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Staphylococcal Cassette Chromosome *mec* (SCC*mec*) typing of clinical isolates of coagulase-negative staphylocci (CoNS) from a tertiary care hospital in New Delhi, India

Sir,

Coagulase-negative staphylococci (CoNS) have emerged as significant aetiological agent of health-care associated infections, particularly in patients with indwelling devices, prosthesis and implants and those subjected to invasive medical procedures. Rise in the incidence of infections with CoNS has also been attributed to the increase in hospitalized immunocompromised patients[1]. Methicillin resistance, mediated by *mec*A gene encoded low affinity penicillin-binding protein (PBP) 2a, has been observed in high proportion of CoNS isolates. Accurate detection of methicillin resistance among CoNS isolates is an important factor in guiding antibiotic therapy. The *mec*A gene is a part of the mobile genetic element Staphylococcal Cassette Chromososme *mec* (SCC*mec*), which acts as a vehicle for horizontal transfer of antibiotic resistance genes to organisms like *Staphylococcus aureus*[1,2]. Hospital-acquired infection associated methicillin resistant CoNS (MR-CoNS) serve as a reservoir of genetically diverse SCC*mec* types, each type being associated with a different antibiotic resistance pattern. The distribution of SCC*mec* types varies with host species of CoNS and with geographical locations[2]. Molecular characterization by SCC*mec* typing of MR-CoNS is an essential epidemiological tool for studying the evolution of these genetic elements and providing useful information regarding the antibiotic resistance pattern in staphylococci. There are limited studies reported from India on speciation and antibiotic susceptibility testing of MR-CoNS by phenotypic methods[3,4]. Thus, the present study was undertaken to determine the distribution of SCC*mec* types in MR-CoNS, isolated from patients admitted to a tertiary care hospital in north India and study its relation with antibiotic resistance.

The study was conducted in the Bacteriology laboratory, department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi, India. The study protocol was approved by the Ethics committee of the Institute. The study included 124 consecutive non-duplicate isolates of CoNS obtained from clinical specimens of patients admitted in various wards between May and October, 2012. Criteria for inclusion of a blood culture isolate of CoNS as clinically significant were isolation of the same strain in pure form from multiple blood culture samples of the same patient taken at different points of time or in presence of central venous catheters (CVCs), isolation of the same strain from culture of catheter segment and peripheral blood along with at least two of the following clinical criteria in absence of evidence of infection at any other site: temperature >38°C or <36°C; heart rate >90/min; respiratory rate >20/min; leucocyte count >12000/µl or <4000/µl or >10 per cent immature neutrophil granulocytes. For CoNS isolated from samples other than blood culture, only strains, which were isolated as pure growth from clinical samples of a patient, more than once, were considered as significant pathogens and included in the study[5].

The CoNS isolates obtained from soft-tissue infection site and pus samples, blood and bones on culturing onto 5 per cent sheep blood agar plates, were screened for CoNS on the basis of colony morphology, Gram stain reaction, catalase, tube and slide coagulase tests. Species identification of CoNS was done based the panel of biochemical reactions described by Kloos

and Bannerman[6]. All CoNS isolates were subjected to antimicrobial susceptibility testing on Mueller-Hinton agar using the Kirby-Bauer disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI)[7] for the following antibiotics: penicillin (10 units), amoxicillin-clavulanic acid (20/10 µg), gentamicin (10 µg), amikacin (30 µg), doxycycline (30 µg), trimethoprim-sulphamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), erythromycin (15 µg), clindamicin (2 µg), and linezolid (30 µg). Susceptibility to vancomycin was performed by microbroth dilution taking the minimum inhibitory concentration (MIC) interpretative criteria of ≤4, 8-16 and ≥ 32 µg/ml as sensitive, intermediate and resistant, respectively[7]. *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 and *S. epidermidis* ATCC 12228 were used as control strains.

