Genotyping of methicillin-resistant *Staphylococcus aureus* isolates from Indian hospitals

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major problem in hospitals around the world, including India. This study was undertaken to genotype clinical MRSA isolates collected from hospitals in different parts of India. One hundred and eighty-six isolates were collected and characterized by phenotypic and genotypic methods using published protocols. Majority of isolates were positive for *mecA* gene detected by PCR and possessed Staphylococcal Cassette Chromosome (SCC)*mec* type III or IIIA cassettes with the exception of two isolates from Nagpur, which had type II cassettes. The allelic patterns of seven housekeeping genes determined by multilocus sequence typing of a select number of isolates, grouped the Indian isolates as predominantly Sequence Type (ST) 239. Two isolates belonged to ST 241, a single locus variant of ST 239. Most of the isolates had the same staphylococcal protein A (*Spa*) types except for a few variations. Although MLST and *Spa* of most isolates were similar, the pulsed field gel electrophoresis patterns were diverse and indicated many short-term genetic changes among these isolates. Our results indicate that Indian isolates are related to the Hungarian and Brazilian MRSA clones. While Japan and Korea have predominantly type II SCC*mec* isolates, India along with China, Indonesia and other Asian countries, has SCC*mec* type III and IIIA isolates.

Keywords: Genotyping, Indian hospital MRSA isolates.

METHICILLIN-resistant *Staphylococcus aureus* (MRSA) is a major hospital-associated as well as a community-associated pathogen causing a wide range of diseases, including endocarditis, osteomyelitis, toxic-shock syndrome, pneumonia, food poisoning and carbuncles[1,2]. In Indian hospitals, MRSA is one of the common causes of hospital-acquired infections and different hospitals have reported anywhere from 30 to 80% methicillin resistance based on antibiotic sensitivity tests[3]. This leaves only vancomycin as the drug of choice in India and vancomycin-resistant MRSA has already been reported in Japan, the United States and several other countries. Nosocomial MRSA isolates are mostly multidrug resistant. Methicillin resistance is due to the presence of *mecA* gene coding for penicillin-binding protein (PBP2A) with a low affinity for β-lactam antibiotics. This gene is carried on Staphylococcal Cassette Chromosome (SCC)*mec*, a unique mobile genetic element, integrated into the staphylococcal chromosome. Several types of SCC*mec* cassettes have been sequenced and primers from different regions have been used to characterize types of cassettes from clinical isolates[4]. Bacterial strain molecular typing is an important clinical tool to investigate hospital outbreaks and to evaluate nosocomial transmission. In addition to tracking outbreaks, genotyping can be used to distinguish between separate episodes of infection and relapse of disease. It also helps in answering an epidemiological query whether the strains causing disease in one geographic area are related to those causing the same disease in other regions[5].

We have genotyped MRSA isolates from two Bangalore hospitals in an earlier study[6], and now we have collected 186 clinical strains from eight hospitals in different cities of India and have done molecular characterization by various established techniques. MRSA isolates from different parts of India have not been genotyped till now and there are no epidemiological studies available on the spread of MRSA in hospitals.

Materials and methods

Hospitals: Isolates were collected from St. Johns Medical College Hospital (SJMH) and Manipal Hospital (MH), Bangalore, Karnataka; Christian Medical College (CMC), Vellore, Tamil Nadu; Raja Muthaiah Hospital (RMH), Annamalai, Tamil Nadu; Hinduja Hospital (HH), Mumbai, Maharashtra; Nagpur Medical College (NMC), Nagpur, Maharashtra; Sir Gangaram Hospital (SGRH), Delhi; and Kolkata Medical College (KMC), Kolkata, West Bengal. MH, HH and SGRH are private, multi-specialty, tertiary-care hospitals. SJMH, CMC, RMH, NMC and KMC are multi-specialty, tertiary, teaching hospitals with medical colleges.

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Samples: Majority of the isolates from all hospitals were cultured from pus swabs. The rest of the isolates were cultured from ear, nose, throat swabs, fluids from CT-guided aspirates, blood and urine. The isolates were inoculated into peptone water or semi-solid nutrient agar deeps, sealed and sent to us.

Bacterial strains: Forty-five clinical isolates were obtained from SJMH, 37 from MH (collected from 2003 to 2004), 24 from CMC, 9 from RMH, 7 from HH, 16 from NMC, 29 from SGRH, and 19 from KMC (collected in 2005).

Growth: Growth and phenotypic characterization of the isolates by Gram staining and detection of catalase, coagulase and DNAase have been described in earlier publications[6,7].

