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Molecular & phenotypic characterization of *Staphylococcus epidermidis* in implant related infections

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***Background & objectives*: The discrimination between the *Staphylococcus epidermidis* colonizing the deep seated indwelling devices and those which are mere commensals has always been a challenge for the clinical microbiologist. This study was aimed to characterize the *S. epidermidis* isolates obtained from device related infection for their phenotypic and molecular markers of virulence and to see whether these markers can be used to differentiate the pathogenic *S. epidermidis* from the commensals.**

***Methods*: Fifty five *S. epidermidis* isolates from various device related infections such as endophthalmitis following intra-ocular lens (IOL) implantation, intravascular (IV) catheter related sepsis and orthopaedic implant infections, were studied for slime production, biotyping, antibiotic sensitivity; and *mec* A and *ica* positivity by the recommended procedures.**

***Results*: Twenty three (41.8%) isolates were multi-drug resistant, 26 (65.2%) were slime producers, 30 (54.5%) were adherent, 23 (41.8%) possessed the intercellular adhesin (*ica*) gene, and 28 (50.9%) harboured the *mec* A gene. Biotypes I and III were the commonest, most members of which were multi-drug resistant. Twenty two (73.3%) of the 30 adherent bacteria were slime producers as opposed to only 4 (16%) of the 25 non-adherent bacteria ($P<0.001$). A vast majority *i.e.* 21 (91.3%) of the 23 *ica* positive organisms were adherent to artificial surfaces in contrast to only 9 (28.1%) of the 32 non-*ica* positive organisms ($P<0.001$). Twenty (86.9%) of the 23 *ica* positive bacteria were slime producers, as opposed to only 6 (18.7%) of the 32 *ica* negative bacteria ($P<0.001$). Of the 23 multi-drug resistant isolates, 19 (82.6%) carried the *mec* A gene.**

***Interpretation & conclusions*: The present findings showed that *ica* AB and *mec* A were the two important virulence markers of *S. epidermidis* in implant infections and slime was responsible for the sessile mode of attachment on the devices.**

Key words Adherence - Biofilms - device associated infections - implant related infections - multi drug resistance - slime - *Staphylococcus epidermidis*

During the last two decades, *Staphylococcus epidermidis* and other coagulase negative staphylococci (CoNS) have emerged as major causative agents of nosocomial infections[1]. These organisms, which constitute the main component of the normal skin and mucosal microflora, are particularly responsible for catheter and other medical device related infections[1,2]. The pathogenesis of *S. epidermidis* in device associated infections mostly relies on the potential of the bacterium to adhere to the device surface[3,4].

Clinical and laboratory evidences support the view that significantly higher numbers of slime producing *S. epidermidis* isolates are adherent to artificial surfaces than the slime negative isolates[5-7]. Recent observations also documented that cell to cell aggregation and biofilm formation, subsequent to the bacterial attachment to the device were mediated by a component of slime *i.e.* the polysaccharide intercellular ahesin (PIA), which was encoded by the chromosomal '*ica*' gene locus[7]. Besides the above virulence factors that are involved in the process of adherence and biofilm formation, the emergence of multidrug resistance, including methicillin resistance amongst the nosocomial *S. epidermidis* is a major threat to the clinician for the patient management[8].

Thus, the purpose of the present study was to characterize the *S. epidermidis* isolates obtained from device related infections in terms of their phenotypic and molecular markers of virulence and to investigate if these markers would differentiate the *S. epidermidis* that cause device infections from those that are mere commensals, and to address if such characterization would eventually benefit in making clinical decisions to ascribe a particular isolate as a pathogen and not merely as a commensal.

Material & Methods

Study design: The study was conducted in the Department of Ocular Microbiology, Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi. The protocol was approved by the ethics committee of the Institute. A total of 91 patients (29 females and 62 males) ranging in the age group of 29 to 67 yr, all with indwelling implant related infections were included in the study during the period from March 2008 to June 2010, after obtaining the informed consent from the subjects.

