

Comparison of Phenotypic and molecular profile of Coagulase negative Staphylococci from clinical isolates and commensals along with Biofilm detection

Faiza Samreen^{1,*}, Anjana Gopi², Prachee Bele Kenge³, Swati Jain⁴

^{1,4}Junior Resident, ²Professor & HOD, Dept. of Microbiology, Kempegonda Institute of Medical Sciences, Bangalore, ³Senior Resident, Bhat Biotech Pvt. Ltd., Bangalore

***Corresponding Author:**

Email: faizasamreen6@gmail.com

Abstract

Background: Coagulase-Negative *Staphylococci* (CoNS) are commensals of the human skin and mucosa. However, *Staphylococcus epidermidis* (*S. epidermidis*) and other CoNS have emerged as nosocomial pathogens. This study aims to identify and characterize clinical and commensal isolates of CoNS, to compare their association with potential virulence factors and to determine their antimicrobial resistance pattern.

Materials and Method: This study was conducted in Department of Microbiology of a tertiary care centre in Bangalore. A total of 100 samples were processed (50 samples isolated from clinical samples were treated as cases and 50 from skin of healthy volunteers were considered as controls). Identification and speciation of CoNS was done by conventional biochemical tests. Antimicrobial susceptibility was tested by Kirby Bauer disc diffusion method. Detection of biofilm by Tissue culture plate method. Detection of *icaAB*, *atlE* genes which code for polysaccharide intracellular adhesin (PIA) that is fundamental for biofilm formation and *mecA* gene which encodes an altered Penicillin binding protein (PBP2a) mediating oxacillin resistance was done by multiplex PCR.

Results: Out of the 50 cases from clinical samples, 21/50 (42%) of the isolates were from blood, 18/50 (36%) from pus, 9/50 (18%) from urine and 2/50 (4%) were from CSF. *S. epidermidis* was the most common species isolated among clinical and commensal isolates. Biofilm was detected in 16/50 (32%) clinical isolates and 05/50 (10%) of controls. 37/50 (74%) cases and 27/50 (54%) controls were tested positive for *mecA* gene. 17/50 (34%) cases and 15/50 (30%) controls were found to express *atlE* gene. 20/50 (40%) cases and 12/50 (24%) controls possessed *icaAB* gene.

Conclusion: Species identification, antimicrobial susceptibility testing and understanding the genetic components involved in pathogenesis are important aids in the diagnosis and management of serious CoNS infections.

Keywords: *atlE* gene, biofilm, Coagulase-Negative *Staphylococci* (CoNS), *icaAB* gene, *mecA* gene.

Introduction

Coagulase-Negative *Staphylococci* (CoNS) are colonisers of skin and mucous membranes. The important role as pathogens and their increasing incidence has been recognized in recent times.^(1,2,3) CoNS account for approximately 30% of all nosocomial blood stream infections.⁽¹⁾ CoNS are by far the most common cause of bacteraemia related to indwelling medical devices. They are also implicated in central nervous system shunt infections, native or prosthetic valve endocarditis, urinary tract infections and endophthalmitis.⁽²⁾ Most of these infections are hospital-acquired. Among CoNS, *S. epidermidis* is the principal cause of infection, other species such as *S. saprophyticus*, *S. haemolyticus*, *S. warneri* and *S. hominis* have also been isolated. The coagulase-negative species *S. saprophyticus* is a recognized pathogen causing primarily acute urinary tract infections in young healthy, sexually active women. Serious infections due to CoNS are not easy to treat because of the risk factors and the multiple drug resistance displayed by these organisms. Among the various virulence factors expressed by CoNS species, biofilm formation on indwelling medical devices is an important virulence factor which confers antimicrobial resistance and ability to evade host immune response.⁽⁴⁾

Most the species of CoNS especially *S. epidermidis* express the *ica* operon which encodes for the major polysaccharide adhesion involved in the formation of biofilm along with *atlE* which is ubiquitously expressed. Resistance of these organisms to wide range of antimicrobial agents is well documented. Approximately 55-75% of nosocomial isolates is methicillin resistant. CoNS were the first organisms in which glycopeptide resistance was recognized.⁽¹⁾ It is important to monitor antibiotic consumption and resistance trends of nosocomial CoNS, and it is necessary to take preventive measures in order to limit the colonization and spread of multi-resistant strains within hospital environment before a nosocomial infection with these organisms starts. Hence, a study was undertaken to characterise CoNS from clinical isolates and to study their phenotypic and genotypic virulence factors in comparison to commensal isolates.

