IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 20, Issue 2 Ser.2 (February. 2021), PP 58-65
www.iosrjournals.org

Biofilm detection and Clinical significance of Coagulase negative Staphylococci isolates in a tertiary care centre

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Abstract:
Background: *Coagulase negative Staphylococci (CoNS) are normal commensals of the human skin and mucous membranes. However, in immunocompromised and debilitated patients, they can cause infections like osteomyelitis, bloodstream infections, surgical site infections, endocarditis and device associated infections. They adhere to indwelling devices and colonise them by formation of biofilm, an important virulence factor. Biofilm associated bacteria are more resistant to host defence, thus making treatment of these infections difficult. The present study was undertaken to know the relation between biofilm production and antimicrobial resistance and its clinical significance.*
Materials and Methods: *A total of fifty non – repetitive isolates of Coagulase negative Staphylococci from clinical samples were studied for biofilm production by 3 methods – Tube method, Congo Red agar method and Microtiter Plate method and they were compared. Antimicrobial susceptibility testing was done by the Kirby Bauer disc diffusion method. The isolates were tested for methicillin resistance using the Cefoxitin disc diffusion method.*
Results: *Among the 50 isolates, 32 (64%) were positive for biofilm production by the Microtiter Plate method, 28 (56%) were positive by the Tube method and 22 (44%) were positive by the Congo Red agar method. The Tube method was found to be superior to the Congo Red agar method. The biofilm forming isolates were found to be comparatively more resistant to the commonly used antimicrobial agents than the non biofilm forming isolates.*
Conclusion: *The Microtiter plate method is a convenient and quantitative method for the detection of biofilm production. The use of a reliable method to determine biofilm production, helps to identify potentially dangerous organisms, and thus guide therapeutic decision making. It also helps to formulate strategies for the prevention of device related infections.*
Keywords: *Coagulase negative Staphylococci; Biofilm; Microtiter plate method; Tube adherence method; Congo Red agar.*

Date of Submission: 25-01-2021 Date of Acceptance: 10-02-2021

I. Introduction

Coagulase negative Staphylococci (CoNS) are normal human commensals, inhabiting the skin and mucous membranes[1]. However, they act as opportunistic pathogens, causing nosocomial infections among the immunocompromised and critically ill patients[2]. Staphylococcus epidermidis and Staphylococcus hemolyticus are the most significant species causing device associated infections, bloodstream infections, peritonitis, surgical site infections and osteomyelitis. Staphylococcus saprophyticus causes urogenital tract infections among young, sexually active females[3].

They are responsible for infections associated with implanted or indwelling medical devices, which are increasingly being used in recent times. CoNS are now ranked the most common infective agent in prosthetic valve associated endocarditis[4]. Slime or biofilm production is one of the most important virulence factors associated with these organisms. It enables them to adhere to various biotic and abiotic surfaces, causing device associated infections[5].

Biofilms are multicellular communities of bacteria, surrounded by an extracellular polymeric matrix. The most important component of Staphylococcal biofilms is the Polysaccharide Intercellular Adhesin (PIA), which mediates cell to cell adhesion[6,7]. Bacteria adhere to medical devices by means of microbial surface components, which interact with adhesive matrix molecules and then they multiply to establish a biofilm[8].

Organisms growing in a biofilm are less susceptible to the host defence mechanism and to antimicrobial agents. This is due to reduced penetration of the antibiotic, high bacterial density, slower growth of the organisms and the higher prevalence of resistance determining genes. This leads to persistence of infections and failure of antibiotic therapy.

Biofilm formation can be determined by phenotypic methods such as Tube adherence method, Congo Red agar method, quantitative Microtiter / Tissue culture plate assay, confocal laser scanning microscopy and genotypic methods such as RNA sequencing, microarray technique and PCR. The test for biofilm production helps in deciding the pathogenicity of CoNS and their detection enables early, appropriate treatment. The classification of CoNS with respect to its biofilm phenotype helps to elucidate the pathogenesis of biofilm formation and to formulate strategies to prevent device related infections[9,10].

