037 were found to be common among carrier isolates of *S. aureus* in this study.

MLST types and clonal complexes

When coupled with SCC*mec* typing, MLST is used to discriminate between different clones of MRSA. In this study, a total of 15 ST were detected among major *spa* types ($n > 10$) subjected to MLST types. The CC and STs included CC1 - (ST1, ST772), CC5 - (ST5), CC6 - (ST6), CC8 - (ST239, ST1208, ST368), CC20 - (ST20), CC22 - (ST22), CC30 - (ST30), CC45 - (ST45), CC121 - (ST120) and CC9 - (ST109). This indicates a high diversity of MRSA and MSSA clone in community as well as in the hospital settings. No clonal complex was found for ST672. This is the first Indian study to compare the ST of *S. aureus* from hospital-associated infections, community associated infection, infections of HIV-infected patients and healthy carriers. The representative CC in this study was compared for their *spa* types, resistance, SCC*mec* types, *agr* types, virulence factors and infections [Table 4].

Clonal complex CC1

ST772 - also called as subcontinent clone or Bengal Bay Clone was the predominant CA - MRSAclone, which is single locus variant of ST-1. MRSA ST-772 clone was found to be highly pathogenic associated with abscess infections and acquired resistance determinants and belonged to single spa type 657. Other members were found to be t347 (pvl MSSA); t1931 (MSSA).

Clonal complex CC8

ST239 was the predominant multidrug-resistant HA–MRSA clone endemic in hospitals almost worldwide. It is the second major MRSA clone among HA infections, detected in this study. It was found to be mupirocin and inducible clindamycin resistant strain.

ST368– another MDR HA–MRSA clone of the study belonging to this clonal complex with *spa* type t425/*agr*-I with a prevalence of 6% of MRSA causing HA infections mostly among diabetic patients. It has been previously reported from Sri Lanka. Other members of this clonal complex include ST1208 MRSA-III and ST1208-MRSA-V with *spa* types – t1223 and t064, respectively. Clones with t064 were found to be typical CA-MRSA and were causing middle ear, skin and soft tissue infections. The clones with spa type t1223 were found to be HA-MRSA and were associated with post-operative wound infections and were recently reported from India.

Clonal complex CC-22

ST22-MRSA-IV was the second major CA-MRSA in this study constituting 9% of total MRSA isolates with spa types – t005/t852 with PVL. ST22is an international clone of CA-MRSA, is found circulating in most parts of the world. In this study, these CA-MRSA isolates were found to be causing skin and soft tissue infections. It is highly prevalent in Europe

and India. We find gentamicin resistance among these MRSA clones.

Clonal complex CC-5

ST5 is highly virulent with super-antigen enterotoxins, leucocidins and is resistant to multiple antibiotics. It is a p*vl* positive MSSA and was found to cause community associated ear, skin and soft tissue infections, belonged to *spa* type t448 and *agr* type III. Along with the *egc*gene cluster, all the 10 isolates of t448 carried the plasmid pIB485 with enterotoxins *sed and sej.* MRSA isolates of this clone were reported from Australia, Ireland and Germany.

Single *pvl*-positive CA-MRSA (CC30; ST30-MRSA-IV) was isolated from breast abscess. It has been previously reported from developed countries.

Clonal complexes of methicillin sensitive *Staphylococcus aureus*

CC9; ST109 clone-A total of 13 methicillin sensitive *S. aureus* isolates were obtained from healthy carriers and were positive for exfoliative toxin B. It has been previously reported predominantly among veterinary isolates.

Clones with methicillin resistant *Staphylococcus aureus* and methicillin sensitive *Staphylococcus aureus*

ST-672-carried both MRSA and MSSA isolates with *spa* type t3841. Five of these isolates were found to be MRSA, which carried SCC*mec*I and were found to be causing HA infections, while one MSSA isolate was from healthy carrier. ST 672 was first reported from South India in 2010.[20]

Conclusion

This study comparing molecular characteristics of resistance and virulence determinants of MRSA from four different populations has shown that the MRSA isolates causing hospital-associated infections were significantly different in their genotypes from MRSA causing community-associated infections, HIV-associated infections and carrier isolates of high-risk community. Virulence factors were significantly high among community and hospital-associated *S. aureus* compared to clinical isolates from HIV-infected patients. Antibiotic resistance was significantly high among HA-MRSA compared to MRSA from community-associated infections and HIV-infected patients. Antibiotic resistance was significantly lower among carrier isolates compared to clinical isolates, but carrier isolates also showed resistance to antibiotics especially to erythromycin, tetracycline and cefoxitin. Genotyping by *spa* typing showed that the carrier *S. aureus* isolates from community were highly diverse compared to clinical isolates. Prevalence of MRSA (2%) among healthy individuals from various high risk communities indicates that CA-MRSA isolates are circulating in the Indian community settings asymptomatically. The emergence of MDR-CA-MRSA among hospitalised patients shows that CA-MRSA has acquired additional drug resistance determinant on entering the hospital settings. This study has shown that in the Indian scenario, CA-MRSA isolates have infiltrated into the hospital settings, acquired multiple drug resistance determinants and have become endemic in hospitals.

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Conflicts of interest

There are no conflicts of interest.

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