Methicillin resistance in CoNS was determined by disc diffusion method using a cefoxitin disc (30 µg) with the interpretative criteria ≤ 24 mm (resistant) and ≥ 25 mm (susceptible) and by *mec*Agene PCR using the published primers *mecA* P4 (5'TCCAGATTACAACTTCACCAGG3') and *mecA* P7 (5'CCACTTCATATCTTGTAACG3')[8]. *S. aureus* ATCC 33591 and *S. epidermidis* ATCC 12228 were used as positive and negative controls for PCR, respectively. Inducible resistance to clindamycin in erythromycin-resistant CoNS isolates was detected by D-test as per CLSI guidelines[7]. Multi-drug resistance was defined as resistance to three or more classes of antibiotics including β-lactams.

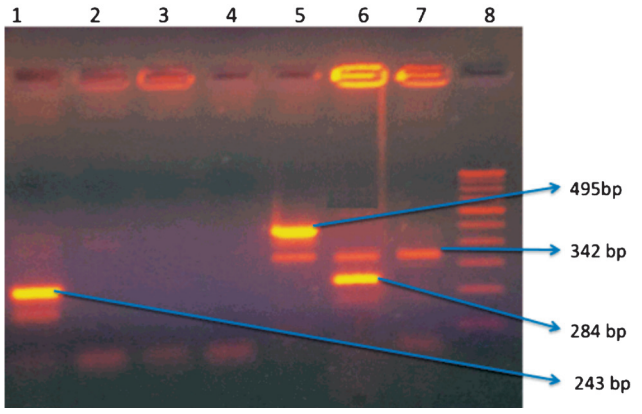

Figure. Multiplex PCR for SCC*mec* typing of CoNS. Lane 1: CoNS isolate harbouring SCC*mec* type III (243 bp). Lanes 2-4: CoNS isolates negative for *mec*A. Lane 5: CoNS isolate harbouring SCC*mec* type I (342 & 495 bp). Lane 6: CoNS isolate harbouring SCC*mec* type II (342 & 284 bp). Lane 7: CoNS isolate harbouring SCC*mec* type IV (342 bp). Lane 8: 100 bp DNA ladder.

Isolates positive for *mec*A were further subjected to SCC*mec* typing (I-IV) by multiplex PCR which included four pairs of primers for locus A (exclusive to SCC*mec* type I), locus B (exclusive to SCC*mec* type II), locusD (common to SCC*mec* types I, II and IV) and locus E (exclusive to SCC*mec* type III) as described by Oliveira *et al*[8]. Identification and differentiation of SCC*mec* types was based on the separation of amplicons by agarose gel electrophoresis of the multiplex PCR products (Figure). The following *S. aureus* strains were used as controls for SCC*mec* typing: 665U (type I), UK EMRSA-16 (type II), UK EMRSA-1 (type III) and UK EMRSA-15 (type IV) (kindly provided by Dr Edet E. Udo, University of Kuwait, Kuwait).

Potential risk factors for CoNS infection in hospitalized patients were evaluated by reviewing the medical records. Statistical analyses were performed using STATA version 11.2 software (STATA Corp LP, College Station, TX, USA) using the Chi-square test.

Of the 124 CoNS isolates, 79 (63.7%) were recovered from infected surgical site soft tissue samples and post-operative wound discharge/pus specimens, followed by blood (43, 34.7%), and bone tissues (2, 1.6%). Seven species accounted for all CoNS isolates: *S. haemolyticus* (n=45, 36.3%), *S. epidermidis* (n=23, 18.5%), *S. schleiferi* subspecies *coagulans* (n=18, 14.5%), *S. schleiferi* subspecies *schleiferi* (n=14, 11.3%), *S. warneri* (n=12, 9.7%), *S. capitis* (n=11, 8.9%) and *S. intermedius* (n=1, 0.8%).

Resistance to methicillin in CoNS was detected in 44 (35.5%) isolates by cefoxitin disc diffusion test. All these CoNS isolates also tested positive for *mec*A gene by PCR. All methicillin sensitive (MS-CoNS) isolates by cefoxitin disc diffusion assay were negative for *mec*A gene PCR.