Materials and Method

A prospective comparative study was conducted in the Department of Microbiology at a tertiary care hospital in Karnataka, India; from January 2015 to June 2016. A total number of 100 isolates were subjected to speciation, virulence typing and antibiotic susceptibility testing. 50 clinically significant strains of Coagulase-

Negative *Staphylococcus* species isolated from urine, pus, blood and CSF samples routinely obtained for culture and sensitivity in Microbiology department were included in the study as cases. 50 strains of Coagulase-Negative *Staphylococci* isolated as normal flora from skin of healthy volunteers served as controls, swabs from hands and nose were collected. The samples were inoculated in BHI broth, Blood agar and Mac conkey agar. Isolates were identified by colony characteristics, Gram stain and catalase test. Bacitracin (0.04 U) and furazolidone (100 µg) susceptibilities were determined to exclude *Micrococcus*, *Planococcus* and *Stomatococcus* spp.⁽⁵⁾ Coagulase test (slide and tube) and mannitol fermentation were done to exclude *Staphylococcus aureus* and other coagulase- positive species. These tests were performed on all samples of *Staphylococcus* as per standard procedures.^(5,6) Speciation was done by conventional biochemical tests, antimicrobial susceptibility testing was performed on Mueller Hinton agar (Hi-media, Maharashtra India) by Kirby Bauer disc diffusion method in accordance with CLSI 2014 guidelines, biofilm detection was done by Tissue culture plate method^(7,8) (18 hr cultures of CoNS were diluted 1:100 with fresh Trypticase soy broth prepared without glucose. Individual wells of sterile polystyrene 96 well fitted Tissue culture plate were filled with 0.2ml aliquots of diluted culture. Tissue culture plates were incubated for 18 hrs at 37°C. The contents were gently aspirated by tipping the plate and wells are washed four times with 0.2 ml PBS (phosphate buffer saline) pH 7.2 Adherent organisms were fixed in place with Bouin's fixative and stained with Hucker's crystal violet. Excess stain is rinsed off, after drying the OD's of adherent films are read with microELISA autoreader at 570nm. Values of < 0.120 are considered as non-producers and values of > 0.240 as producers. Detection of *icaAB*, *mecA* and *atlE* gene by multiplex PCR was carried out at Bhat Biotech, Bangalore, India.

The Primers used for PCR,

1. *ica* (product size 546 bp)

Forward *ica* – TTATCAATGCCGAGTTGTC

Reverse *ica* – GTTTAACGCGAGTGCGCTAT

2. *mecA* (product size 310 bp)

mecA1 – GTAGAAATGACTGAACGTCCGATAA

mecA2 – CCAATTCCACATTGTTTCGGTCTAA

3. *atlE* (product size 682 bp)

Forward *atlE* – CAACTGCTCAACCGAGAACA

Reverse *atlE* – TTTGTAGATGTTGTGCCCCA

Results

A total number of 100 isolates (50 clinical strains considered as cases and 50 isolates from skin of healthy volunteers were considered as controls). Among the clinical strains obtained 21/50 (42 %) of the isolates were from blood samples of patients with clinical history of sepsis and high total leucocyte counts and positive C-Reactive Protein values, 18/50 (36%) isolates were from pus samples, 9/50 (18%) were from urine samples out of which two samples were from catheterised patients and 2/50 (4%) were from CSF samples of pediatric age group of which both were having a ventriculo-peritoneal shunt (VP shunt) in situ. 29/50 (58%) of the isolates were from clinical samples which were obtained from patients who had an indwelling device such as IV cannula, Drain tip, CVP tip, Foley's catheter and VP shunts.

S. epidermidis was the predominant isolate among both cases 39/50 (78%) and controls 27/50 (54%). *S. saprophyticus* 7/50 (14%) and *S. hominis* 4/50 (8%) were isolated among cases only. *S. hemolyticus* 18/50 (36%) and *S. warneri* 5/50 (10%) were isolated controls only. *S. saprophyticus* species isolated from urine were from female patients in the reproductive age group.

