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PHENOTYPIC CHARACTERIZATION AND BIOFILM DETECTION OF COAGULASE NEGATIVE STAPHYLOCOCCI ISOLATED FROM CLINICAL SPECIMENS IN KOTA REGION

Dr. Anita E. Chand — Senior professor and Head, Department of Microbiology, GMC, Kota

Dr. Danish — III[rd] year PG Resident, Department of Microbiology, GMC, Kota

Dr. Pradhuman Singh Chauhan — Senior Demonstrator, Department of Microbiology, GMC, Kota

ABSTRACT

Introduction: Coagulase negative staphylococci are most often associated with chronic infections of implemented medical devices. The biofilm-forming ability of CONS helps to resist the conditions of the surrounding environment with high levels of tolerance to antibodies, antibiotics, disinfectants and phagocytic inflammatory cells. So CONS may emerge as important cause of nosocomial infection.

Aims & Objectives: To detect prevalence of CONS and their ability to form biofilm by using three different screening methods. Also to determine antimicrobial susceptibility pattern among these isolates and correlate antibiotic susceptibility pattern of CONS and biofilm production.

Material and Method: A total of 100 CONS isolated from various clinical samples were subjected to antibiotic susceptibility testing and screened for biofilm production by Microtitre plate method, Tube method and Congo red agar method.

Results: CONS were most commonly isolated from pus samples (39%). Staphylococcus epidermidis (62%) was most commonly isolated CONS species. Maximum number of CONS isolates were resistance to ampicillin (67%) followed by Cotrimoxazole (66%). Maximum biofilm production was detected by MTP method (63%), followed by Tube method (52%) and CRA method (16%). Maximum biofilm producers were isolated from surgical wounds (pus) (20%). The Biofilm producing CONS isolates showed higher antibiotic resistance than non-biofilm producers.

Conclusion: The virulence of CONS is directly related to its capability to form biofilms. There is association between biofilm production with persistent infection and antibiotic failure. Hence, in small microbiological laboratories where PCR cannot be done, Microtitre plate method can be recommended which is simple and cost effective. Due to biofilm formation very soon CONS may emerge as one of the leading nosocomial pathogen and should no longer be considered as a harmless commensal.

KEYWORDS : CONS- Coagulase-negative staphylococci, EPS-Extracellular polymeric substances, PIA-Polysaccharide intercellular adhesion, MTP-Microtitre plate method, TM-Tube method, CRA-Congo Red Agar method, PBS-Phosphate buffer saline

INTRODUCTION

Coagulase negative staphylococci (CONS) are gram-positive spherical cocci belonging to the genus *Staphylococcus*. Microscopically, these bacteria appear in pairs, clusters or short chains in arrangement. They are catalase-positive, non-motile, non-spore-forming, noncapsulated. The absence of secreted enzyme coagulase is the key characteristic of CONS, differentiating them from coagulase-positive staphylococci, such as *Staphylococcus aureus*, which is able to clot plasma and is generally accepted as a pathogenic bacterium related to acute infections.[1]

Historically CONS have been considered as saprophytes with little pathogenic potential. They cause infections in debilitated or immunocompromised patients, also in patients with indwelling urinary catheters, cardiac valves and artificial joints.[2] Numerous species of CONS have been recognized as pathogens. *Staph. epidermidis* is the CONS species most frequently isolated from infections. *Staph. epidermidis* has been implicated as the etiological agent in infections of wound, urogenital tract, respiratory tract, meninges, conjunctiva and skin.[3,4] *Staph. saprophyticus* is second to coliforms as the most common cause of the acute urethral syndrome and UTI in women.[5,6]

Some strains of CONS produce a viscous extracellular material or slime (biofilm). These strains adhere to various biotic and abiotic surfaces.[7] Property of adhesion by CONS was first observed by Bayston and Penny in 1972. They observed mucoid colonies of CONS from CSF shunts.[8]

Biofilms are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced, and exhibited an altered phenotype with respect to growth rate and gene transcription.[9] Biofilm is made up of extracellular matrix which comprises polysaccharides, proteins, enzymes, DNA, bacterial glycolipids, water and aggregates of microorganisms.[7] Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing.[10]

Infections related to biofilms are generally associated with the use of catheters and other medical devices.[11] Bacterial cells can detach from mature biofilms and spread to other organ systems.[12,13] As a result, biofilms become sources of persistent and chronic infections.[11] The increased use of indwelling medical devices had considerable impact on the role of *staphylococci* in clinical medicine.[1]

In *Staphylococcus epidermidis* polysaccharide intercellular adhesin (PIA) is responsible for intercellular adhesion. Recently, PIA homologs are identified in many pathogens with biofilm formation ability, which shows that the three-dimensional matrix formation plays an important role in bacterial virulence. Biofilm protects CONS, against both; antibiotics used to treat infections and host immune system responses.[7]

Hence, the present study is undertaken to study the biofilm production and antibiogram of CONS.