Subjects and case definitions: Vitreous samples were collected from 50 subjects with post-operative endophthalmitis following intra-ocular lens (IOL) implantation. Intravascular catheters/canulas were collected from 24 patients with catheter related sepsis, and pus/wound aspirates were taken from 17 subjects having infections due to implanted orthopaedic devices. Additionally, blood samples were collected from 3 of the 24 patients with catheter infections and from 8 of the 17 with orthopaedic implant infections amounting to a total of 102 samples from 91 patients (Table I).

Individuals with features of endophthalmitis such as increasing pain and redness, decreasing visual acuity, flare in the anterior chamber, corneal oedema, hypopyon, and poor glow within 4-6 wk following IOL implantation were termed as the cases of late onset post-operative endophthalmitis (POE)[9]. The 24 patients with indwelling central venous catheters/intravascular canulas had clinical evidences of infection (catheter related sepsis) characterized by fever >38^{o}C, pulse rate of more than 90/min, respiration rate of more than 20/min and WBC count of >12000/µl of blood. In addition, subjects with localized infection at the exit of the truncated tract were also considered as having catheter related infections[10].

Those having either localized inflammatory signs at the implant site or signs of sepsis, as defined above, owing to implantation of joint prostheses, nails, plates and bone cements were designated to have orthopaedic implant associated infections.

The criteria for sample size and sample selection were based upon the number of patients available at that particular point of time, fulfilling the aforementioned parameters of the case definitions. Decision to collect pus or aspirate was in accordance with the type of the lesion. Additional blood samples were taken from patients having infections with orthopaedic or intravascular devices as and when the patients developed signs and symptoms of sepsis.

Patients with POE: About 0.1 ml of vitreous fluid was collected with the help of sterile tuberculin syringe and 26 gauge needle. The beveled tip of the needle was closed with a sterile rubber bung and was transported to the laboratory immediately. The vitreous fluid was stained and cultured according to the standard procedures described earlier[11].

Patients with intravascular implants: The tips of intravascular catheters/canulas and/or central venous catheters were collected aseptically, in sterile test tubes, and were immediately inoculated onto blood agar

plates according to the rolling technique improvised by Maki *et al*[12] and subsequently onto trypticase soy broth (TSB). The blood agar plates and TSB tubes were incubated at 37°C maximum up to 48 h. For blood culture, the sample of blood was inoculated directly onto the broth.

Patients with orthopaedic implants: Aspirate was collected with sterile syringe and needle after properly disinfecting the surrounding skin. Pus or discharge was collected by rubbing the bed of the ulcer with sterile cotton tipped swab. If the material was insufficient, then wound was squeezed and the exuded purulent material was collected. Intra-operative pus, if obtained, was directly inoculated onto TSB. Blood was also collected for culture if there was indication of sepsis. Additionally, the pus, aspirate, discharge were subjected to Gram staining. The material inoculated into TSB was incubated at 37°C. Growth from TSB was subcultured onto blood agar, MacConkey's agar and Chocolate agar plates, which were incubated at 37°C.

After overnight incubation at 37°C, those colonies showing Gram-positive cocci on smear examination were processed further. The organisms were confirmed as CoNS by their catalase positive reaction from growth on nutrient agar, ability to ferment glucose and inability to coagulate rabbit plasma both by the slide and the tube coagulase tests[13]. All isolates were speciated according to the Baird-Parker's modified scheme[14] of the original method of Kloos and Schleifer[15], and those identified as *S. epidermidis* were stored at -20°C as nutrient agar stab cultures until further testing. In addition, 26 commensal *S. epidermidis* from the hands and the conjunctival swabs of healthy volunteers isolated and identified exactly by the same procedure as mentioned above were also included. The volunteers who provided the commensal organisms did not have any contact to medical facilities or hospital settings. These commensals were also subjected to the same phenotypic and molecular characterization methods in order to look for any discrimination between the deep seated and the commensal *S. epidermidis*.

