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Molecular characterisation of methicillin-resistant *Staphylococcus aureus* isolated from patients at a tertiary care hospital in Hyderabad, South India**Ganapuram J Archana1, Akhauri Yash Sinha2, Madhavi Annamanedi3, Kandala Pavan Asrith3, Satyajit B Kale4, Nitin V Kurkure4, Swapnil P Doijad5, Kammili Nagamani1, Nagendra R Hegde3,** 1 Department of Microbiology, Gandhi Medical College and Hospital, Secunderabad, Telangana, India2 Ella Foundation, Genome Valley, Turkapally, Shameerpet Mandal, Telangana, India3 National Institute of Animal Biotechnology, Hyderabad, Telangana, India4 Department of Veterinary Pathology, Nagpur Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra, India5 Division of Veterinary Public Health, ICAR Research Complex for Goa, Old Goa, Goa, India**Correspondence Address**:Dr. Nagendra R HegdeNational Institute of Animal Biotechnology, Opposite Journalist Colony, Extended Q City Road, Near Gowlidoddi, Gachibowli, Hyderabad - 500 032, TelanganaIndia

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Abstract**Context:** Infections with methicillin-resistant *Staphylococcus aureus* (MRSA) greatly influence clinical outcome. Molecular characterisation of MRSA can help to predict their spread and to institute treatment and hospital protocols. **Aim:** The aim of this study is to understand the diversity of MRSA in a tertiary care hospital in Hyderabad, India. **Settings and Design:** Samples collected at Gandhi Medical College, Hyderabad, and designed to assess hospital-or community-associated MRSA (HA-MRSA or CA-MRSA). **Subjects and Methods:** MRSA were subjected to antibiotic susceptibility testing, pulsed-field gel electrophoresis (PFGE), *spa* typing, multi-locus sequence typing and staphylococcal cassette chromosome–mec (SCCm*ec*) typing. **Statistical Analysis Used:** Discriminatory index and 95% confidence interval. **Results:** Of the 30 MRSA, (a) 18 and 12 were HA-MRSA and CA-MRSA, respectively, and (b) 23.3% and 6.6% displayed induced clindamycin and intermediate vancomycin resistance, respectively. Genetic diversity was evident from the presence of (a) 20 pulsotypes, (b) eight *spa* types, with the predominance of t064 (*n* = 9) and (c) seven sequence types (ST), with the preponderance of ST22 and ST8 (9 each). ST22 and ST8 were the most prevalent among HA-MRSA and CA-MRSA, respectively. SCC*mec* type IV was the most frequent (*n* = 8). 44.4% of HA-MRSA belonged to SCC*mec* IV and V, whereas 33.3% of CA-MRSA belonged to SCC*mec* I and III; 33.3% (5/15) of the isolates harbouring the *pvl* gene belonged to SCC*mec* IVC/H. **Conclusions:** ST8 was a dominant type along with other previously reported types ST22, ST239, and ST772 from India. The observations highlight the prevalence of genetically diverse clonal populations of MRSA, suggesting potential multiple origins.