The present study was carried out to determine biofilm production by 3 phenotypic methods & to elucidate its relation to the antibiotic resistance profile of Coagulase negative Staphylococci isolates from different clinical samples received in the microbiology laboratory of a tertiary care hospital.

II. Material & Methods

The cross-sectional study was carried out in Osmania General Hospital, Hyderabad from October 2019 – December 2019. Institutional ethics committee approval was obtained prior to the study.

Study Design: Cross – sectional study

Study Location: Department of Microbiology, Osmania General Hospital & Osmania Medical College, Hyderabad.

Study Duration: October 2019 – December 2019 (3 months duration)

Sample size: 50 non-repetitive clinical isolates of Coagulase negative Staphylococci obtained from various clinical samples, like blood, urine, central venous catheter tips, endotracheal tube aspirate, pus and exudates.

Inclusion Criteria

1. Coagulase negative Staphylococci isolates from clinical samples of patients admitted to various inpatient wards of Osmania General Hospital. .
2. Clinical samples like blood, urine, central venous catheter tips, endotracheal tube aspirate, pus and exudates.

Exclusion criteria

1. Coagulase negative Staphylococci isolates from outpatients.
2. Repetitive isolates from the same patient.
3. Patients not giving consent.

Procedure methodology

Identification of Coagulase negative Staphylococci

Isolates from various clinical samples like blood, central venous catheter tips, endotracheal tube aspirate, urine, pus and exudates were identified as Coagulase negative Staphylococci by standard microbiological methods (Gram staining, colony morphology, slide and tube coagulase test and biochemical reactions). Repeat isolations (twice) from blood cultures with pure growth were given significance.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was done by Kirby Bauer's modified disc diffusion method on Mueller Hinton agar (Hi-Media). Susceptibility was tested according to the CLSI guidelines using the following antimicrobial discs - Ciprofloxacin (5µg), Erythromycin (15µg), Clindamycin (2µg), Cotrimoxazole (1.25/23.75µg), Gentamicin (10µg), Cefotaxime (30µg), Cefoperazone Sulbactam (75/30µg) and Linezolid (30µg). All the isolates were subsequently screened for Methicillin resistance, based on Kirby-Bauer disc diffusion method using Cefoxitin disc (30µg) from Hi-Media Laboratories Pvt. Ltd.

Detection of biofilm production

Biofilm production of Coagulase negative Staphylococci isolates was determined by 3 methods - Tube adherence method, Congo Red Agar method and the Microtiter plate method.

Tube adherence method - A loopful of bacterial suspension from overnight culture was inoculated into 10 ml of Trypticase Soy broth with 1% glucose in a test tube and incubated at 37℃ for 24 hrs. The broth from tubes was gradually poured down and washed with phosphate buffer solution (pH 7.3). The tubes were then dried in inverted position. After drying, the tubes were stained with 0.1 % crystal violet for half an hour. Excess stain was removed, by washing with distilled water and the tubes were then dried in inverted position. It was then observed for biofilm formation. A visible film lining the walls & bottom of the test tube was taken as positive for biofilm production.

Congo Red Agar method - CoNS isolates were plated on Congo Red Agar medium and incubated aerobically at 37°C for 24 hrs. The medium was composed of Brain heart infusion - 37g, Sucrose - 50g, Congo red dye -

0.8 g and Agar - 10g (Hi-Media) in 1 litre of distilled water. The Congo red dye was prepared as an aqueous solution in 200ml of distilled water and the rest of the constituents were added in 800ml of distilled water. They were autoclaved separately and the Congo red solution was added to the other constituents when it cooled to 55^0C. Black coloured colonies with a dry, crystalline consistency, indicated strong biofilm production, whereas red colonies indicated non biofilm producers.