MATERIAL AND METHOD

The present study was conducted in Department of Microbiology, Government Medical College, Kota (Rajasthan) from January 2017 to December 2017. Total 100 consecutive CONS isolated from

various clinical samples like urine, blood, pus, sputum, intravenous catheter tips, urinary catheter, endotracheal aspirate and body fluids (pleural, ascitic, peritoneal fluid and CSF) etc. were processed for the study.

All specimens were processed according to standard operating procedure of laboratory. Organism identification done by Gram's staining, motility, cultural characteristics and biochemical reactions. All CONS isolates were subjected to antibiotic susceptibility testing by Kirby Bauer disk diffusion method according to CLSI guidelines using Mueller-Hinton agar medium and commercial antibiotic discs (HIMEDIA).

Detection of biofilm formation

1.Microtitre plate method (MTP)[14]

Overnight growth from solid media was diluted 1 in 100 in fresh medium. Individual wells of microtitre plates wells were filled with 200 µl aliquots of the diluted cultures (uninoculated broth served as control) and incubated for 24 hours at 37°C then content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2). Biofilms formed in plate were fixed with sodium acetate (2%) and stained with safranine (0.1% w/v). After 20 min of staining wells were washed with PBS. Finally, 33% glacial acetic acid was added. The mean OD value obtained from control well was deducted from all the test OD values.

Interpretation:-

Mean OD values	Adherence	Biofilm formation
<0.120	Non	Non/weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

1.Tube method (TM)[15]

Brain heart infusion (BHI) with 2% sucrose (10ml) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation.

Interpretation:-

Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation.

Tubes were examined and the amount of biofilm formation was scored as:-

0-absent,
1-weak,
2-moderate,
3-strong

2.Congo Red Agar method (CRA)[16]

This method requires brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C.

Interpretation:-

Strong slime producers- Black colonies with a dry crystalline consistency

Moderate slime producers- Darkening of the colonies with absence of a dry crystalline colony.

Weak slime producers- Pink (occasional darkening at the centres of colonies was observed)

OBSERVATION AND RESULTS

In present study CONS were most commonly isolated from surgical wounds (pus) (39%) (table-1) with maximum prevalence in 41-60 years age group (34%). Mean age of study group was 49.57 years. 62% CONS isolates were prevalent in male and 38% in female with male:female ratio 1.63:1. CONS infection were more common in IPD cases (68%) than OPD cases (32%). The most commonly isolated CONS species were *Staphylococcus epidermidis* (62%) followed by *Staphylococcus haemolyticus* (22%), *Staphylococcus saprophyticus* (15%) and *Staphylococcus lugdunensis* (1%) (table-2). Maximum number of CONS isolates were resistant to ampicillin (67%) followed by Cotrimoxazole (66%) (table-3).

Maximum biofilm production was detected by MTP method (63%), followed by TM method (52%) and CRA method (16%). Maximum biofilm producers were from surgical wounds (pus) (20%) followed by urine (19%) (table-1). Biofilm production had a strong association with medical device related to orthopaedic implant, urinary catheterization and intravenous catheters. Maximum biofilm production was found in *Staph. epidermidis* (61.90%), followed by *Staph. hemolyticus* (20.63%), *Staph. saprophyticus* (15.87%) and *Staph. lugdunensis* (1.60%) respectively (table-2). The biofilm producing CONS isolates showed higher antibiotic resistance than non-biofilm producers. Among the biofilm producer highest antibiotic resistance were noted against cefoxitin (88.88%), followed by cotrimoxazole (80.85%). No isolates were resistant to vancomycin and linezolid (table-3).

Table-1:Sample wise biofilm production by MTP method in CONS

Type of sample	Number of Isolates N=100 (%)	Biofilm	
		Producers	Non-Producers
Pus from surgical wounds	39(39.00%)	20	19
Urine	28(28.00%)	19	9
Urinary catheters	10(10.00%)	8	2
Intravenous catheters	7(7.00%)	5	2
Body fluids	6(6.00%)	2	4
Implants	5(5.00%)	5	0
Blood	4(4.00%)	4	0
Ear swab	1(1.00%)	0	1
Total	**100 (100%)**	**63**	**37**

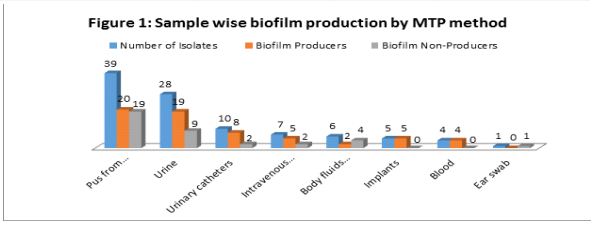


Table-2: Species wise biofilm production in CONS

CONS species isolated	Number of Isolates N=100 (%)	Biofilm Producers (N=63)	Non-Producers (N=37%)
Staph. epidermidis	62 (62.00%)	39 (61.90%)	23 (62.16%)
Staph. hemolyticus	22 (22.00%)	13 (20.63%)	9 (24.32%)
Staph. saprophyticus	15 (15.00%)	10 (15.87%)	5(13.52%)
Staph. lugdunensis	1 (1.00%)	1 (1.60%)	0 (0.00%)
Total	**100 (100.00%)**	**63 (100%)**	**37 (100%)**