05/50 (10%) of the clinical isolates were resistant to tetracycline, 06/50 (12%) to gentamycin, 07/50 (14%) to linezolid, 15/50 (30%) to clindamycin, 17/50 (34%) to cefoperazone, 24/50 (48%) to cefoxitin, 29/50 (58%) to cefepime, 27/50 (54%) to co-trimoxazole, 25/50 (50%) to ciprofloxacin, 36/50 (72%) to amoxycylav, 43/50 (86%) to erythromycin. However, none of the isolates were resistant to vancomycin.

All the 100 isolates (including cases and controls) were screened for biofilm formation. As depicted in Graph 1 and Table 1, biofilm formation was detected in 16/50 (32%) of the clinical isolates and 5/50 (10%) of controls.

Graph 1: Biofilm formation among cases and controls

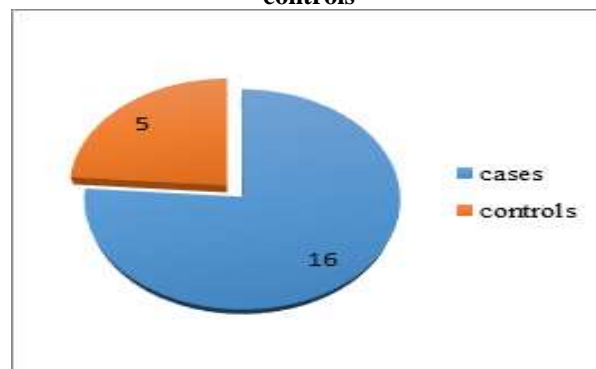


Table 1: Biofilm formation among different species of cases and controls

Species among cases	Total isolated (50)	Biofilm producers (16)	Species among controls	Total isolated (50)	Biofilm producers (05)
<i>S. epidermidis</i>	39	15	<i>S. epidermidis</i>	27	05
<i>S. saprophyticus</i>	07	01	<i>S. hemolyticus</i>	18	00
<i>S. hominis</i>	04	00	<i>S. warneri</i>	5	00

20(40%) of the clinical isolates showed the presence of the genes responsible for intercellular adhesion (*icaAB*) in comparison to 12 (24%) of controls, 17 (34%) of the clinical isolates showed presence *atIE* gene against 15 (30%) of controls which showed presence of *atIE* gene and 37 (74%) of cases and 27(54%) of controls showed presence of *mecA* gene. The calculated probability was found to be significant for genotypic expression of methicillin resistance among clinical isolates as compared to commensal isolates.

Graph 2: Graphical representation of comparison of gene detection among cases and controls

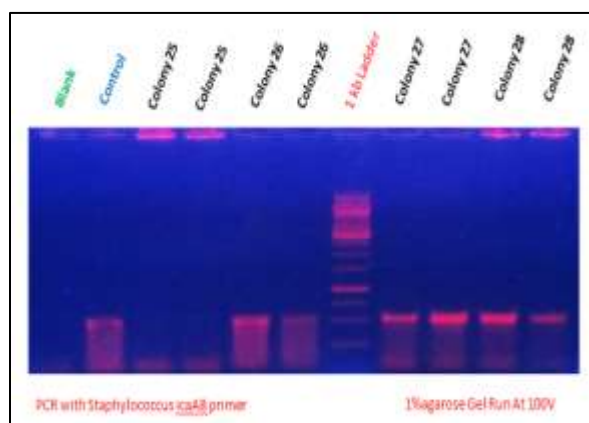
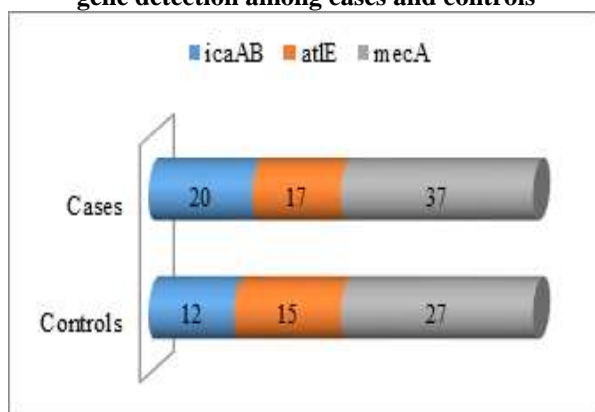