Microtiter plate method - A loopful of bacterial suspension from overnight culture was inoculated in Trypticase Soy broth with 1% glucose and incubated at 37°C for 24 hrs. The culture was diluted 1:100 with fresh medium. Then 200 µL of the culture was filled into individual wells of a sterile, 96 well flat – bottomed, polystyrene microtiter plate. It was incubated at 37°C for 24 hrs. After incubation, the contents of each well were removed by gently tapping the plates. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.3) four times, to remove the planktonic bacteria. Then, 0.2 ml of 2% sodium acetate was added to each well and allowed to stand for half an hour for fixation. It was then poured out and 0.2 ml of Crystal violet (0.1%) was added to each well and allowed to stand for half an hour for staining. The isolates which formed biofilm were stained uniformly with crystal violet. The wells were washed with distilled water to remove excess stain and then kept for drying. Optical density (O.D) of stained adherent bacteria was determined using micro ELISA autoreader at a wavelength of 570 nm. A negative control well containing sterile medium was also set up and O.D was calculated. The mean O.D value for each isolate was determined by subtracting the negative control O.D value from the test O.D value.

Mean O.D value of each isolate = Test O.D value – Control O.D value.

The mean O.D values were an index of bacterial adherence and biofilm formation. They were graded as follows, according to Christensen et al. (Table 1)

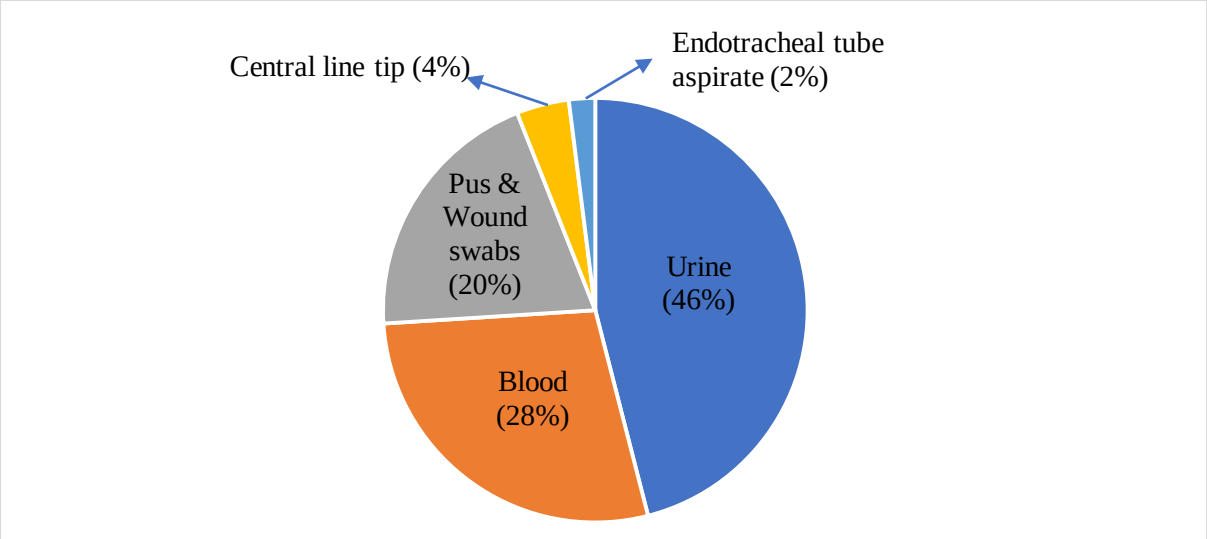
Table no 1: Grading of biofilm formation, according to the O.D values in Microtiter plate method.

MEAN O.D VALUES	ADHERENCE	BIOFILM FORMATION
< 0.120	None	None / Weak
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

III. Result

Among the 50 Coagulase negative isolates studied, 23 (46%) were isolated from urine, followed by blood (28%), pus and wound swabs (20%), central line tips (4%) and endotracheal tube aspirate (2%). Among the isolates from urine, 74% were from patients on urinary catheter, whereas 26% were from non - catheterised patients.