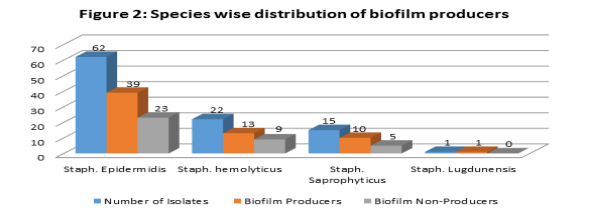
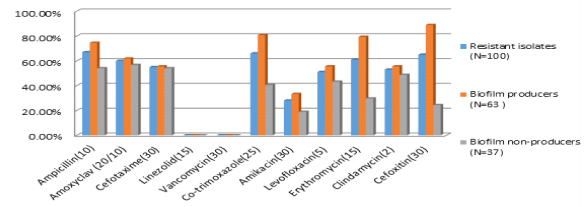


Table-3: Antibiotic resistance pattern of biofilm producer and non-producer isolates

Antibiotic (concentration µg/disk) (HIMEDIA)	Resistant isolates (N=100)	Biofilm producers (N=63)	Biofilm non-producers (N=37)
Ampicillin(10)	67(67.00%)	47(74.60%)	20(54.05%)
Amoxycillin-clavulinic acid (20/10)	60(60.00%)	39(61.90%)	21(56.76%)
Cefotaxime(30)	55(55.00%)	35(55.55%)	20(54.05%)
Linezolid(15)	00(0.00%)	00(0.00%)	00(0.00%)
Vancomycin(30)	00(0.00%)	00(0.00%)	00(0.00%)
Co-trimoxazole(25)	66(66.00%)	51(80.85%)	15(40.54%)
Amikacin(30)	28(28.00%)	21(33.33%)	7(18.91%)
Levofloxacin(5)	51(51.00%)	35(55.55%)	16(43.24%)
Erythromycin(15)	61(61.00%)	50(79.37%)	11(29.73%)
Clindamycin(2)	53(53.00%)	35(55.55%)	18(48.65%)
Cefoxitin(30)	65(65.00%)	56(88.88%)	9(24.34%)

Figure 3: Antibiotic resistance pattern



DISCUSSION

CONS have been major cause of nosocomial infections. Biofilm producing bacteria are difficult to eradicate. Taking into consideration, the increased frequency of isolation of CONS from clinical specimens, they must be individually evaluated as potentially true pathogens. CONS are known to cause chronic infections, the major virulence factor determining the pathogenicity of CONS has found to be biofilm production.[17]

In present study CONS were most commonly isolated from surgical wounds (pus) (39%) which is comparable with the study of Samant Sharwari et al[18] (43%), while in study of Usha MG et al[19] CONS were commonly isolated from blood (52%). In present study CONS were most commonly isolated from 41-60 years age group (34%) which is comparable with study of Puja Gupta et al[20] (39%). While in study of SS Vijayasari et al[21] CONS were most common in 21-40 years age group (31.81%).

In present study Male:Female ratio for CONS isolates was 1.63:1 which correlated with study by SS Vijayasari et al[21] (1.70: 1), whereas Puja Gupta et al[20] reported 1.1: 1. In present study CONS were more commonly isolated form inpatients (68%) than outpatients (32%) this finding correlates with study by SS Vijayasari et al[21] who reported more inpatients (70%) than outpatients (30%), whereas Puja Gupta et al[20] reported inpatients (95%) and outpatients (5%).

In present study *Staph. epidermidis* (62%) was most commonly isolated species which correlate with the study of Priyanka Mane et al[22] and Samant Sarvari et al[18]. There is wide variation in antibiotic sensitivity pattern in all studies, which may be due to selective pressure of antibiotic used in particular area. The present study showed that MTP method was best method (63%) for detection of biofilm production which correlates with study of Riyaz et al[23](64%). While in study of Usharani et al[24] best method of biofilm production was CRA method because unavailability of ELISA OD reader, MTP method findings were interpreted manually in qualitatively manner. In present study biofilm production had a strong association with artificial medical device related infections (81.81%) which correlates with study of Samant Sarawari et al[18](90%) and Mulla et al[25](77.77%).

Biofilm production by various CONS species varies considerably between different studies. In present study, *Staph. epidermidis* (61.90%) were most common biofilm producers species, which was comparable with the study of SS Vijayasari et al[21] (66.66%).

In present study, antimicrobial resistance were higher among biofilm producers in comparison with non-biofilm producers which was similar to study done by Roberto et al[26].

CONCLUSION

The virulence of CONS is directly related to its capability to produce biofilms and clinical significance of CONS is increasing day by day in device related infections, urinary tract infections, endocarditis so very soon CONS may emerge as one of the leading nosocomial pathogen.

Among various phenotypic methods MTP method showed high sensitivity and specificity with cost effectiveness and can be readily used where molecular methods are not available.
Prophylactic antibiotic therapy should be given before any invasive intervention and insertion of implant which slows progression of biofilms formation on biomaterials.

Early detection of CONS with biofilm formation can decrease morbidity and mortality of patients.

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