Biotyping and antibiotic sensitivity testing: Biotyping was done by the standard protocol using Voges-Proskauer test, phosphatase test, and fermentation of lactose, maltose and mannitol[16]. Antibiotic sensitivity testing was performed by the standard Kerby Bauer disc diffusion method[17]. The antibiotics (Hi-media, Mumbai, India) and their antimicrobial content/disc (μg) were tetracycline (30), chloramphenicol (10), genatmicin (10), cloxacillin (1), ciprofloxacin (5), gatifloxacin (5), moxifloxacin (5), tobramycin (10), vancomycin (30), cephazolin (30), and ceftazidime (30). Bacteria showing resistance to 3 or more antibiotics were labelled as multidrug resistant[18].

Slime test: Test for slime production was carried out by the Congo Red Agar (CRA) plate method described earlier by Freeman *et al*[19].

Quantitative slime test for adherence: Adherence of each isolate to smooth surfaces was determined quantitatively by the method of Christensen *et al*[6] with only a very minor modification that quartz cuvettes were used in place of polysterine micro-titre well plates. Briefly, overnight cultures of bacteria in TSB were diluted 1 in 100 in fresh TSB and 1 ml volume of each was put into separate cuvettes. After overnight incubation at 37°C, the cuvettes were washed four times with phosphate buffered saline (PBS), fixed using Bouin's fluid (saturated solution of picric acid 75 parts, formaldehyde 25 parts and glacial acetic acid 5 parts) and then stained with Hucker's crystal violet. Excess stain was removed by decanting the cuvettes and then rinsing them very gently with tap water. The optical density (OD) of the stained bacterial biofilm was read spectrophotometrically (Spectrocolorimeter 103, Systronics, Baroda, India) at 570 nm. The cut-off OD was calculated which was three times the standard deviation (SD) above the mean OD of 10 blanks stained exactly by the same procedure.

DNA extraction: Bacterial DNA was extracted by the standard protocol improvised earlier, with some modifications[1,7]. Briefly, the isolates were grown on Mueller-Hinton agar (Himedia, India) at 37°C overnight. An aliquot (0.1 ml) from this culture (10^8 cfu) was prepared and pelleted by centrifugation (5000xg for 5 min). The bacterial pellet was resuspended in 450 μl of lysis buffer [150 μl of 0.1M Tris-HCl, pH 7.5, 30 μl of 10% SDS, 100 mM EDTA (Merck, India)] containing 30 μl of 100 μg/ml lysostaphin (Sigma, USA), 30 μl of 100 μg/ml proteinase K (Calibiochem, USA). Samples were treated with phenol-chloroform mixture (25:24) and centrifuged at 704 g for 5 min. The aqueous layer was separated and to it were added 1/10 volume of Na acetate and 0.6 volume of isopropanol, followed by washing with 70 per cent ethanol, and centrifugation at 7826 g for 5 min. The pellet containing the DNA was then suspended in 200 μl of TE buffer.

Polymerase chain reaction (PCR) for amplification of ica AB gene: The intercellular adhesin gene '*ica*'

responsible for the phenotypic character like biofilm, was amplified using the following primers; forward 5'-TTA TCA ATG CCG CAG TTG TC and reverse 5'-GTT TAA CGC GAG TGC GCT AT[20]. The previously employed method[20] was followed after standardization as per our own laboratory conditions and with minor modifications. PCR reagent mixture consisted of 200 µM each of dATP, dTTP, dCTP and dGTP, 1.25 U of Taq polymerase and 10 pM of each PCR primer, in a reaction volume of 25 µl. A thermal amplification programme for *ica*A gene included the following parametrers: an initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min) and final extension at 72°C for 5 min. Amplification products (10 µl of each) were run on 2 per cent agarose gel electrophoresis along with molecular weight marker [50 base pair DNA ladder (Fermentas Life Sciences, Bangalore, India)].