Rates of methicillin resistance among all species of MR-CoNS were highest in *S. haemolyticus* (n=29, 64.4%) followed by *S. epidermidis* (n=6, 26.1%) and *S. schleiferi* (n=6, 18.7%) isolates. MR-CoNS showed higher level of resistance to all non β-lactam antimicrobials tested as compared to MS-CoNS, difference being significant for amikacin (18.2 vs 6.2%, P=0.04), ciprofoxacin (68.2 vs 37.5%, P<0.01) and clindamycin (36.4 vs 15%, P<0.01). Multi-drug resistance was observed in 72.7 per cent (32/44) of MR-CoNS isolates and was most frequently detected among methicillin-resistant *S. haemolyticus* isolates. Of all CoNS isolates, highest rates of antibiotic resistance were observed in *S. hemolyticus*. Resistance

to erythromycin, ciprofloxacin, clindamycin, co-trimoxazole, doxycycline, amikacin and gentamicin were present in 51.1, 48.9, 31.1, 19.6, 20.0, 15.6 and 4.4 per cent of all *S. haemolyticus* isolates, respectively.

All CoNS isolates included in our study were susceptible to vancomycin and linezolid. All MS-CoNS were uniformly susceptible to penicillin and amoxicillin-clavulanic acid. None of erythromycin-resistant CoNS isolates expressed inducible resistance to clindamycin.

The 44 MR-CoNS isolates were tested for SCC*mec* types using the multiplex-PCR for SCC*mec* types I, II, III and IV and 27 (61.4%) isolates were identified with SCC*mec* type I, three (6.8%) with SCC*mec* type II, seven (15.9%) with SCC*mec* type III and five (11.4%) with SCC*mec* type IV. Two (4.5%) isolates of MR-CoNS were not typable using this method. Approximately 60 per cent of SCC*mec* type I positive CoNS isolates (16/27) belonged to *S. haemolyticus* species. The distribution of SCC*mec* types among methicillin-resistant *S. haemolyticus*, the most frequently isolated MR-CoNS species, indicated that 55.2 per cent harboured SCC*mec* I, 20.7 per cent SCC*mec* III, 13.8 per cent SCC*mec* IV and 6.9 per cent SCC*mec* II (Table I).

An analysis of resistance rates was performed for the susceptibility profile of MR-CoNS to non-β-lactam antimicrobials among different SCC*mec* types (Table II). It was observed that isolates with SCC*mec* type I, the most frequently identified SCC*mec* type, exhibited high rates of resistance to ciprofloxacin, rifampicin, erythromycin, clindamycin, doxycycline and co-trimoxazole (70.3, 59.2, 51.7, 37.1, 33.3 and 25.9% of isolates, respectively).

No significant association was observed between isolation of MR-CoNS and variables like duration of mean hospital stay, presence of prosthetic devices and intravenous catheters and history of admission to intensive care units (ICUs).

In our study, *S. haemolyticus* was the commonest species of CoNS followed by *S. epidermidis*. This is in contrast to studies from developed countries where *S. epidermidis* is the most frequently isolated species of CoNS from hospitalized patients, thereby highlighting the fact that epidemiology of health-care associated

Table I. Distribution of MR-CoNS according to sample types and distribution of SCC*mec* type among different MR-CoNS species (n=44)