Antibiotic susceptibility testing: Antibiotic susceptibility was performed by Kirby–Bauer disc diffusion according to the guidelines recommended by the NCCLS on Mueller–Hinton agar plates at 37°C using antibiotic discs containing penicillin, gentamicin, erythromycin, oxacillin, tetracycline, methicillin and vancomycin (HiMedia). Minimum inhibitory concentration (MIC) for oxacillin was determined by the broth dilution method in Mueller–Hinton broth after 24 h of incubation at 37°C in micro titre plates[8].

Preparation of chromosomal DNA: Cells from an overnight culture in BHI broth were collected by centrifugation and processed as described earlier[6].

Multiplex PCR: This was performed according to the procedure of Oliveira *et al.*[9]. PCR for detection of *mecA* (gene coding for the penicillin binding protein 2A) and *femA* (factor essential for methicillin resistance) genes were used as internal controls for confirmation of MRSA isolates. The conditions for PCR were as published in Mehrotra *et al.*[10] and the sizes of the amplified products for *mecA* and *femA* were 163 and 132 bp respectively. The remaining primers for detecting type I, II and III *SCCmec* cassettes, conditions of PCR and size of the amplified products were as described in Oliviera *et al.*[9].

PCR for typing Cassette Chromosome Recombinases (*ccrAB*): PCR was performed for detection of type 1, 2, and 3 *ccrAB* system among the various strains with primers and conditions as described by Hanssen *et al.*[11].

MLST and spa typing: MLST (multi locus sequence typing) was performed as described by Enright *et al.*[12]. Internal fragments of the seven housekeeping genes, namely carbamate kinase (arcC), shikimate dehydrogenase (aroE), glycerol kinase (glpF), guanylate kinase (gmK), phosphate acetyltransferase (pta), triosephosphate isomerase (tpi) and acetyl coenzyme A acetyltransferase (yqiL) were amplified by PCR with the specified primers. The PCR products were purified (QiaQuick PCR purification kit, Qiagen GmbH, Germany) and sequenced using ABI Prism 377 DNA sequencer. Consensus sequences were assembled from both orientations. *Spa* typing was performed according to the procedure of Shopsin *et al.*[13,14] using the forward (5′-GAACAACGTAACGGC TTCATCC-3′) and reverse (5′-CAGCAGTAGTGCCGT TTG-3′) primers and consensus sequences were assembled from both forward and reverse sequences.

PFGE: PFGE (pulsed field gel electrophoresis) was done as described by McDougal *et al.*[15], with minor modifications as described in Arakere *et al.*[6]. The plugs were digested with *Sma*I restriction enzyme (Promega Corporation, Madison, WI) for 3 h. Restriction fragments were separated on a 1·5% gel (Bio-Rad PFGE agarose) with an initial switch time of 5 s and final switch time of 35 s, voltage of 6 V/cm, included angle of 120° and running time of 21 h using the CHEF-DRIII device (Bio-Rad). After electrophoresis, the gels were stained using ethidium bromide, destained in water and photographed under UV light with the GelDoc system (Bio-Rad).

Dendrogram: PFGE pattern of strain NCTC 8325 was used as a standard with assigned molecular weights according to Tenover *et al.*[16], and the dendrogram based on the similarities was derived from the unweighted pair group method using arithmetic averages and Dice coefficients using quantity one software (Bio-Rad).

Results and discussion

Data on two Bangalore hospitals have been published earlier and it is only included as a part of the analysis of samples from different Indian hospitals[6]. Majority of isolates were derived from pus, although the percentage of isolates from various other sites varied amongst hospitals. The SCC*mec* types of all the isolates collected from the hospitals were determined using various primers specific for types I, II, III, IIIA and IV by two multiplex PCR reactions. Figure 1 depicts the multiplex PCR patterns of

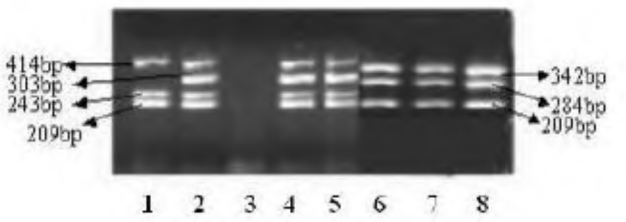

Figure 1. Multiplex PCR of MRSA isolates: Lanes 1, 2, Isolates from SJMH; lanes 4, 5, Isolates from MH; lane 3, Methicillin-sensitive *Staphylococcus aureus*; lanes 6, 7, Type II isolates from NMC; lane 8, Type II DNA from N315.