Figure no 1: Sample – wise distribution of Coagulase negative Staphylococci isolates



All the isolates were studied for biofilm production by the Tube method, Congo Red agar method and the Microtiter plate method. By the Tube method, 28 isolates (56%) were found to be positive for biofilm production, whereas the remaining 22 (44%) were found to be non - biofilm producers (Figure no 2). By the

Congo Red agar method, 22 isolates (44%) were positive for biofilm formation and 28 isolates (56%) were negative for biofilm formation (Figure no 3).

Figure no 2: Detection of biofilm formation by Tube method. A – Positive for biofilm production, B – Negative control

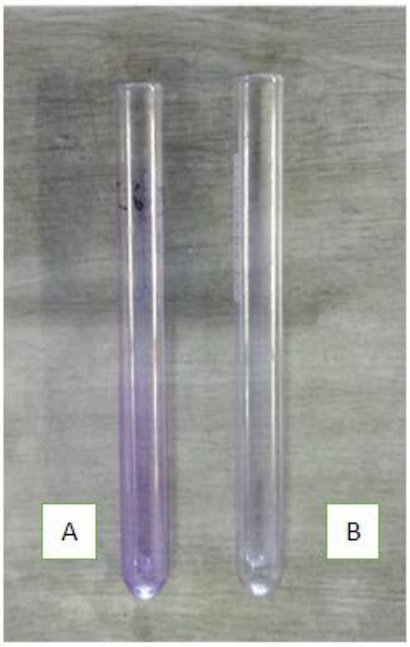
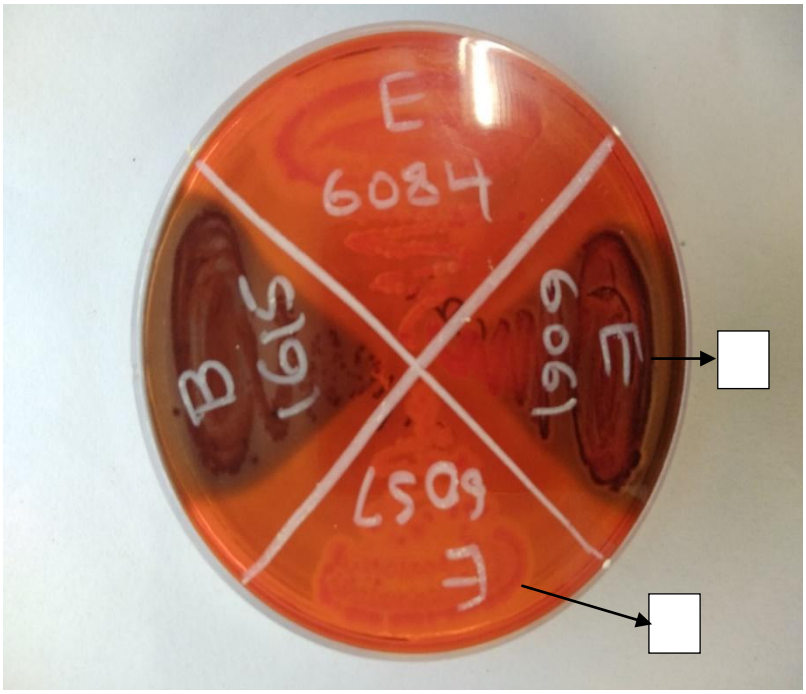
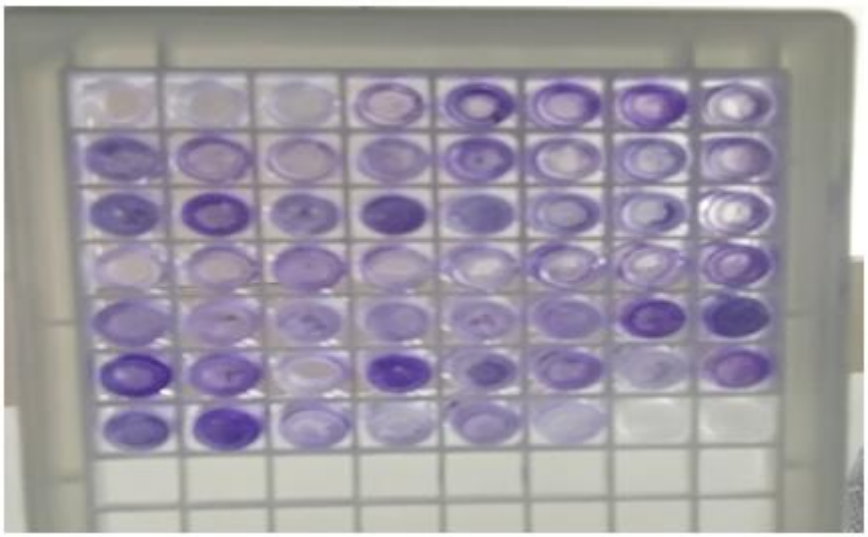