Distribution of MR-CoNS according to sample types						
	Infected soft tissue sample & pus/ discharge (n=29)		Blood (n=15)			
Total no. of MR-CoNS isolates	Surgical site infection/peri-operative wound discharge	Infected tissues from prosthetic device infection patients	Bone-marrow transplant recipients	Patients undergoing chemotherapy	Post-surgical patients admitted to ICU	Patients admitted to ICU due to medical reasons other than malignancy
44	18 (40.9)	11 (25)	1 (2.3)	8 (18.2)	3 (6.8)	3 (6.8)
Distribution of SCC*mec* types between MR-CoNS species						
SCC*mec* types (no. of isolates)	*S. haemolyticus* (n=29)	*S. epidermidis* (n=6)	*S. schleiferi* (n=6)	*S. capitis* (n=2)	*S. warneri* (n=1)	
I (27)	16 (55.2)	5 (83.3)	5 (83.3)	1 (50)	0 (0)	
II (3)	2 (6.9)	0 (0)	0 (0	1 (50)	0 (0)	
III (7)	6 (20.7)	0 (0)	0 (0)	0 (0)	1 (100)	
IV (5)	4 (13.8)	1 (16.7)	0 (0)	0 (0)	0 (0)	
Non-typable (n=2)	1 (3.4)	0 (0)	1 (16.7)	0 (0)	0 (0)	

Percentages within brackets denote proportion of MR-CoNS isolates of a particular species harbouring a given SCC*mec* type

Table II. Distribution of SCC*mec* types in MR-CoNS isolates (n=44) according to resistance pattern to non β-lactam antibiotics

Antibiotics	Type I (n=27)	Type II (n=3)	Type III (n=7)	Type IV (n=5)	Non-typable (n=2)
Amikacin	4 (14.8)	1 (33.3)	2 (28.6)	1 (20.0)	0 (0)
Gentamicin	2 (7.4)	0 (0)	1 (14.3)	0 (0)	0 (0)
Ciprofloxacin	19 (70.3)	3 (100)	5 (71.4)	2 (40.0)	1 (50.0)
Clindamycin	10 (37.1)	2 (66.7)	0 (0)	3 (60.0)	1 (50.0)
Co-trimoxazole	7 (25.9)	0 (0)	2 (28.6)	1 (20.0)	1 (50.0)
Erythromycin	15 (51.7)	2 (66.7)	1 (14.3)	4 (80.0)	1 (50.0)
Doxycycline	9 (33.3)	0 (0)	2 (28.6)	2 (40.0)	1 (50.0)
Rifampicin	16 (59.2)	2 (66.7)	1 (14.3)	2 (40.0)	1 (50.0)

Percentages within brackets denote proportion of MR-CoNS of one particular SCC*mec* type resistant to a given antibiotic.
Since one isolate of a given SCC*mec* type can be resistant to more than one antibiotic, percentages within brackets do not add to give a value of 100

CoNS infections varies according to geographical locales[2,9]. Our finding was in concordance with a previous study from north India where *S. haemolyticus* was the commonest species of CoNS isolated from blood cultures[4]. The high prevalence of *S. haemolyticus* could be related to its ability to adapt to selective pressures such as antimicrobials and biocides present in the hospital environment[10]. Hospital-acquired CoNS act as reservoir of antibiotic resistance genes, with increase in resistance rates to multiple antibiotics being reported all over the world[11]. Our study also highlights a high prevalence of multi-drug resistance CoNS with clear dominance of resistance in *S. haemolyticus*.

In the present study, methicillin resistance was detected in a high percentage of CoNS isolates (34.4%). High rates of methicillin resistance ranging from 52 to 66 per cent in Indian isolates of CoNS from patients with health-care associated infections have been previously reported[3,12]. These studies have also reported high rates of penicillin resistance in MS-CONS isolates (81-100%). In contrast, none of our MS-CoNS isolates showed resistance to penicillin and amoxicillin-clavulanic acid, implying that these drugs remained the preferred antibiotics for treating infections with MS-CoNS in our setting. The difference in penicillin resistance in MS-CoNS isolates can be explained by the variations in antibiotic selection pressure caused by different antibiotic policies in different hospital settings.