selected isolates (*mecA*- and *femA*-positive by PCR) from SJMH, MH and NMC to determine the SCC*mec* types. All the isolates from SJMH and MH gave SCC*mec* type of III (presence of 209, 243, 303 and 414 bp amplification products) or IIIA (209, 243 and 414 bp) and none of them had type I, II or IV SCC*mec* cassettes. Two isolates from NMC gave amplification products of 209 bp (primers from the region common to types II and III SCC*mec* cassettes), 342 bp (from the region common to types I, II and IV) and 284 bp from a region specific for type II SCC*mec*. There were 82 strains from Bangalore and 104 from all the other hospitals. Seventy-five isolates from Bangalore typed III (65%) or IIIA (35%). Forty-six isolates out of 104 from all the other hospitals had SCC*mec* type III or IIIA and their percentages were 76 and 24 respectively. Sixteen isolates were *mecA* gene-positive, but did not have one or the other amplification products out of the four found in type III *SCCmec* cassette and were referred to as partial cassettes of type III. The rest (34) of the isolates had borderline resistance to oxacillin and were *mecA*-gene negative. Two isolates from NMC gave amplification products characteristic of a type II SCC*mec* cassette and four isolates may have variation of a type I cassette from preliminary PCR studies. Type I and II SCC*mec* cassettes have not been identified from isolates of any of the other hospitals in this study.

All type III and IIIA isolates were resistant to penicillin, methicillin, oxacillin, tetracycline, gentamicin and erythromycin and sensitive to vancomycin, with the exception of one strain from KMC, which exhibited methicillin sensitivity despite the presence of the *mecA* gene. An isolate from Japan (N315) that was found to be *mecA* gene-positive and containing type II *SCCmec* cassette but sensitive to methicillin has been reported. It has been named as pre MRSA and this phenomenon is explained by repression of the *mecA* gene transcription by *Mec*I, resulting in methicillin sensitivity. This phenomenon has not been reported in any isolate containing a type III or IIIA cassette and needs further work.

Most Asian countries have reported that their type III and IIIA isolates are related to the Hungarian and Brazilian clones by PFGE patterns and classified as ST 239 and ST 241 from MLST data[6,17]. Hungarian and Brazilian isolates were included in the PFGE analysis along with isolates from Indian hospitals.

Figure 2 *a* shows the PFGE patterns of *Sma*I macro restriction fragments of selected strains from KMC, SGRH, RMH, HH and CMC along with standard strains – NCTC 8325 (MSSA), HUSA 304 (Hungarian clone, type III cassette) and HSJ 216 (Brazilian clone, type IIIA) and the lambda ladder. Figure 2 *b* shows the relatedness of the Indian isolates to the Hungarian and Brazilian strains. The relatedness of the Bangalore hospital strains to the Hungarian and Brazilian clones has been reported earlier[6]. This relatedness was confirmed by MLST and *Spa* sequence analysis data for a selected number of isolates which are presented in Table 1. Isolates were divided into different groups based on their MIC values and a representative strain was chosen for performing MLST. Isolates with MIC range of 200–400 and 16–100 μg/ml of oxacillin made up for 79% of the total. Most strains had an allelic profile of 2-3-1-1-4-4-3 for the seven house-