PCR for amplification of mec A gene[20]: In order to verify the presence of the *mec* A gene, PCR was carried out using the following primers: *mec* A - F; GTA GAA ATG ACT GAA CGT CCG ATA A *mec* A- R ; CCA ATT CCA CAT TGT TTC GGT CTA A

The assay was performed in a final volume of 25µl reaction mixture, which consisted of 200 µM each of dATP, dTTP, dCTP and dGTP, 10 pM of *mec* A forward and reverse primers and 1.25 U of Taq polymerase. PCR reaction mixture was subjected to the following thermal cycling programme: initial denaturation for 4 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min and with a final extension for 5 min. 10 µl of the amplified products were run on 2 per cent agarose gel electrophoresis along with the molecular marker (50 bp DNA ladder).

Statistical analysis: Chi-square test was employed to analyze the data to find out the significant associations amongst the various phenotypic and molecular characteristics of the isolates.

Results

Overall culture positivity was found in 66 (64.7%) samples, out of which 55 grew *S. epidermidis* (as pure growth in 51 samples and as mixed growth in 4; Table I). Other organisms recovered as pure growth were, 5 isolates of *Diphtheroids* from the vitreous fluid; 2 *Pseudomonas aeruginosa*, 1 *Candida albicans* and 2 *Viridans Streptococci*, all from intravascular catheters/canulas; and a single isolate of *Staphylococcus aureus* from a pus sample in a patient with orthopaedic implant. All three blood cultures in patients with intravascular devices were sterile. Of the 8 blood samples received from patients with orthopaedic implants, 6 yielded *S. epidermidis.*

Other organisms isolated as mixed growth along with *S. epidermidis* were *Escherichia coli* in two specimens (one from catheter and the other from pus), *S. aureus* in one pus specimen and *P. aeruginosa* plus *E. coli* in another pus specimen (Table I).

Table I. Culture findings and details of samples collected in patients with various implants

Implant type (no. of patients) Nature of the specimen	Number of samples	Culture positive	Culture positive for *S. epidermidis* alone	Culture positive for other organisms as pure growth (No.)	Mixed growth (No.) along with *S. epidermidis*
IOL implant Vitreous	50	30	25	Diphtheroids (5)	nil
Indwelling intravascular device Catheter/canula	24	14	8	*Pseudomonas aeruginosa* (2) *Candida albicans* (1) Viridans streptococci (2)	*Escherichia coli* (1)
Blood	3*	nil	nil	nil	nil
Orthopaedic implants Pus/wound aspirate	17	16	12	*Staphylococcus aureus* (1)	*E. coli* (1) *S. aureus* (1) *P. aeruginosa* & *E. coli* (1)
Blood	8**	6	6	nil	nil
Total	102	66	51	11	4

IOP, intra ocular lens; *belong to the group of 24 patients with indwelling catheters/canulas; **belong to the group of 17 orthopaedic patients

Phenotypic characteristics of S. epidermidis: Production of slime by all the isolates under study was assessed by culture on CRA plates. Slime producing organisms exhibited black shiny colonies with a metallic tinge, whereas nonslime producers looked pink. Of the total of 55 *S. epidermidis* isolates studied, 26 (47.2%) were found to be slime positive (Table II). A total of 30 (54.5%) isolates were adherent to artificial surfaces, as determined by our quantitative slime test, and 23 (41.8%) isolates were multi-drug resistant. A majority (22 of 30 *i.e.* 73.3%) of the adherent bacteria were slime producers, as compared to the non-adherent bacteria (only 4 out of 25; 16% being slime producers), and this difference was statistically significant ($P<0.001$).

Our observation on the distribution of *S. epidermidis* biotypes colonizing the various implants revealed that biotypes I and III isolates were the commonest (47.2 and 32.7%, respectively) , and majority of these [15 of 26 (57.7%) biotype I , and 10 of 18 (55.5%) biotype III] were multi-drug resistant (data not shown).