In our study, MR-CoNS isolates were significantly more resistant to ciprofloxacin, clindamycin and amikacin than MS-CoNS isolates. In addition, more than 70 per cent of MR-CoNS isolates were multi-drug resistant. None of our CoNS isolates were resistant to vancomycin and linezolid. Given the high proportion of MR-CoNS resistant to the different non-β-lactam antibiotics, vancomycin and linezolid should be included in empirical therapy for patients with MR-CoNS infections. However, empirical usage of vancomycin and linezolid should be avoided in treating CoNS infections before recording results of methicillin resistance testing, since overusage of these antibiotics will lead to emergence of glycopeptide and oxazolidinone resistant CoNS strains. CoNS isolates with linezolid resistance and decreased susceptibility to vancomycin have already been reported[13].

In our study, *S. haemolyticus* was the most frequent MR-CoNS with >60 per cent being methicillin resistant. Barros *et al*[14] also observed that 87 per cent of *S. haemolyticus* isolates from various clinical samples were methicillin resistant. High methicillin resistance rates ranging from 75-90 per cent in *S. haemolyticus* isolates from blood cultures have also been reported[15]. High level of antimicrobial resistance in *S. haemolyticus* has been attributed to the significant number of insertion sequence elements, which represent hotspots for acquisition of antibiotic resistance genes[15].

Among all MR-CoNS isolates, SCC*mec* type I was the commonest followed by SCC*mec* type III and 55 per cent of *S. haemolyticus* isolates harboured SCC*mec* type I. SCC*mec* types I and III have been previously reported as the predominant SCC*mec* types in hospital-acquired isolates of MR-CoNS[2]. SCC*mec* type III is harboured predominantly in methicillin-

resistant *S. epidermidis* isolates and SCC*mec*type I is more common in methicillin-resistant *S. haemolyticus* isolates[16]. The predominance of SCC*mec* type I in our isolates could be attributed to the higher number of methicillin-resistant *S. haemolyticus* isolates compared to methicillin-resistant *S. epidermidis*. Low proportion of isolates for SCC*mec* elements such as IV can be explained by the fact that all our isolates were from patients with hospital-acquired infections and SCC*mec* type IV is predominantly detected in community-acquired MR-CoNS isolates.

The present study had limitations. The multiplex PCR used for SCC*mec* typing was capable of detecting only SCC*mec* types I-IV. With evolution of genetically diverse newer SCC*mec* elements, more than ten SCC*mec* types have now been reported in CoNS[2]. Non-typability of two isolates in our study was probably due to the fact that they harboured SCC*mec* genes different from types I-IV. Previous studies have demonstrated that majority of clinically significant MR-CoNS isolates from patients with health-care associated infections harbour SCC*mec* types I-IV[17-19]. In a study from Turkey, it was observed that more than 70 per cent of MR-CoNS isolates from blood culture harboured SCC*mec* types I-IV either alone or in combination[18]. Characterization of SCC*mec* gene of MR-CoNS isolates from a neonatal intensive care unit in Brazil also demonstrated a high prevalence of SCC*mec* types I-IV with an overall predominance of SCC*mec* type I[17].

It has been have shown that the frequency of SCC*mec* among CoNS is increasing due to the wide dissemination of the microorganism in hospital-acquired infections, which is associated with the potential genetic transfer of resistance elements among *S. aureus*[11]. We observed a high frequency of SCC*mec* I and III in our isolates. Majority of MRSA clinical isolates, that have been previously characterized in our hospital, possessed SCC*mec* type III and were significantly more resistant to antibiotics[19].

In conclusion, presence of a particular SCC*mec* type inhospital-acquired CoNS can be a predictor of antibiotic resistance pattern of the isolates. Since the pattern varies from one health-care setting to another, molecular typing of this genetically diverse group of organisms and establishing an association with antibiotic resistance need to be individualized for each laboratory where genotyping facilities are available. This can generate useful data elaborating the molecular epidemiology of nosocomial CoNS isolates particularly in countries like India where genotyping studies for drug-resistant CoNS are lacking. Since CoNS are capable of transmitting drug-resistance genes to organisms like *S. aureus*, molecular typing in adequately large number of CoNS isolates and prospectively monitoring their drug resistance can be helpful in designing antibiotic policies and hospital infection control strategies.

***Conflicts of Interest*:** None.

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