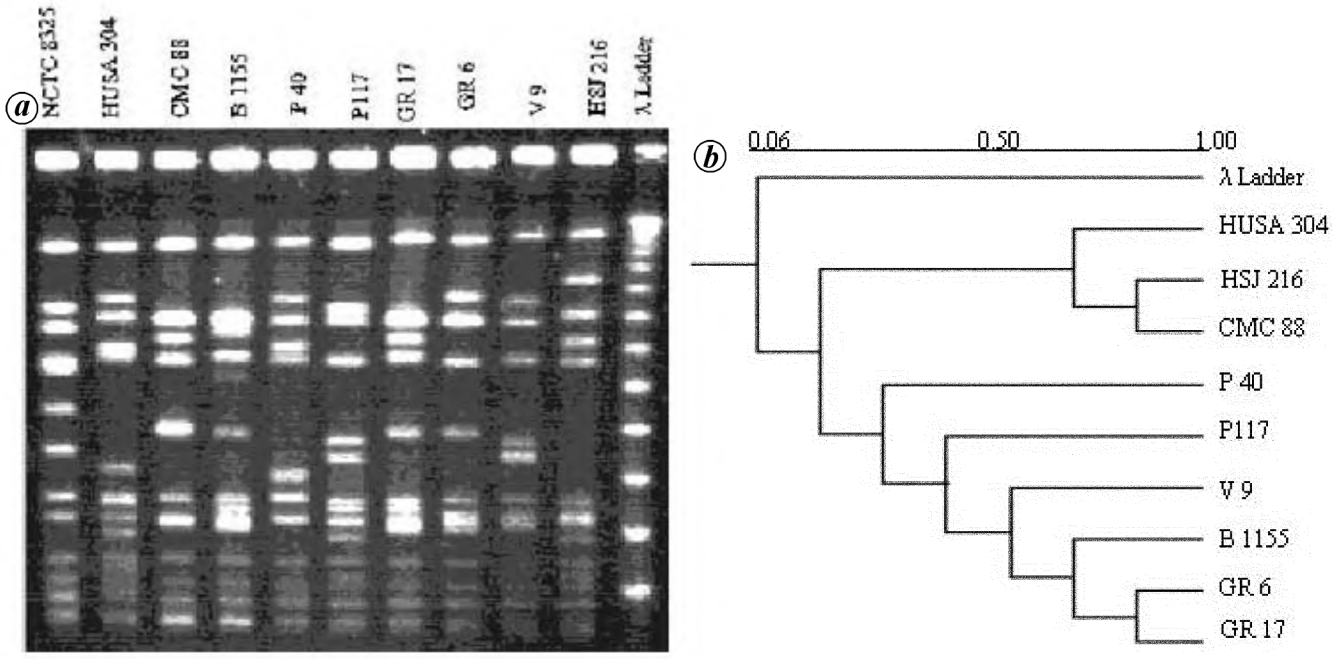

Figure 2. ***a***, PFGE patterns of *Sma*I digests of genomic DNA from MRSA isolates in different hospitals. ***b***, Dendrogram of PFGE based on similarities derived from the UPGMA and dice coefficients using Quantity One software. The scale at the top represents similarity.

Table 1. Genotyping of selected strains from different hospitals

Oxacillin MIC (μg/ml)	Percentage of total strains	SCC*mec* type	*Spa* type[f]	MLST[g]	ST[h]
1–15[a]	8	III (PC)[b]	WKAOMQ	2-3-1-1-4-4-3	239
16–100	23	III, IIIA	WGKAOMQ	2-3-1-1-4-4-3	239
200–400	56	III, IIIA	WGKAOMQ	2-3-1-1-4-4-3	239
		III		2-3-1-1-4-4-30	241
>800	12.8	III	WGKAOMQ	2-3-1-1-4-4-3	239
N105[c]		II	TJMBMDMGMK	1-4-1-4-12-1-10	5
HUSA 304[d]		III	WGKAOMQ	2-3-1-1-4-4-3	239
HSJ 216[e]		IIIA	WGKAOMQ	2-3-1-1-4-4-3	239

[a]Range of MICs obtained for different isolates.
[b]Partial cassette of type III (209, 303).
[c]Type II strain from NMC with an MIC of 100 μg/ml.
[d]HUSA 304 (ATCC BAA-39) is the Hungarian clone.
[e]HSJ 216 (ATCC BAA-43) is the Brazilian clone.
[f]DNA sequence of the repeat region of protein A gene.
[g]Allelic profile based on DNA sequences of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqil*).
[h]Sequence type.

keeping genes that were sequenced (the forward and reverse sequence for each enzyme is entered in the MLST website from which particular allelic profile is derived, which in turn is used to assign a ST) and belonged to ST 239. One isolate had MLST pattern of 2-3-1-1-4-4-30 and a ST of 241 and is a single locus variant of yqil 3. The *spa* types of all the isolates had the same repeat sequences (Kreiswirth nomenclature), except for one that had one repeat unit missing (WKAOMQ). This isolate has a partial cassette of type III and is being sequenced. The MLST and *spa* patterns of one of the type II isolates from NMC are 1-4-1-4-12-1-10 and TJMBMDMGMK, and it belongs to ST 5. HUSA 304 and HSJ 216 also have the same MLST and *spa* sequences as most of the type III and IIIA Indian isolates in this study. The allelic pattern and the *spa* type of the type II isolate are typical of the patterns of isolates belonging to ST 5.