Figure no 3: Detection of biofilm formation by Congo Red agar method. A – Black colonies, positive for biofilm production, B – Red colonies, negative for biofilm production.



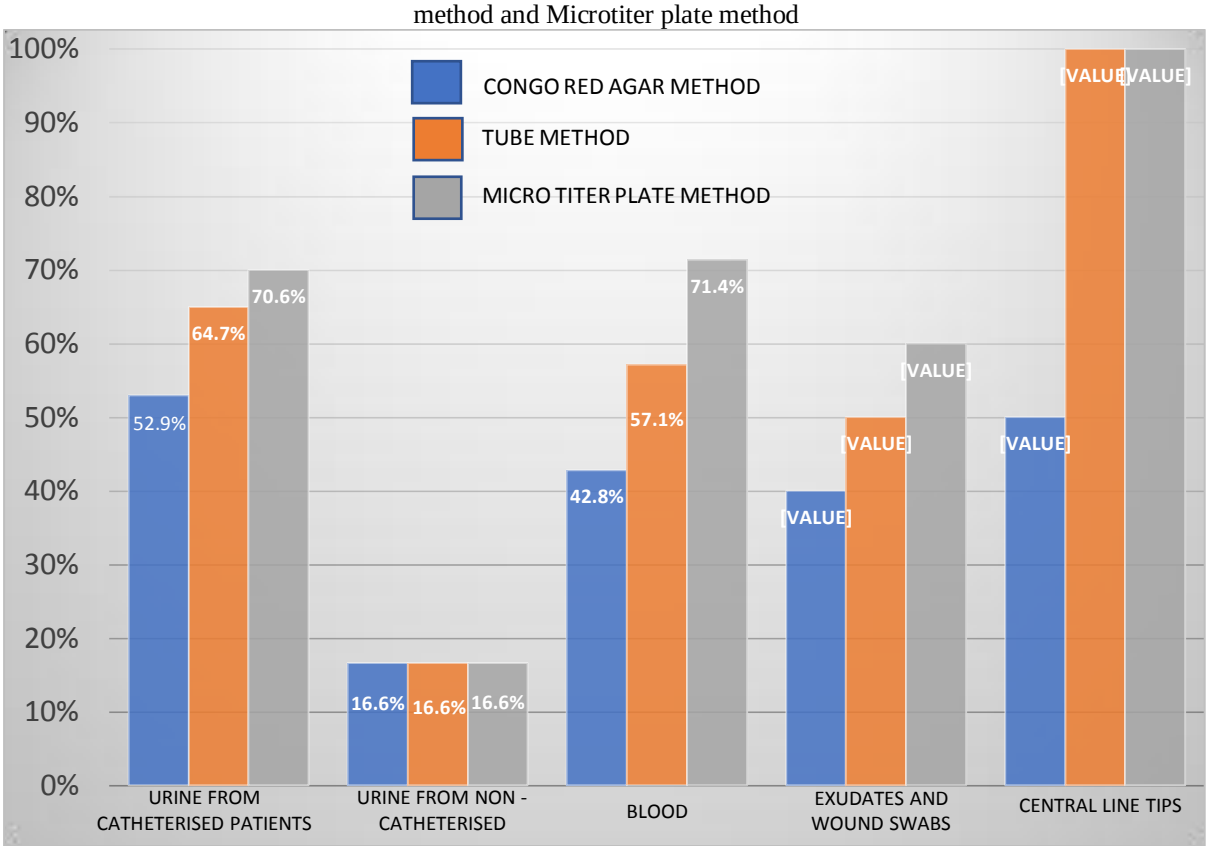
By the microtiter plate method, 26% of the isolates were found to be high biofilm producers and 38% were found to be moderate biofilm producers. Hence, biofilm production was positive in 64% of the isolates. The remaining 36% of isolates were negative for biofilm production (Figure no 4).