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Full Text**Introduction**Methicillin-resistant Staphylococcus aureus (MRSA), which is frequently responsible for nosocomial infections, was initially reported in hospitals (hospital-associated or HA-MRSA), but was later also observed in communities (community-associated or CA-MRSA). Indiscriminate use of antibiotics has led to the emergence of MRSA resistant to multiple antibiotics leaving vancomycin and clindamycin as a last resort for treating β-lactam-resistant S. aureus infections. However, vancomycin-resistant and vancomycin-intermediate S. aureus (VRSA and VISA) have been reported globally, including from different parts of India,[1],[2],[3],[4],[5] and resistance to clindamycin has also been observed.[6] Multi-drug-resistant S. aureus poses a challenge for treatment outcomes; therefore, tracking the prevalence and dissemination of such strains is important.Techniques with high discriminatory power are necessary for tracking and molecular epidemiology of MRSA. The most commonly used techniques are pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and spa typing. These are based on the analysis of (a) genomic DNA fragments as a result of digestion with rare restriction enzymes, (b) variable repeat region sequence of staphylococcal protein A gene and (c) sequence of specific fragments of seven housekeeping genes, respectively. As of 7th July 2020, 6121 MLST (https://pubmlst.org) and 19,492 spa types (https://spa.ridom.de/spatypes.shtml) have been identified. Furthermore, MRSA can be grouped according to staphylococcal cassette chromosome (SCCmec) locus, a mobile genetic element that harbours the mec A gene, which enables resistance to methicillin, into eleven different Types (I to XI); of these, only types I to V are globally distributed while others appear to exist among local/regional strains.[7]The HA-MRSA and CA-MRSA have been reported to have distinctive phenotypic features and molecular epidemiology. HA-MRSA is typically multi-drug resistant and have SCCmec Type I, II and III, while CA-MRSA are susceptible to non-ß-lactam antibiotics, harbor SCCmec Types IV or V, and tend to express the Panton–Valentine leucocidin.[8] However, recent studies have shown penetration of CA-MRSA into hospital settings and vice versa, and occurrence of HA-MRSA with SCCmec type IV or V and CA-MRSA with SCCmec type I, II or III have been reported worldwide, including from India.[9],[10]Molecular epidemiology studies of MRSA are limited in India; studies have shown a predominance of ST239 among the isolates from New Delhi, ST772 from Karnataka and ST22 from Mumbai.[11] However, more epidemiological information is required for accurate characterisation of the prevalent MRSA clones and their resistance patterns for appropriate prognostication and therapy as well as for devising hospital protocols. In this perspective, we characterised MRSA isolates from a tertiary care hospital located in Hyderabad, a metropolitan city in South India, by PFGE, spa typing, MLST and SCCmec typing along with antibiogram studies for vancomycin and clindamycin. Of the 110 S. aureus isolates obtained, 30 were MRSA, with 60% HA-MRSA and 40% CA-MRSA. Induced clindamycin and intermediate vancomycin resistance was observed with 23.3% and 6.6% of the MRSA, respectively. Further, the MRSA could be categorised into 20 pulsotypes, eight spa types, with the predominance of t064 and seven sequence types (ST), with the preponderance of ST22 and ST8, which were the most prevalent among HA-MRSA and CA-MRSA, respectively. SCCmec type IV was the most frequent and 44.4% of HA-MRSA belonged to SCCmec IV and V whereas 33.3% of CA-MRSA belonged to SCCmec I and III. The data show clonal diversity among the small number of isolates obtained over a short period of time, as well as cross-over of HA- and CA-MRSA, and suggest that a wider analysis is required.**Subjects and Methods**SubjectsNon-duplicate skin, wound, blood and sputum samples of infected patients [Table 1] were collected for routine microbiology testing, during January 2012–May 2013, following due procedures set forth by Gandhi Medical College Hospital, Hyderabad, as per the ethical guidelines of the Indian Council of Medical Research.