Fig. 2: Genotypic detection of *icaAB* gene

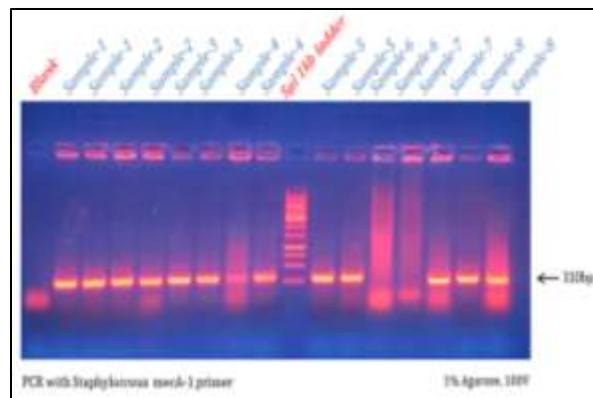


Fig. 3: Genotypic detection of *mecA* gene

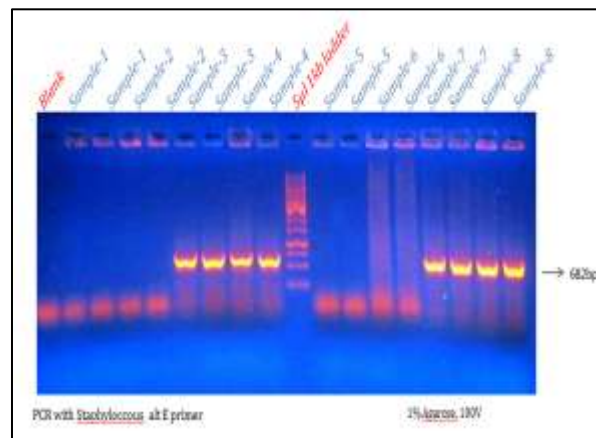


Fig. 4: Genotypic detection of *atIE* gene

Discussion

CoNS are widespread on the human body and are capable of producing very large populations, distinguishing the etiologic agent(s) from contaminating flora is a serious challenge. Species identification is important in monitoring the reservoir and to understand the pathogenic potential of the species. However, a stronger and definitive diagnosis can be made for the identification of a CoNS etiologic agent if the same strain is repeatedly isolated from a series of specimens as opposed to the isolation of different strains of one or more species.

21/50 (42%) of the clinical strains in our study were isolated from blood, we compared our findings with a study done by Usha et al⁽³⁾ which showed 52% isolation from blood samples and in a study by Natoli et al⁽⁹⁾ where the rate of isolation from blood of ICU patients was 47.3% and similarly a study done by

Anjana Gopi *et al*⁽¹⁰⁾ demonstrated a high rate of isolation of CoNS from blood stream infections in neonates.

29/50 (58%) of the isolates were from clinical samples which were obtained from patients who had an indwelling medical device such as IV cannula, Drain tip, CVP tip, Foley's catheter and VP shunts.

CoNS have predilection to bind to polymer surfaces forming microcolonies in a exopolysaccharide matrix and have been found to be one of the most frequently isolated microorganisms in the context of medical device-related infections (e.g., catheters systems, pacemakers, prosthetic joints and heart valves and a range of other polymer and metal implants).^(11,12) They account for approximately 30% of health care-associated bloodstream infections.⁽¹³⁾

S. epidermidis was the most common species isolated among cases 39/50 (78%) and 27/50 (54%) controls, our findings correlated with a study by M. M. A. Khan *et al* who showed that 75.8% of their isolates were *S. epidermidis*.⁽¹⁴⁾ Also in a study by Roopa *et al* and Rajyalakshmi Gunti *et al* *S. epidermidis* was the most common isolate (50.8%) and (42%) respectively.^(15,16)

CoNS are gaining more importance due to methicillin resistance. Methicillin-resistant Coagulase-Negative *Staphylococci* (MRCoNS) have become predominant organism in hospitalised patients. Studies carried out by SENTRY Antimicrobial Surveillance Program, have reported about 70-75% of CoNS are resistant to methicillin.⁽¹⁴⁾ 48% of our clinical isolates showed methicillin resistance. In comparison with our study, the incidence of methicillin-resistance among significant CoNS isolates was 38% in a study by Keim *et al*.⁽¹⁷⁾ The incidence of oxacillin resistance in a study by Anjana Gopi *et al*⁽¹⁰⁾ is 69.7%. The widespread use of antibiotics in hospitals has provided a reservoir of antibiotic resistant genes.