Figure no 4: Micro titer plate method



Among the isolates from urine of catheterised patients, 70.6% were positive for biofilm formation by the microtiter plate method whereas 64.7% and 52.9% were positive by the Tube method and Congo Red agar method, respectively. Among isolates from urine of non – catheterised patients, 16.6% of the isolates were positive for biofilm formation, by all 3 methods. 71.4% of isolates from blood, 60% of isolates from exudates and wound swabs and all isolates from central line tips and endotracheal tube aspirate were positive for biofilm production, by the microtiter plate method.

Figure no 5: Detection of biofilm production according to the sample type, by Congo Red agar method, Tube method and Microtiter plate method



Considering the Microtiter Plate method as gold standard, the Tube method and Congo Red agar method were compared. The sensitivity and specificity of the Tube method was 84.4% and 94.4% respectively, whereas for the Congo Red agar method, it was 62.5% and 88.9%. The positive predictive value of the Tube

method was 96.4%, compared to 90.9% for the Congo Red agar method. The tube method had a negative predictive value of 77.3%, while Congo Red agar method had 57.1%. Thus, the Tube method was found to be superior to the Congo Red agar method as it had a higher sensitivity and negative predictive value (Table no 2).

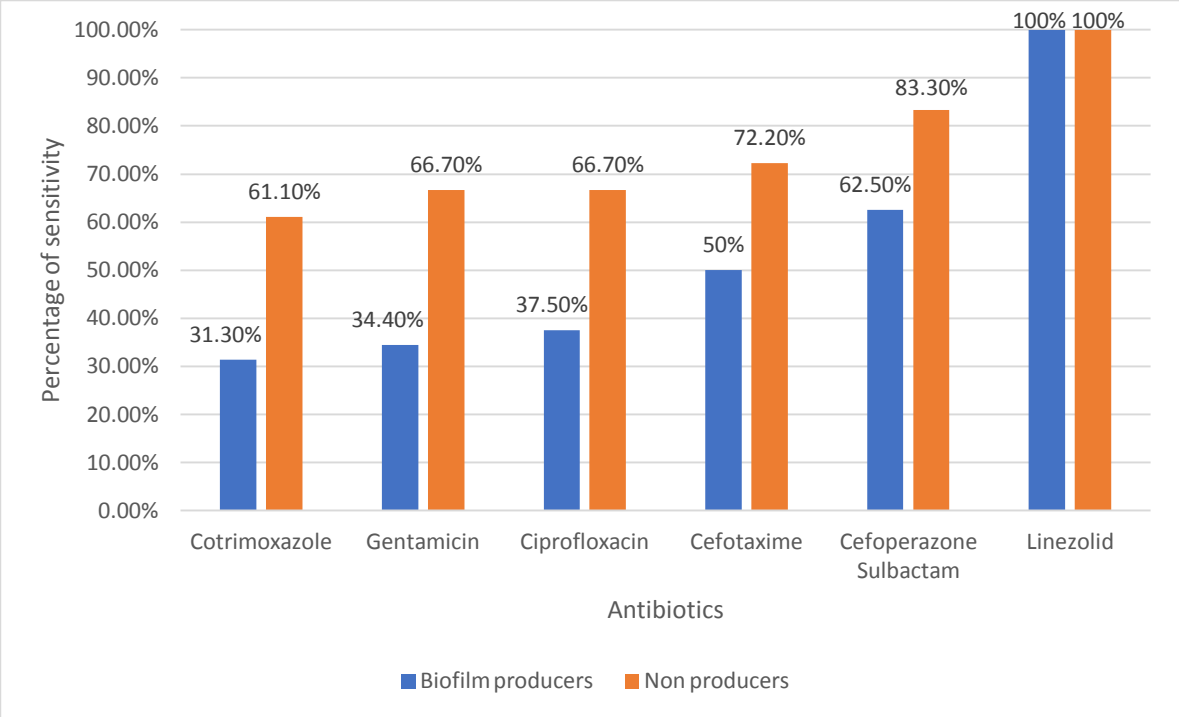
Table no 2: Comparison of Tube method and Congo Red agar method