{Table 1}Isolation and identification of bacteriaThe samples were subjected to standard microbiological investigation including Gram's stain, catalase test, slide and tube coagulase tests, DNase test, OF-Glucose fermentation and growth on mannitol salt agar (all media and reagents were obtained from HiMedia Laboratories, Mumbai). Through pure culture techniques, 110 isolates of S. aureus were obtained [Table 1] and classified as HA-MRSA or CA-MRSA as per the guidelines set forth by the World Health Organisation (WHO).Antibiotic susceptibility testingThe isolates were subjected to disc diffusion test using commercial discs (HiMedia Laboratories), following CLSI guidelines.[12] Based on resistance to cefoxitin, 30 isolates were identified as MRSA and were further subjected to testing for resistance to vancomycin using the Vitek-2 system (Biomeriuex India Pvt Ltd., New Delhi) or to clindamycin employing the standard D test (HiMedia Laboratories).Extraction of DNA from bacterial isolates and polymerase chain reactionGenomic DNA was extracted from 5 mL of tryptic soy broth (HiMedia Laboratories) culture of each isolate, using the HiYield Genomic Mini Kit (Real Genomics, Taiwan), and stored at −20°C. As described previously, polymerase chain reaction (PCR) (for nuc and mec A genes) was used confirm the isolates as MRSA [13],[14] as well as to assess the carriage of Panton-Valentine leucocidin (lukS/F-PV) genes.[15] For SCCmec typing (I to V), a multiplex PCR was performed as described previously.[16]Pulsed-field gel electrophoresis, Spa typing and multi-locus sequence typingPFGE was performed as per the Centre for Disease Control and Prevention Pulse net protocol.[17] Genomic DNA was subjected to Sma I (New England Biolabs, Imperial Life Sciences, Gurugram) digestion and separated on 1% Seakem Gold agarose (Lonza, Mumbai, India) using PFGE-CHEF system (Bio-Rad Laboratories India Pvt Ltd., Chennai) at 200 volts and 14°C with an initial switch of 5 s and a final switch of 40 s, and a run time of 21 h. Data were analysed using Bionumerics software (Applied Maths N.V., Saint-Martens-Latem, Belgium) employing the unweighted pair group method with arithmetic averages (UPGMA) method.Spa typing was performed by employing the protocol as described previously [18] and elaborated in the RidomSpa server (http://www.spaserver.ridom.de/). The sequence analyses were performed through Spa typer (http://spatyper.fortinbras.us/) as well as RidomSpa server.Multi-locus sequence typing was performed as per the guidelines of MLST database (http://saureus.mlst.net). Briefly, the seven housekeeping genes, acetyl coenzyme A acetyltransferase (yqi L), carbamate kinase (arc C), glycerol kinase (glp F), guanylate kinase (gmk), phosphate acetyl-transferase (pta), shikimate dehydrogenase (aro E) and triosephosphate isomerase (tpi) were amplified by PCR with the specific primers as described previously.[19] The amplified fragments were gel purified and sequenced. The sequences were analysed using the PubMLST database to assign the sequence types (STs). Clonal complexes (CCs) were assigned by comparing the STs with the entire MLST database using the eBURST V3 program. Default stringency, where every member of the CC shares six or seven alleles with at least one other member of the complex, was used for the analysis.Statistical analysisDiscriminatory index and 95% confidence interval were calculated as described.[20]**Results**Distribution of methicillin-resistant Staphylococcus aureus in samplesOf the 110 S. aureus isolates obtained, 30 were confirmed as MRSA both by resistance to cefoxitin and by PCR for mec A (data not shown). MRSA were most prevalent in pus exudates (73.3%; n = 22) and blood samples (20%; n = 6) and least abundant in sputum (6.7%; n = 2). The median age for patients was 28 years (range, 4 days to 55 years), and 50% (n = 15) and 30% (n = 9) of the MRSA isolates were from male and female patients, respectively, whereas gender was not recorded for six clinical samples. Of the 30 MRSA isolates, 60% (n = 18) were HA-MRSA and 40% (n = 12) were CA-MRSA [Table 2] as per the WHO guidelines.