Molecular characterization of S. epidermidis: The *ica* AB amplified product was a 516 bp fragment (Fig. 1) and 23 (41.8%) of our isolates were positive in the *ica* PCR assay (Table II). On analyzing retrospectively, it was found that 20 (76.9%) of the 26 slime producing organisms were " *ica* "positive. Contrary to this, there were only in 3 (10.3%) of the 29 non-slime producers which were "*ica*" positive. This difference was statistically significant ($P<0.001$), suggesting thereby that a vast majority of the bacteria carrying the *ica* genes possessed the capability to produce slime on colonization to the indwelling devices. It was also noted that majority of the bacteria positive for the *ica* AB PCR were adherent to artificial surfaces as compared to the PCR negative strains (Table II, $P<0.001$).

The *mec* A gene product was amplified as a 310 bp fragment (Fig. 2). A total of 28 (50.9%) isolates were *mec* A positive (Table II) and as would be expected most (19 of 28; 67.8%) *mec* A positive isolates were multi-drug resistant as compared to the *mec* A negatives (Table II, $P<0.001$).

Virulence markers amongst the deep vs. commensal isolates: There were statistically significant differences in the *ica*, *mec* A gene positivities and in the presence of the phenotypic markers like slime and adherence; between the device associated *i.e.* the deep and the commensal organisms (Table III). Multi-drug resistance, however, was not found to be significantly higher amongst the device associated bacteria as compared to the commensal bacteria.

Discussion

During the last two decades, *S. epidermidis* has emerged as an important cause of infections in patients with indwelling medical devices[21]. Slime as a virulence factor of *S. epidermidis* in medical implant/device related infections was documented in the past[1,4,22]. It was also shown that comparatively higher number of slime producing isolates of *S. epidermidis* from the keratitic lesions were adherent to artificial surfaces as compared to the commensal isolates from the eye[5]. In addition,

Table II. Virulence markers of the 55 implant associated *S. epidermidis* isolates

Isolation source (No.)	Multi drug resistance (resistance to >3 antibiotics)	Slime**	Adherence**	Positivity for '*ica* AB'PCR*	Positivity for '*mec A*' PCR+
Intra-ocular (25)	7	13	14	10	13
Intravascular (9)	6	6	6	5	3
Orthopaedic (21)					
pus (15)	7	7	8	6	8
blood (6)	3	0	2	2	4
Total (55)	23 (41.8%)	26 (47.2%)	30 (54.5%)	23 (41.8%)	28 (50.9%)

*21 (91.3%) of the 23 *ica* PCR positive bacteria and only 9 (28.1%) of the 32 PCR negative bacteria were adherent to artificial devices ($P<0.001$); **22 (73.3%) of 30 adherent bacteria and only 4 (16%) of the 25 non-adherent bacteria were slime producers ($P<0.001$); +19 (67.8%) of the 28 *mec* A positive organisms, as opposed to 4 (14.8%) of the 27 *mec* A negative organisms were multi-drug resistant ($P<0.001$)