The pathogenesis of *S. epidermidis* catheter related infections mostly relies on adherence to polymer surfaces. After adherence, the next step in colonisation of surfaces by *S. epidermidis* involves the production of glycocalyx, which forms a biofilm on plastic surfaces. This biofilm is also produced by *S. saprophyticus*, *S. simulans* and *S. lugdunensis*. Once the biofilm has formed it acts as a penetrating barrier to antibiotics and has significant biological activities, including inhibition of host defence mechanism and interferes with intracellular killing.⁽¹⁸⁾

Polysaccharide synthesis is mediated by *ica* operon which encodes an N-acetylglucosaminyl transferase enzyme that catalyses the synthesis of capsular polysaccharide from N-acetylglucosamine. *atlE* gene encodes vitronectin binding cell surface protein involves in primary attachment. *mecA* gene controls the synthesis of additional penicillin-binding protein PBP2a which codes for methicillin resistance in *Staphylococcus*.⁽¹⁹⁾

Phenotypically, 32% of the cases and 10% of the controls were found to be biofilm producers with a significant p value of < 0.05 which was computed by Chi square test which showed (*p value of 0.006*).

Genotypic expression of *icaAB* gene responsible for intercellular adhesion was seen in 20(40%) of the clinical isolates in comparison to 12 (24%) of controls, 17 (34%) of the clinical isolates showed presence *atlE* gene against 15 (30%) of controls and 37 (74%) of cases and 27(54%) of controls were positive for *mecA* gene expression. The results for *icaAB* and *atIE* combined gene detected among cases and controls were calculated by Z test, the Z score is 1.8641. The (*p value is 0.031*) as depicted in Graph 2 and Fig. 2, 3, 4. The CoNS isolates which expressed the *icaAB* gene were also found to be positive for biofilm production and were resistant to multiple antibiotics phenotypically. Similar to our study, a study by Sujatha Prasad *et al* 41.8% of clinical isolates possessed the intercellular adhesin (*ica*) gene and (50.9%) harboured the *mecA* gene.⁽²⁰⁾ In a study by Sharma *et al* 58% of isolates expressed *icaAB* gene, 80% showed expression of *mecA* gene and *atlE* gene was expressed ubiquitously in all isolates.⁽¹⁹⁾

Conclusion

Important virulence factors of CoNS such as biofilm need to be elucidated and research should focus on elaborating genetic determinants among both commensal and clinical strains. Adapting preventive measures to avoid CoNS infections should be our main focus clinically. As infections are mostly transferred through instruments and procedures, there is a need to practice sterile procedures strictly and use proper instruments. Health care workers should be offered regular training and awareness programs related to such infections.

References

1. Piette A, Verschraegen G. Role of Coagulase-Negative *Staphylococci* in human disease. *Vet Microbiol*.2009 Feb 16;134(1-2):45-54. (pubmed).
2. Huebner, J and D. A. Goldmann. 1999. Coagulase-Negative *Staphylococci*: role as pathogens. *Annu. Rev. Med.* 50:223-236. (pubmed)
3. Usha MG, Shwetha DC, Vishwanath G. Speciation of Coagulase-Negative *Staphylococcal* isolates from clinically significant specimens and their antibiogram. *Indian J Pathol Microbiol* 2013;56:258-60.
4. Otto M Virulence factors of the Coagulase-Negative *Staphylococci*. *Front Biosci.* 2004 Jan 1;9:841-63.(pub med)
5. P.M, tille. "*Staphylococcus*, *Micrococcus* and Similar Organisms". Bailey and Scott' S Diagnostic Microbiology. 13th ed. Elsevier, 2014. 232.
6. D. Baird. *Staphylococcus*. In: Collee JG, Marmion BP, Simmons A, Fraser AG, (eds) In :J.G C, A.G F, B.P M, A S. Mackie and McCartney Practical Medical microbiology. 14th ed. Elsevier; 2014.p 245-261.
7. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fathma T, Rattan A. Detection of biofilm formation among clinical