{Table 2}Antibiotic susceptibility testingMost of the 30 MRSA were multi-drug resistant, but all were susceptible to linezolid and tigecycline [Table 1]. Five isolates showed a minimum inhibitory concentration (MIC) of ≥2 μg/mL, and two (isolates 5 and 22) were considered as vancomycin-intermediate S. aureus [VISA; MIC 4 μg/mL; [Table 2]. Seven isolates showed induced clindamycin resistance, including two (isolates 8 and 22) which were less susceptible to vancomycin [Table 2].Molecular typingTyping by PFGE revealed 23 different banding patterns with 20 different pulsotypes [designated A to T; [Figure 1]. All the isolates with >80% similarity in the banding pattern were placed under a single pulsotype and were considered as a cluster. Five clusters were identified, K being the largest (16.7% of the isolates; n = 5), followed by M and O (10%; n = 3 each). F and G were the smallest clusters (6.7%; n = 2 each), while the rest of the fifteen isolates (50%) were singletons without any clones (100% similarity) or subtypes. The PFGE analysis revealed that the ST8/t064 isolates belonged to three pulsotypes: (a) O and N (with >75% similarity) with SCCmec NT or type V; (b) G (SCCmec NT), H (SCCmec V) and I (SCCme c NT), with ~60% similarity between G and H-I clusters and >70% similarity between H and I clusters; and (c) B (SCCmec NT), which was a singleton.{Figure 1}The 30 isolates belonged to eight different spa types [Table 1]; t064 was the most prevalent (30.0%; n = 9) followed by t037 (23.3%; n = 7) and t05 (16.7%; n = 5). t12224 included three isolates, while t5122 and t425 included two isolates each. Two single isolates were identified as t852 and t657.MLST categorised MRSA isolates into seven different STs [Table 2]. ST8 and ST22 were the most abundant (30%; n = 9 each).The predominant SCCmec type was IV (IVC/H) (n = 8; 26.7%) followed by III (n = 7; 23.3%), and V (n = 5; 16.6%) [Table 1] and [Figure S1]. Notably, one MRSA isolate (number 12) showed the presence of two amplicons, for both type I and III (I + III) [Table 2], [Figure S1] and [Figure S2]. There were no type II strains, and 10 isolates (33.3%) were non-typeable (NT).[INLINE:1][INLINE:2]Among the HA-MRSA isolates, three (16.6%) were Type III, whereas four (22.2%) each were Type IV (C/H) and V, and six were NT. Among the CA-MRSA isolates, four (33.3%) belonged to Type IVC/H, whereas three (25%) belonged to Type III and one to Type V. Four (33.3%) CA-MRSA isolates were NT [Table 1].Among the MRSA, 15 (50%) were pvl positive, of which eleven (73.3%) were HA-MRSA and the rest were CA-MRSA [Table 2].Combinatorial analysisCombining spa type with PFGE revealed that all the ST8 isolates had a common spa type of t064, while ST239 had t037. Contrarily, ST22 had more diverse spa types with t05 (n = 5) as the majority followed by t12224 (n = 2) and t852 (n = 1). PFGE clusters G and O were associated with ST8/t064, while in cluster F and M, ST239/t037 was prevalent. All of the isolates in cluster K belonged to ST22 but were distributed among t05 (n = 4) and t12224 (n = 1), the latter being NT by SCCmec typing [Figure 1] and [Table 3]. It is to be noted that t12224 is a triple locus variant of t05 (two changes and one deletion). Interestingly, two different pulsotypes, C and P, both belonged to ST580/t5122 and SCCmec NT. Several STs and spa types were widely distributed among the singleton pulsotypes. The discriminatory power and 95% confidence interval of PFGE, spa typing and MLST are shown in [Table 4].{Table 3}{Table 4}ST22 and ST8 constituted the most abundant (n = 9 each) STs, followed by ST239 (n = 7). Majority (n = 7) of the ST8 isolates were NT by SCCmec typing while two isolates belonged to Type V. Majority (n = 8) of the ST22 isolates belonged to SCCmec IV, whereas the other one was NT. Among the ST239 isolates, four belonged to SCCmec III and one belonged to SCCmec I + III. Two isolates belonged to ST580, whereas ST30, ST368 and ST772 were represented by single isolates. The eBURST analysis revealed that ST8 and ST239, which belong to clonal complex 8 (CC8), were clustered into one group; indeed, ST239 is a single locus variant (SLV) of ST8. In addition, ST368, which is an SLV of ST239 and a double locus variant of ST8, was also clustered along with ST239. Similarly, ST772, which belongs to CC1 along with ST1[21] was represented as SLV of ST1. ST22 (CC22), ST30 (CC30) and ST580 were represented as independent groups with their SLVs [Figure 2].{Figure 2}The MRSA isolates designated 8, 23 and 24 with vancomycin MIC of 2 ug/mL belonged to t657/ST772/SCCmec V, t05/ST22/SCCmec IVC/H and t037/ST239/SCCmec III, respectively. The two VISA (MIC of 4 ug/mL) isolates (designated 5 and 22) belonged to t12224/ST22/SCCmec IVC/H and t037/ST239/SCCmec III, respectively [Table 2].The MRSA isolates with induced clindamycin resistance (designated 1, 8, 19, 20, 21, 22 and 30) belonged to t852/ST22/SCCmec IVC/H, t657/ST772/SCCmec V, t064/ST8/SCCmec NT,), t5122/ST 580/SCCmec NT, t05/ST22/SCCmec IVC/H, t037/ST239/SCCmec III and t037/ST 239/SCCmec III, respectively [Table 2].Nine (60%) pvl -positive isolates were of SCCmec IV and V, three isolates (20%) were type III, and one was type I + III, while two (13.3%) isolates were NT [Table 2].