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Molecular characterization of community- & hospital-acquired methicillin-resistant & methicillin-sensitive *Staphylococcus aureus* isolates in Sikkim

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***Background & objectives*: The two major genotypic markers that distinguish community acquired (CA) from hospital acquired (HA) methicillin resistant *Staphylococcus aureus* (MRSA) isolates are the architecture of mobile genetic element (SCC*mec* type) and presence of panton valentine leukocidin (PVL) toxin. This study was conducted to determine the molecular characteristics of CA- and HA- MRSA and methicillin sensitive *S. aureus* (MSSA) isolates in Sikkim.**

***Methods*: A total of 150 clinical isolates of *S. aureus* isolated from various clinical specimens were subjected to duplex (*mec-A* and *pvl* gene) and multiplex (SCC*mec* typing) PCR.**

***Results*: Of the 150 isolates, 53 (35.33%) and 66 (44%) were positive for *mec-A* (MRSA) and *pvl* genes, respectively. Thirty eight (25.33%) met the definition of CA-MRSA and 15 (10%) of HA-MRSA and the remaining 63 (42%) and 34 (22.66%) as CA- and HA-MSSA, respectively. No significant difference was seen in the distribution of PVL toxin in MRSA and MSSA isolates, but it was significantly (P<0.001) high in overall MRSA isolates than in MSSA. The majority of the MRSA isolates showed a double amplification band of SCC*mec* type III plus V (54.71%), and only a fewer isolates were amplified by single DNA fragments of type I (1.88%), III (3.77%), IVa (1.88%) and V (11.32%). SCC*mec* types I, III, IVa, were found only in HA-MRSA isolates, whereas type V in both the CA- and HA-MRSA. AST pattern showed that 18.42 per cent (7/38) and 46.66 per cent (7/15) were multidrug resistant (MDR)-CA-MRSA and MDR-HA-MRSA, respectively.**

***Interpretation & conclusions*: The present results show that SCC*mec* type V MRSA has been on the rise, and genotypic markers such as *pvl* gene detection used for the differentiation of these clinically distinct isolates of MRSA may not be reliable.**

Key words CA-MRSA - CA-MSSA - HA-MRSA - HA-MSSA - *pvl* gene - SCC*mec* element

The architecture of staphylococcal cassette chromosome *mec* (SCC*mec*) type and presence of panton valentine leukocidin (PVL) toxin are the two important genotypic markers that differentiate community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains from hospital-acquired (HA)-MRSA[1]. Based on SCC*mec* types, HA-MRSA usually carried large SCC*mec* element; typess I, II and III (34-67kb)[2] but CA-MRSA harboured newly described smaller SCC*mec* element type IV (24kb)[3] or less frequently V or a variant V_T[4]. The PVL toxin is widely associated with the presence of SCC*mec* types IV and sporadically with SCC*mec* type V or V_T but not with SCC*mec* type, I, II or III[1]. High association of PVL toxin in CA-MRSA strains is thought to have evolved via methicillin-sensitive *S. aureus* (MSSA) strains acquiring the *lukS-PV* and *lukF-PV* genes for PVL production and the resulting PVL positive MSSA gaining methicillin resistance through integration of the smaller, more mobile SCC*mec* types IV or V[1].

The present study was undertaken to compare the molecular characteristics of community- and hospital-acquired MRSA and MSSA isolates in Sikkim by detecting two major genotypic markers; presence of *pvl* gene and SCC*mec* type, and to determine the diversity of SCC*mec* type of MRSA strains circulating in this region.

Material & Methods

Settings and bacterial isolates: A total of 150 *S. aureus* isolates obtained from the various clinical specimens of blood (10), sputum (1), throat swab (1) and pus (138) submitted to the department of Microbiology of Sikkim Manipal Institute of Medical Sciences (SMIMS) and Sir Thutob Namgyal Memorial (STNM) hospital at Gangtok, Sikkim, during the period from September 2009 to March 2011, were studied. The study was approved by the ethics committee of SMIMS.

Confirmation and storage of S. aureus isolates: All these isolates were confirmed as *S. aureus* by using standard techniques[5]. The isolates were inoculated into the semi-solid nutrient agar and stored at -20ºC until further study.