- isolates of *Staphylococci*: an evaluation of 3 different methods. Indian J med Microbiol.2006;24(1):25-29.
8. Christensen GD, Simpson WA, Baddour LM, Younger JJ et al. Adherence of Coagulase-Negative *Staphylococci* to plastic tissue culture plates: a quantitative model for adherence of *Staphylococci* to medical devices. J Clin Microbiol 1985;22 996-1006.
 9. Natoli S, Fontana C, Favaro M, Bergamini A, Testore GP, Minelli S, et al. Characterization of Coagulase-Negative staphylococcal isolates from blood with reduced susceptibility to glycopeptides and therapeutic options. BMC Infect Dis 2009;9:83.
 10. Dr. Anjana Gopi, Dr. Syeda Misbah ul khair and Dr. Hitha, T. K. A study of Coagulase-Negative *Staphylococci* associated blood stream infections in pediatric patients in a tertiary care hospital. Int J of Cur Res, May2016;8(05):30766-30769.
 11. Durando, P., M. Bassetti, G. Orengo, et al. Hospital-acquired infections and leading pathogens detected in a regional university adult acute-care hospital in Genoa, Liguria, Italy: results from a prevalence study. J Prev Med Hyg, 2010;51(2): 80-86.
 12. Kollef, M. H., L. M. Napolitano, J. S. Solomkin, et al. Health care-associated infection (HAI): a critical appraisal of the emerging threat-proceedings of the HAI Summit. Clin Infect Dis, 2008;47(02): S55-99; quiz S100-101.
 13. Otto, M. *Staphylococcus epidermidis*- the 'accidental' pathogen. Nat Rev Microbiol,2009;7(8):555-567.
 14. Mohammad Mubashir Ahmad Khan, Aftab Faiz, Ahmad Mohammad Ashshi. Clinically significant Coagulase-Negative *Staphylococci* and their antibiotic resistance pattern in a tertiary care hospital. J Pak Med Ass, 2014;10(24):1171-1174.
 15. C. Roopa and Sunilkumar Biradar. Incidence and Speciation of Coagulase-Negative *Staphylococcus* Isolates from Clinically Relevant Specimens with their Antibiotic Susceptibility Patterns Int. J. Curr. Microbiol. App. Sci (2015) 4(9):975-980.
 16. Rajyalakshmi Gunti, Durgarani Arava, Rajasekhar Koppada Speciation of Coagulase-Negative *Staphylococci* and Their Antibiogram Isor journal Jan2016; Volume 15(1) Ver X:28-31.
 17. Luiz S. Keim, Sylvio R. Torres-Filho, Patricia Vollú Silva, and Lenise A. Teixeira: Prevalence, Aetiology and Antibiotic Resistance Profiles of Coagulase-Negative *Staphylococci* Isolated in a Teaching Hospital. Braz J Microbiol. Jan-Mar 2011;42(1):248–255.
 18. Winn WC, Allen SD, Janda WM, Koneman EW, Procop CW, Schreckenberger PC, et al. Gram positive cocci Part I: *Staphylococci* and related Gram positive cocci. In: Koneman's colour atlas and Textbook of diagnostic Microbiology. 6th ed. USA: Lippincott Williams and Wilkins; 2006. p. 623-73.
 19. Sharma P, Lahiri K K, Ketoki Kapila. Conventional and molecular characterization of Coagulase-Negative *Staphylococcus* in hospital isolates. Indian J of Patho Microbiol. Jan-March 2011;54(1):84-89.
 20. Sujata Prasad, N. Nayak, G. Satpathy, H.L. Nag, P. Venkatesh et al. Molecular & phenotypic characterization of *Staphylococcus epidermidis* in implant related infections. Indian J Med Res. 2012 Sep; 136(3): 483–490.