METHOD	SENSITIVITY	SPECIFICITY	POSITIVE PREDICTIVE VALUE	NEGATIVE PREDICTIVE VALUE	ACCURACY
Tube method	84.4%	94.4%	96.4%	77.3%	88%
Congo Red agar method	62.5%	88.9%	90.9%	57.1%	72%

The antimicrobial sensitivity pattern of the biofilm forming isolates were compared with that of the non – biofilm producers. 61.1% of the non – biofilm producing isolates were found to be sensitive to Cotrimoxazole, while only 31.3% of the biofilm formers were sensitive ($p<0.05$). Among the isolates negative for biofilm production, the percentage of sensitivity was 66.7% to both Gentamicin and Ciprofloxacin, whereas it was 34.4% and 37.5%, respectively for the biofilm producers ($p<0.05$). When tested against Cephalosporins, for biofilm producers, 50% of the isolates were sensitive to Cefotaxime and 62.5% were sensitive to Cefoperazone Sulbactam. For non - biofilm producers, the percentage of sensitivity was 72.2% to Cefotaxime and 83.3% to Cefoperazone Sulbactam). All the isolates were sensitive to Linezolid (Figure no 6).

Among the 32 isolates positive for biofilm production, 15 were found to be methicillin resistant (46.9%), by the Cefoxitin disc diffusion method. Among the non – biofilm producers, 6 out of 18 isolates were found to be methicillin resistant (33.3%).

Figure no 6: Comparison of antibiotic sensitivity of Biofilm producing isolates and non – biofilm producing isolates



IV. Discussion

Coagulase negative Staphylococci are important causative agents of implanted device related infections, endocarditis, bloodstream infections, urinary tract infections (UTIs), ophthalmitis, and soft tissue infections. Biofilm formation is an important virulence factor in Staphylococcal species, associated with the infection of biomedical devices. The ability of Coagulase negative Staphylococci to form biofilm makes them adherent to these devices and enables them to establish infection.

Detection of biofilm formation amongst clinical isolates is of significance as the biofilm constitutes a reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections. A

reliable and simple method for their diagnosis is necessary to facilitate early initiation of treatment. It also paves the way for development of anti – adhesive coatings for biomedical devices and for drug development[11].

In the present study, biofilm formation was determined by 3 phenotypic methods, the Tube adherence method, Congo Red agar method and Micro titer plate method[12,13,14]. Among 50 clinical isolates of Coagulase negative Staphylococci, 32 (64%) were found to be positive for biofilm production, by the Microtiter plate method. The Tube method and Congo Red agar method detected biofilm production in 56% and 44% of the isolates respectively.