Table 2 depicts the PFGE patterns assigned to all the isolates from different hospitals. There were predominantly three patterns – A, B, and C. One or two band variations within a pattern were assigned A1, A2, etc. The SJMH and MH samples from Bangalore were spread between patterns A1 and A4 while the Vellore samples had patterns A2–A4 and KMC had pattern A3. Isolates from RMH, SGRH and HH did not have any pattern A variants. Isolates from all hospitals possessing patterns B and C had more diversity evident from the SJMH and MH (Bangalore) isolates, which had variations from B1 to B11 and C1 to C8. The CMC isolates fell within the same patterns, while RMH isolates had new variants of patterns, B and C, namely B13, B14 and C9 in addition to B2. Isolates from SGRH had new variants B12, B15 and C10 in addition to pattern B3. HH isolates had pattern B14 in addition to B1 and C7 and KMC isolates had A3 and C9 patterns. Two NMC isolates with SCC*mec* type IIIA had a different pattern compared to the common patterns A, B and C and we have called it pattern F. The PFGE patterns of two type II SCC*mec* isolates from NMC were identical and different from all the type III isolates. We have designated it as pattern Y (not shown in Figure 2 *a*). The common patterns amongst the other NMC isolates were B3, B7 and C1.

As there was substantial diversity in the PFGE patterns of isolates although they contained type III or IIIA *SCCmec* cassettes, PCR was carried out to detect the presence of type 3 *ccr*AB in all the isolates. All type III or IIIA SCC*mec* containing isolates had *ccr*AB type 3 and the two isolates from NMC with type II SCC*mec* cassettes were positive for *ccr*AB type 2 by PCR (data not shown).

We are reporting the first molecular characterization of Indian MRSA isolates from hospitals in various cities. The results are similar to what we had seen with two Bangalore hospitals earlier with the exception of six isolates from NMC. In the past year, several reports on the genotypes of MRSA prevalent in Asian countries have been published[17–20]. Kwan Soo Ko *et al.*[17] collected five or more isolates from the following Asian countries – Korea, Japan, China, India, Indonesia, Philippines, Saudi Arabia, Singapore, Sri Lanka, Taiwan, Thailand and Vietnam and genotyped them. They reported that Korea and Japan had exclusively SCC*mec* type II containing isolates and the remaining countries had isolates with type III or IIIA cassettes, IIIA being the majority. Among the Indian isolates we have analysed, 65–75% comprised of type III and the rest IIIA. A more recent publication from the same authors[18] from a survey of samples collected from different hospitals in Korea reports that type III and IIIA containing isolates are surfacing in Korea although the dominant type is still type II. In a recent publication, Ip *et al.*[19] have reported presence of *SCCmec* types I and II from

Table 2. PFGE patterns of MRSA isolates from different hospitals

Hospital	PFGE pattern		
	A	B	C
SJMH + MH, Bangalore	A1–A4	B1–B11	C1–C8
CMC, Vellore	A2–A4	B2–B7	C1–C3
RMH, Annamalai		B2, B13, B14	C9
SGRH, Delhi		B3, B12, B15	C1, C10
KMC, Kolkata	A3		C9
HH, Mumbai		B1, B14	C7
NMC, Nagpur		B3, B7	C1

Hong Kong along with type III among contemporary MRSA clones.

Although the majority of Asian isolates have origins in the Hungarian and Brazilian clones, a wide variety of short-term genetic changes have taken place as is evident from the PFGE patterns. In addition, a few isolates containing type I and II cassettes which have origins in the Pediatric/Iberian and New York/Japan clones have been detected in Hong Kong and also now in India. Similarly, in Korea, a few isolates containing type III cassette have been found along with majority of type II isolates. NMC is one of the largest teaching hospitals in India and it is possible that travellers from elsewhere could have brought these strains to the hospital.

MRSA isolates belonging to ST 239 (type III and IIIA) represent a distinct branch within the clonal complex CC8 in the evolutionary model of emergence of MRSA[21,22]. ST 5 (type II isolate from NMC) belongs to clonal complex CC5 from which several isolates possessing different SCC*mec* types have emerged. Genotyping data from large international studies have shown that a few clones of MRSA are responsible for disease in various parts of the world. During the last decade, five major internationally spread MRSA clones, the Iberian, Brazilian, Hungarian, New York/Japan and pediatric clones were identified using a combination of molecular techniques. MRSA infections in the hospitals and communities are becoming a problem of serious proportions and pose a challenge to the healthcare system. Epidemiological studies on the spread of MRSA infections at the local, national and international levels will help in designing strategies for preventing the spread.

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