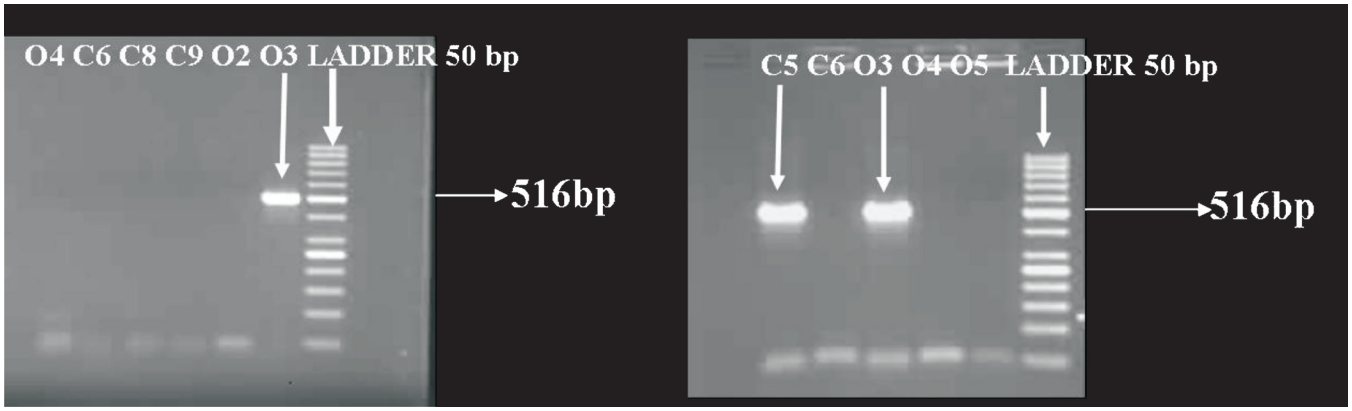

Fig. 1. *ica* gene PCR; Lane C5: Isolate from IV catheter +ve; Lane C6: isolate from IV catheter -ve; Lane 3: isolate from pus +ve; Lane 4 & 5: isolates from pus -ve; Ladder 50 bp.

the gene responsible for biofilm production *i.e.* the *ica* operon was identified in the majority of *S. epidermidis* isolates from catheter related sepsis, whereas none of the commensal isolates from skin and mucosa of healthy volunteers possessed this intercellular adhesin gene[7]. This however, was an isolated report on catheter related infections only, and not on any other device associated conditions.

The present study showed that 47.2 per cent of the isolates were slime positive. This is in good agreement with the reports of Arciola *et al*[7] who noted that 48.5 per cent of their clinical isolates of *S. epidermidis* were slime positive. We also observed previously that approximately 43 per cent of the corneal ulcer isolates of *S. epidermidis* were slime producers[5]. The marginally higher rate of slime positivity amongst our present isolates as compared to those in our previous studies[5,23] on keratitis, suggests that *S. epidermidis* colonizing the indwelling device may be more virulent than those colonizing the corneal surface. Whereas Arciola *et al*[7] studied slime production on intravascular catheter isolates only; ours were not only from vascular devices, but also from other sources such as IOLs and orthopaedic implants.

Our results indicated that bacteria carrying '*ica'* operon mostly possessed the quality of slime production. These organisms, upon colonization to indwelling devices having the potential to adhere, could trigger the intriguing pathway of slime production, intercellular adhesion leading eventually to biofilm formation that is very crucial for the rigid sessile form of attached bacteria onto the device[1,24].

Adherence of coagulase negative Staphylococci was earlier analysed by Muller *et al*[25] who observed that more number of polysaccharide adhesin (PS/A) positive organisms bound to 1.5 cm segments of silicon-elastomer catheters after 15 min of exposure, than did the PS/A negative isolates. Zmantar and colleagues[26] reported the association between slime production and the presence of *ica* A/D genes by PCR assay in 46 clinical isolates of *S. aureus*. The gene profile of all the clinical isolates of *S. epidermidis* from orthopaedic prostheses associated infections was compared with the phenotypic biofilm forming ability evaluated by CRA method[27] and it was noted that 57 per cent of the biofilm producing isolates turned out to be *ica* positive. Duggirala and colleagues[28] reported that *ica* AB was positive in 69.64 per cent of their clinical isolates of *S. epidermidis* from cases of keratitis and endophthalmitis. But they did not compare *ica* positivity of their isolates with biofilm forming ability, though biofilm production was studied as a separate entity. Unlike others[27], we noticed that 70 per cent of the biofilm forming (adherent) organisms in our study were *ica* positive, all these isolates being from various clinical implants, and not merely from orthopaedic prostheses. This might suggest that in different *in vivo*