**Discussion**Dissemination of MRSA from hospitals to community and vice versa and emergence β-lactam-resistant strains is a cause of significant concern worldwide. However, there have been very few reports from India on molecular profiling of MRSA isolates collected from human patients in the hospital or community settings.Recent evidence suggests that MRSA isolates have developed increased resistance to vancomycin and clindamycin. Variable proportions of VRSA and VISA isolates have been reported earlier from tertiary care hospitals from North [3],[5] and South [1],[2],[22] India, including Hyderabad.[4] Worldwide, VISA isolates have been reported to be of different strain/clonal lineages in different countries. In India, a previous study [22] reported VISA to be associated with ST239/CC5, indicating a possible origin from the Brazilian clone with the same genotype, reported earlier.[22],[23] The two VISA isolates from our study displayed t12224/ST22/SCCmec IV and t037/ST239/SCCmec III, respectively. The former genetic composition has not been reported earlier, and we believe that this is a novel strain and is a possible variant of epidemic MRSA-15. The second isolate ST239/SCCmec III/t037 might be a variant of the Brazilian/Hungarian clone described previously.[24] It is possible that these strains originated from elsewhere, and spread to India.The proportion (23.3%) of clindamycin resistance of our isolates was similar to that reported from India and elsewhere;[25],[26],[27] however, the diversity was more than that reported earlier, where only ST239/SCCmec III/t037 was found to be associated.[28] The higher diversity in our strains could be due to dissemination of new clones and/or a result of indiscriminate use of antibiotics.Further analysis of our isolates indicated the existence of four clonal complexes CC8, CC22, CC30 and CC1. Slightly more than half of the isolates (56.6%) belonged to CC8, which includes ST8, ST239 and ST368. We found that ST8 and ST22 were the predominant (30% each) MRSA clones followed by ST239. Previous studies from various parts of India have reported the prevalence of ST1, ST5, ST6, ST20, ST22, ST30, ST45, ST109, ST113, ST120, ST238, ST239, ST368, ST772, ST1208 and ST2371 distributed among CC1, CC5, CC6, CC8, CC20, CC22, CC30, CC45 and CC121,[29],[30],[31],[32],[33],[34],[35],[36],[37] with the largest diversity reported from Chennai in a recent study.[29] Infections associated with ST772 have also been recorded in Irish neonatal ICU isolates, and have been attributed to connections with India through travel or ethnic background.[38]ST8 belongs to CC8 and primarily comprises of USA300, USA500 and Archaic and Iberian clones, which typically harbor t008/SCCmec IV, t064/SCCmec IV or II and ST8/t064, respectively.[39],[40] Our MRSA isolates belonged to ST8/t064 and were mainly SCCmec NT or V, indicating that these isolates are possible variants of the above-mentioned clones. As far as ST22 is concerned, our data of ST22/SCCmec IV/t005 (n = 5), t852 (n = 1) are in concordance with previous reports which indicated that it is the most prevalent type in tertiary care hospitals in India.[10],[37],[41] On the other hand, ST22/SCCmec IV/t12224 (n = 3) has not been reported previously. The other prominent ST from our study was ST239/t037, with SCCmec III (n = 6) or SCCmec I + III (n = 1), similar to those reported from outpatients and inpatients in hospital and community settings from various parts of India.[10],[24],[28]Our results indicate that the infectious isolates of ST22/t005 may be of same or similar clone/clones while ST8/t064 and ST239/t037 have variation in their clonal type. Further, the results indicate the diversity of infectious MRSA clones within a small group of patients. On the other hand, only three of our isolates did not belong to ST8, ST22 and ST239; two were ST580/t5122/SCCmec NT, and one each were ST30, ST368 and ST772/SCCmec V/t657. The latter has been reported as the predominant type in hospital settings of India by previous studies; on the other hand, the single ST30 isolate from our study belonged to t425/SCCmec V and did not conform to t021/SCCmec V as reported earlier.