Case definition : HA-MRSA was defined as one cultured from a clinical specimen obtained ≥72 h after patient's hospital admission or whose sources of isolation were associated with risk factors for HA-MRSA infection (*e.g*. recent hospitalization, recent surgery, residence in a long-term care facility, drug use)[6], within one year of MRSA isolation date. CA- MRSA isolate was defined as one cultured < 72 h of a patient's hospital admission, or whose sources of isolation were not associated with risk factors for HA-MRSA infection. In a similar manner HA- and CA-MSSA were defined[8].

Exclusion criteria: Duplicate isolates from the same patients, even if the site of infection was different during the sample collection time frame were excluded from the study.

DNA isolation: The DNA was extracted by using the HiPurA™ Bacterial and Yeast Genomic DNA Miniprep Purification Spin kit (Hi-Media, Mumbai) as per the manufacturer's instructions.

Duplex PCR for detection of mec-A and pvl genes: The primer pairs (Desalted) for *mec-A* and *pvl* genes were taken from the published sequence by Oliveria *et al*[7] and McClure *et al*[8], respectively. Primers were blasted and commercially obtained from Sigma-Aldrich Pvt. Ltd., Bangalore. PCR was performed by using Multiplex PCR kit (Qiagen, Hilden, Germany) with slight modification of final reaction volume of 25 µl (12.5 µl mastermix, 2.5 µl primer mix, 3µl of DNA template and 7µl of RNase-free water). Thermocycling conditions and visualization of products were done as per the manufacturer's instructions. Reference strains ATCC 43300 and 25923 were used as positive and negative controls for *mec-A* gene, respectively and ATCC 43300 was used as negative control for *pvl* gene.

Multiplex PCR:

Primers - Primers were selected from the published sequence of the SCC*mec* types I-III (Oliveria *et al*)[7], IVa -IVb (Okuma *et al*)[9], IVc-IVd (Hisata *et al*)[10], V (Zang *et al*)[11] and were commercially obtained from Sigma-Aldrich Pvt., Ltd., Bengaluru.

Preparation of primer mix - The preparation of primer mix was done as per manufacturer's instructions of multiplex PCR kit (Qiagen). For multiplex PCR, the primers were divided into two sets: set A was designed to amplify SCC*mec* type I, II, III, V and *mec-A*, whereas set B was designed to amplify SCC*mec* IVa, IVb, IVc, IVd and *mec-A*. The *mec-A* gene was included in the protocol as an internal positive control.

SCCmec typing - To ensure the individual primer pairs were adequate for the amplification of all loci (gene fragments), the single-target PCR protocol[11] with each individual primer pair was conducted prior to the multiplex PCR optimization. Then PCR was performed by using Qiagen Multiplex PCR kit with slight

modification. Reaction was run in two sets with two different sets of primers mix. Multiplex PCR with set A primers consisting of 12.5 µl mastermix, 2.5 µl primer mix (set A), 3µl of DNA template and 7µl of RNase-free water with total reaction volume of 25µl. Multiplex primer set B included the same constituents as in set A except for the primers mix (set B). Thermocycling conditions and visualization of products were done as per the manufacturer's instructions.

Antibiotic susceptibility testing of MRSA isolates- Kirby-Bauer disc diffusion method[12] was performed with following antibiotics discs (Hi-Media, Mumbai); penicillin-G (10 units), co-trimoxazole (25 µg), erythromycin (15 µg), ofloxacin (5 µg), gentamicin (10 µg), linezolid (30 µg/ml), rifampicin (5 µg), chloramphenicol (30 µg), fusidic acid (30 µg). Five discs in one and four in another agar plate were tested. The testing conditions and interpretation of the test was done as per Clinical and Laboratory Standards Institute (CLSI) criteria[13]. MRSA isolates resistant to ≥ three non-beta lactam antibiotics were classified as multidrug-resistant MRSA (MDR-MRSA)[14].

Statistical analysis: Categorical variables were analyzed using Chi-square and Fisher's exact tests using IBM SPSS Statistics 20 (IBM Corporation, USA).