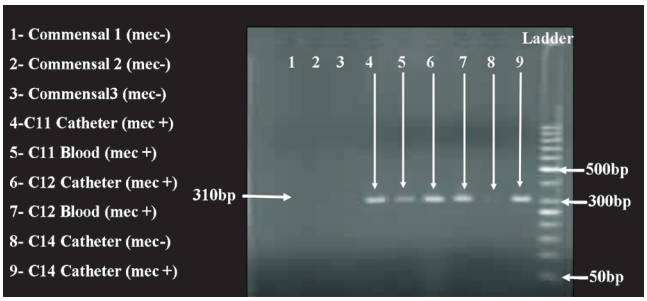

Fig. 2. *mecA* gene PCR showing amplification of 310bp fragments in lanes 4, 5, 6, 7 and 9. Lanes 1,2 and 3 denote commensal *S.* epidermidis. Ladder 50bp.

Table III. Phenotypic and molecular markers amongst device associated and commensal bacteria

Source of the organisms	No. of organisms	Number (%) positive for			
		Slime	Adherence	*ica* PCR	*mec* A PCR
Device related	55	26** (47.2)	30** (54.5)	23** (41.8)	28* (50.9)
Commensals	26	4 (15.3)	6 (23.0)	3 (11.5)	4 (15.3)

*P**<0.05, ***<0.01 compared to commensals

conditions and in the presence of varying colonizing surfaces, the organism may behave differently with varying capabilities for the expression of phenotypic and genotypic characters[22,24].

S. epidermidis biotypes I and III were the most common and virulent forms, exhibiting multi drug resistance patterns associated with the deep seated device related organisms. This is in contrast to corneal ulcer isolates most of which belonged to biotype II and I[5] and no definite multi drug resistance pattern could be ascribed to these. If such predisposition between certain *S. epidermidis* strain types and particular device colonization can be established, in future, by analyzing a large number of invasive clinical isolates, this will have an impact on the patient management.

The present study showed significantly higher number of device associated organisms to be slime producers and adherent as compared to the commensals. In addition, the amplification of *ica* AB and *mec* A genes revealed striking differences between the two groups. These results are in agreement with those of other investigators[29], who demonstrated that *S. epidermidis* recovered from clinical materials behaved differently from the commensal *S. epidermidis* not only by the presence of the *ica* A and the *ica* B genes, but also by their tendency towards phase variation, adherence to polymer surfaces, and capabilities for slime production. Another study documented that majority of the infecting strains (sepsis and catheter related infections) of *S. epidermidis* possessed the *ica* and *mec*A genes as compared to the contaminating strains[20].

Although phenotypic markers such as slime and adherence reflect the potential for virulence[5,6] and may be easier to be demonstrated than the molecular markers, the determination of such phenotypes is often hampered by phase variation exhibited by the organism, reliability in interpreting the test, and subjective variation in recording the results. Therefore, while guiding clinical decisions, detection of the *ica* and *mec* A genes may be more reliable tools than the phenotypic characters, to discriminate between virulent and avirulent/commensal bacteria.

In the present study, *mec* A was found in almost half of the deep isolates, as compared to the commensals. Most of *mec* A positive bacteria in our study were multi drug resistant, which was a true reflection of the nosocomial isolates[8]. However, in 15.3 per cent of our commensal organisms, we detected the *mec* A gene, which could suggest the presence of methicillin resistant *Staphylococcus epidermidis* (MRSE) in the general population. Whether the presence of the *mec* A gene in *S. epidermidis* from the healthy individuals is a reflection of the dissemination of hospital strains to the community or the role of antibiotics in food remains to be elucidated[20].

In conclusion, our study results showed that the distinction between colonizing and infecting *S. epidermidis* with the help of molecular markers *ica AB* and *mec A* may help in clinical judgment in the overall management of patients with indwelling device associated infections.

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