[10],[37],[42]PFGE analyses suggest that isolates within the major groups are from the same or similar strains or clones. ST22 isolates could be divided into one major cluster (K; four with t005 and one with t12224) and four singletons, (A/t12224, S/t12224, T/t852 and M1/t005, with ~ 60% similarity between S and T), and ST239/t037 isolates could be divided into two small clusters (F/SCCmec III, M/SCCmec III or I + III) and three singletons (D, Q, J, all with SCCmec III). The prevalence of singletons over the clusters shows the diversity in MRSA strains. Our observations on STs as well as PFGE types support the existence of a plurality of pulsed-field electropherotypes observed among isolates from various settings at various places from India.[30],[31],[43],[44],[45],[46],[47],[48]By SCCmec analysis, our isolates belonged predominantly to type IVC/H (n = 8; 26.7%) followed by III (n = 7), V (n = 5) and I + III (n = 1). This is on concordance with other studies, which have reported the predominance of types IV and V, followed by III and II, and low prevalence of type I in India.[29],[31],[33],[35],[36],[48],[49] The presence of two SCCmec types from a single isolate (double amplicon) has previously been reported, albeit with a frequency of 1.1% in one case and 73.58% in the other.[9],[50] A large proportion of our isolates were NT (n = 10, 33.3%), in contrast to the previous reports from India, wherein the frequency of NT isolates was 4% or 7.6%,[9],[24] while >80% Taiwanese isolates were found to be NT.[51] The high proportion of NT isolates may be due to clones endemic to the region, but a larger sample size from the South Asian region would be required to come to a definitive conclusion.In general, HA-MRSA carry SCCmec I, II and III while CA-MRSA carry IV and V. However, recent findings have blurred this distinction. Our HA-MRSA and CA-MRSA showed the predominance of SCCmec type IV and V, and type IV and III, respectively, whereas 1/3rd of the isolates were NT in each case. These findings are in concordance with previous reports on the frequency of types III, IV and V,[10],[24],[37],[52] and the penetration of SCCmec IV and V among HA-MRSA, and I and III among CA-MRSA [10] in India. Similar findings have also been reported from neighboring Asian countries.[53],[54] Surprisingly, majority of pvl positive isolates from our study were HA-MRSA (n = 11; 73.3%), and primarily harbored SCCmec IVC/H or V (40%; n = 4) followed by SCCmec III, V and NT (20%; n = 2 each). This further indicated the penetration of CA-MRSA characteristics, to HA-MRSA, as observed by others.[10]ST8 clones are distributed among both CA-MRSA and HA-MRSA.[55],[56] Only one of our ST8 isolate was CA-MRSA while the rest were HA-MRSA [Table 2]. Furthermore, only two of the ST8 isolates were pvl positive. ST8 MRSA and the related USA-300-like strains have not been reported frequently from India,[35],[36] and the emergence of ST8 in community, as well as hospital settings and the diversity among the clones, needs wider investigation. In addition, ST22, which is known to be prevalent in hospital settings, was also isolated, buttressing the reports of the spread of ST22 SCCmec IV from hospital to community settings and vice versa in India.[10]**Conclusion**The study demonstrates marked genetic diversity among MRSA isolates, along with resistance to vancomycin and clindamycin, within a small sample size, raising public health concerns. Additional studies with a larger sample size are required to analyse the genotypic and antibiotic resistance patterns among MRSA strains prevailing in the region. Whereas PFGE provides the highest discriminatory index [Table 4], owing to the requirement of an expensive equipment and analytical software, using either spa typing or MLST would be better for the analysis of diversity among isolates. Because seven different genes will need to be amplified and their sequence analysed for MLST, spa typing is the simplest for clonal analysis of S. aureus.Financial support and sponsorshipA small part of this work was accomplished through funds available from a grant (No. BT/PR11245/ADV/90/165/2014) from the Department of Biotechnology, Ministry of Science and Technology, Government of India, to NRH.Conflicts of interestThere are no conflicts of interest.References

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