Mathur et al[15] conducted a study among Staphylococcus species in which Tissue culture plate method detected biofilm production in 53.9% of isolates. The Tube method and Congo Red agar detected biofilm formation in 41.4% and 5.3% of the isolates respectively. The Congo red agar method was the least effective in detection of biofilm formation, compared to the other 2 methods.

In the study by *Shrestha et al*[16] among Coagulase negative Staphylococci isolates, 70.4%, 59.2% and 56.3% of the isolates were positive for biofilm production by the tissue culture plate method, Tube method and the Congo Red agar method, respectively. *Agarwal et al*[17] showed that 45.9% of the CoNS isolates studied, were positive for biofilm formation. Higher rates of biofilm production were reported by *Oliveira and Cunha Mde*[18] (81%).

In the present study, by the Microtiter plate method, 26% were found to be strong biofilm producers and 38% were moderate biofilm producers. Strong biofilm producing isolates were isolated more frequently from patients on indwelling medical devices like central line, intravenous catheters and urinary catheters. *Abdel Halim et al*[19] showed that 43.3% of the isolates of Staphylococcus species were strong biofilm producers and 30.7% were positive for moderate biofilm production.

Based on the available literature[20], the Microtiter plate method was considered to be the gold standard method of biofilm detection. The statistical parameters of the other 2 methods were calculated and compared accordingly. The Tube method had a higher sensitivity, accuracy and negative predictive value than the Congo Red agar method. The Tube method had a sensitivity and specificity of 84.4% and 94.4%, respectively and an accuracy of 88%. It correlated well with the strong biofilm producing isolates, while it was less effective in differentiating the moderate biofilm producers from the non – producers. According to *Rampelotto et al*[21], the Tube method showed a sensitivity of 78%, specificity of 89% and accuracy of 85%. *Mathur et al*[15] showed that Tube method had a higher sensitivity and accuracy than the Congo Red agar method.

In the current study, 42% of the CoNS isolates were methicillin resistant. Among the MR - CoNS, 71.4% were biofilm producers. *Abdel Halim et al*[19] showed that 87.5% of the CoNS isolates were methicillin resistant and among the MR CoNS, 71.4% were positive for biofilm formation. According to the study by *Kitti et al*[22] among MR-CoNS, 38.2% of the isolates were positive for biofilm formation.

On comparing the in vitro antibiotic sensitivity pattern, the biofilm producers were found to be significantly less susceptible to the commonly used antibiotics like ciprofloxacin, gentamicin and cotrimoxazole, than the non biofilm producers. 46.9% of the biofilm producers were methicillin resistant, whereas among non biofilm producers, only 33.3 % were methicillin resistant. This can be explained by the altered gene expression and the higher prevalence of resistance determining genes among biofilm – associated bacteria. All the isolates were sensitive to Linezolid.

In the study by *Agarwal et al*[17], 57.1% of the biofilm producing isolates were sensitive to ciprofloxacin, while 77.8% of the non - producers were sensitive. *Shrestha et al*[16] showed that 57% of non biofilm producers were sensitive to cotrimoxazole but only 28% of biofilm formers were sensitive. *Abdel Halim et al*[19] showed that 53.2% of biofilm producing isolates of Staphylococci were resistant to Gentamicin, whereas only 23.6% of non biofilm producers were resistant. . Biofilm forming strains showed higher resistance to most of the antimicrobials tested than the non biofilm producers.

V. Conclusion

Microtiter plate method is a convenient, reliable and quantitative method for detection of biofilm production. It finds application in routine clinical laboratories as a screening method to determine the biofilm phenotype of the infecting organism. Among the other 2 methods, the Tube adherence method was found to be superior to the Congo Red agar method.

The biofilm producers were less susceptible to commonly used antimicrobial agents than the non – producers. Earlier detection of the biofilm phenotype of the organism, will help in guiding further treatment to prevent the risk of chronic infections and failure of antimicrobial therapy. It also helps to formulate strategies to prevent biofilm formation on indwelling medical devices.

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