The ethical clearance was taken from the Institutional ethics committee, SMIMS.

Results

Duplex PCR: Out of 150 *S. aureus* isolates, 53 (35.33%) and 66 (44%) showed amplifications with *mec-A* and *pvl* genes, respectively (Fig. 1). Among 150 *S. aureus* isolates, 38 (25.33%) met the definition of CA-MRSA and 15 (10%) of HA-MRSA and rest of CA-MSSA and HA-MSSA (Table I). All MRSA isolates were positive for *pvl* barring two HA-MRSA isolates. Among MSSA, 12.37 per cent (12/97) and 3.09 per cent (3/97) were *pvl* positive CA-MSSA and *pvl* positive HA-MSSA, respectively. The presence of PVL toxin was significantly higher (P<0.001) in overall MRSA population (77.27% or 51/66) than that in MSSA (22.72% or 15/66).

SCCmec typing: SCC*mec* typing patterns of MRSA isolates (Figs 2 and 3), and its distribution in two groups of MRSA (CA- and HA-MRSA) is shown in Table II. All MRSA isolates tested were found to be positive for internal control (*mec-A*) included in every batch of testing. The SCC*mec* types distributions in HA-MRSA patients were as follows: *(i)* patients developed

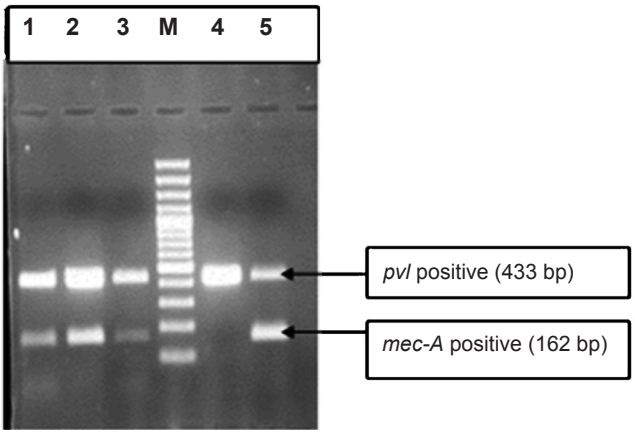

Fig. 1. Duplex PCR (*mec-A* and *pvl* gene). Lanes 1,2,3,5 = Positive *mec-A* (162bp) and *pvl* (433bp), M= Marker (100bp DNA ladder), Lane 4 =Negative *mec-A* (162bp) positive *pvl* (433 bp).

infections ≥ 72 h of hospital admission (n=3), one each type I, II and II+III *(ii)* patients with previous history of hospital admission (n=9), one each type III, IVa, V, II+III, non-typeable and four isolates harboured combination band of III+V *(iii)* patients with previous history of antibiotic intake (n=3), III+V (two) and non-typeable (one).

Antimicrobial susceptibility pattern of MRSA isolates: The results of *in vitro* susceptibility testing of CA- and HA-MRSA isolates are given in Table III. High percentage of CA-MRSA (92.10%) and all isolates of HA-MRSA were resistant to penicillin and co-trimoxazole. Among CA- and HA-MRSA, 18.42 per cent (7/38) and 46.66 per cent (7/15) were found to be multi-drug resistant (MDR), respectively. SCC*mec* types of MDR-HA-MRSA isolates were as follows: type I (one), III (two), V (one), II+III (one) and III +V (two). Similarly, SCC*mec* types of MDR-CA-MRSA were type V (two), II+III (one), II+V (two) and III+V (two).

Table I. Distribution of *pvl* gene in CA- and HA-MRSA and MSSA isolates

S. aureus group	Total	*pvl* positive (%)	*pvl* negative (%)
CA-MRSA	38	38 (100)	0 (0)
HA-MRSA	15	13 (86.66)	2 (13.33)
CA-MSSA	63	12 (19.4)	51 (80.95)
HA-MSSA	34	3 (8.82)	31 (91.17)
Total	150	66 (44)	84 (56)

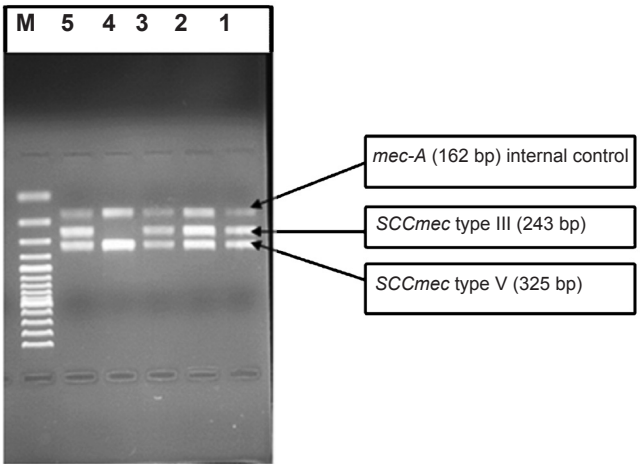

Fig. 2. SCC*mec* typing of MRSA isolates. Lanes 1,2,3,5 = *mec-A* (162 bp), SCC*mec* type III (243 bp) and type V (325 bp) positive. Lane 4= *mec-A* (162bp) and SCC*mec* type V (325 bp) positive.

Discussion

In the present study, we evaluated the presence of PVL toxin as a marker of CA-MRSA isolates. Several studies have reported the *pvl* as a reliable marker for CA-MRSA strains[15,16] and a few studies[17,18] have reported the presence of the PVL toxin more in MSSA than MRSA. Our finding is in agreement with the reports from Ireland[19] and Finland[20].

In this study, SCC*mec* typing revelead that MRSA isolates with double DNA fragment bands (73.58%) were much higher than single fragment (18.86%), majority (54.71%) had the combination of SCC*mec* type III plus V. Similarly, Zang *et al*[11] reported the presence of double bands in 1.1 per cent of clinical isolates of MRSA of these combinations; SCC*mec* type III +IVc (one), type I+II (two) and type II+IVc (two isolates). The percentage of non-typeable MRSA isolates in our study was 7.54 per cent, which was higher than the earlier reports from Korea (1.35%)[21], Canada (1.77%)[11], India (4%)[22]. In contrast, a study from Taiwan reported non-typeable MRSA as high as 80.95 per cent[23].

SCC*mec* type III has been reported to be the predominant MRSA in Asian continent except Korea and Japan[22,25]. The present study showed low occurrence of type III MRSA isolates in Sikkim (3.8%) compared to that reported from other parts of India[22, 25-27]. D'Souza *et al*[16] from Mumbai, India, reported that SCC*mec* type V (41.01%) was higher than type III (24.55%). Our study suggests that SCC*mec* type V seems to be an emerging MRSA in this part of India.

In our study only one HA-MRSA isolate was found to be positive for type IVa. On the contrary, a study from Mumbai, India, reported presence of SCC*mec* type IV in 34 per cent of MRSA isolates collected during a three year period. In earlier studies[24,27] conducted on the MRSA isolates collected from Indian hospitals have not reported type IV MRSA isolates.

The association of specific SCC*mec* type in CA- and HA-MRSA isolates has been well characterized, and formed the basis of molecular definition[1]. In the present study, the SCC*mec* types IV and V which are typically associated with CA-MRSA, were also found in HA-MRSA isolates. Yu *et al*[28] reported type IV in HA-MRSA isolates (12.5%). Gonzalez *et al*[29] also reported CA-MRSA isolates (SCC*mec* type IV) as an important cause of healthcare-associated infections. This indicates that HA-MRSA with typical molecular characteristics of CA-MRSA (SCC*mec* type IVa, V

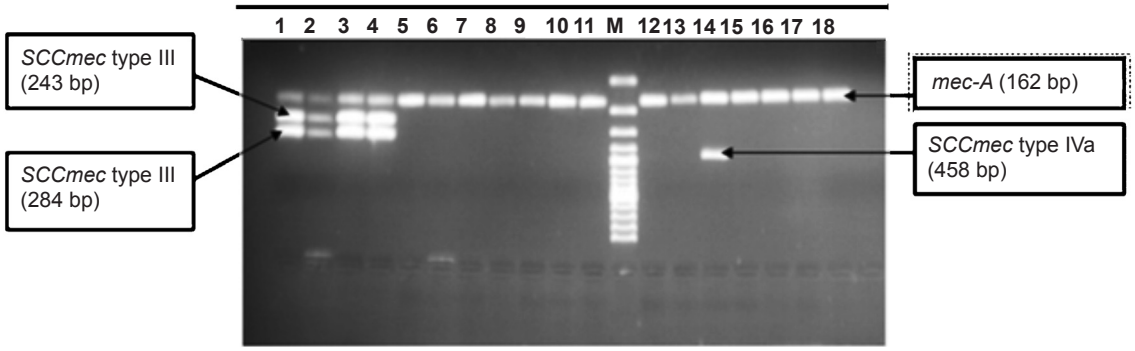

Fig. 3. SCC*mec* typing of MRSA isolates. Lanes 1,2,3,4 (reaction mixture of Set A primer mix run): Positive for *mec-A* (162 bp), SCC*mec* type II (284 bp) and type III (243 bp). Lane 5-18 (reaction mixture of Set B primer mix run)= Positive for *mec-A* gene. Lane 14= Positive for *mec-A* (162bp) and SCC*mec* type IVa (458 bp).

Table II. SCC*mec* type of CA- and HA-MRSA isolates

MRSA group	SCC *mec* type No. (%)									
	I	II	III	IVa	V	II+III	II+V	III+V	NT	Total
CA-MRSA	0	0	0	0	5 (13.15)	2 (5.26)	6 (15.78)	23 (60.52)	2 (5.26)	38
HA-MRSA	1 (6.66)	1 (6.66)	2 (13.33)	1 (6.66)	1 (6.66)	2 (13.33)	0	6 (40)	2 (13.33)	15
Total	1 (1.88)	1 (1.88)	2 (3.77)	1 (1.88)	6 (11.32)	1 (7.54)	6 (11.32)	29 (51.71)	4 (7.54)	53

NT, non-typeable (amplification with *mec-A* gene only)

and PVL positive) have emerged as an important cause of healthcare-associated infections. Therefore, differentiation of MRSA based on these genotypic markers would not be reliable in near future. However, these findings need to be validated on a large number of isolates.

Apart from genotypic markers, MRSA is also categorized based on the susceptibility pattern to various antibiotics[30]. Based on this definition, CA-MRSA has wider spectrum of susceptibility to antibiotics compare to HA-MRSA[31] and multidrug resistance is a phenotypic marker for nosocomial strain[14]. In support of this a study has reported 33 per cent of MRSA isolates as MDR-MRSA, where CA-MRSA isolates were less likely to be resistance to antibiotics than HA-MRSA isolates[14]. Fey *et al*[31] reported 87.5 per cent of HA-MRSA as MDR, whereas no MDR was found among the CA-MRSA isolates. In our study also, occurrence of MDR-MRSA was more in HA-MRSA than CA-MRSA.

In conclusion, *pvl* may no longer be a reliable marker for CA-MRSA isolates, rather all MRSA may be important reservoir of PVL toxin, and SCC*mec* type V MRSA is emerging as predominant isolates over most prevalent type III/IIIA. Multidrug resistance seen among CA-MRSA indicates the changing epidemiological and microbiological characteristics of MRSA in the community and hospitals in this part of India.

Table III. Antibiotic resistance pattern of CA- and HA-MRSA isolates

Antibiotic (µg/ml)	CA-MRSA (n=38)	HA-MRSA (n=15)
Penicillin (10)	35 (92.10)	15 (100)
Co-trimoxazole (25)	35 (92.10)	15 (100)
Erythromycin (15)	8 (21.05)	5 (33.33)
Ofloxacin (5)	9 (23.68)	3 (20)
Gentamicin (10)	5 (13.15)	3 (20)
Linezolid (30)	4 (10.52)	3 (20)
Chloramphenicol (30)	1 (2.63)	2 (13.33)
Fusidic acid (30)	1 (2.63)	0
Rifampicin (5)	1 (2.63)	0

Values in parentheses are percentages

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