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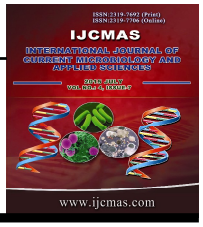

Original Research Article

Association between Biofilm Formation and Methicillin Resistance in Coagulase Negative *Staphylococci*

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Coagulase negative *Staphylococci*, Biofilm, Methicillin resistance

A B S T R A C T

Coagulase negative staphylococci (CoNS) have emerged as frequent cause of bloodstream and nosocomial infections. The major virulence factor of CoNS is believed to be the ability to produce biofilm. The present study aims to determine the sensitivity patterns of CoNS as well as the probable relationship between antibiotic resistance and biofilm formation with special reference to methicillin resistance. A total of 100 CoNS isolates from various clinical specimens like, pus (47), urine (32), blood (21) were included in the study. The isolates were identified by standard microbiological techniques. Biofilm formation was detected by two methods tube and the microtitre plate method. All isolates were tested for their antiobiotic susceptibility pattern to first line drugs like Erythromycin (E),Ciprofloxacin (Cip), Amoxycillin (Am), Cotrimoxazole (Co) and Cefoxitin (Cn) and higher antibiotics like Vancomycin (Va), Amoxycillin/clavulanic acid (Amc), Tigecycline (Tgc), Teicoplanin (Te) and Linezolid (Lz) by the Kirby-Bauer disc diffusion method according to CLSI guidelines. Among the 100 CoNS isolates, 92 showed biofilm formation by both the methods. In the tube method, out of the 74 biofilm forming isolates, 42 showed strong biofilms, 34 showed moderate biofilms. By the plate method, 45 isolates were shown to be strong biofilm producers and 30 were shown to be moderate biofilm producers. Among the first line antibiotics tested, 47 isolates were susceptible to Erythromycin, Cotrimoxazole, 56 to Ciprofloxacin and 31 isolates were susceptible to Amoxycillin. On screening for methicillin resistance using Cn, 55 isolates were found to be methicillin resistant. Among the second line antibiotics, all isolates were found to be susceptible to Vancomycin, Linezolid and Tigecycline, 72 isolates were sensitive to Teicoplanin and 31 to Amoxyclav. This study shows a definite association between biofilm production and antibiotic resistance. Hence the phenotypic detection of biofilm in CoNS should be emphasized routinely. Finally, a combination of effective detection and good infection control practices play a viital role in the battle against virulent multidrug resistant CoNS.

Introduction

Coagulase negative staphylococci are generally considered to be contaminants having little clinical significance. In recent years however, these organisms have become recognized as important agents of human disease particularly hospital acquired infections (Huebner Huebner and Goldmann, 1999).

When clinical findings are correlated with the isolation of coagulase –negative staphylococci, Staphylococcus epidermidis is by far the most frequently recovered organism, accounting for 50% to over 80% of isolates (Oliveira and Cunha, 2010).

Virulence attributes of CoNs include ability to form biofilms, presence of fibrinogen binding protein and production of fatty acid modifying enzyme, lipases (Huebner and Goldmann, 1999).

The biofilm formation is mediated by Polysaccharide Intercellular Adhesin (PIA) which is a β-1,6 linked N-acetylglucosamine polymer responsible for cell-cell attachment (Gerke *et al.*,1998).

Biofilm producing organisms exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilms, decreased growth rate and expression of resistance genes (Kim, 2001).

Multiple antibiotic resistance is characteristic of hospital strains of CoNS which tend to carry many plasmids when freshly isolated. Methicillin resistance and Vancomycin resistance have already emerged in CoNS and resistance to newer glycopeptides is not uncommon (Baird, 1996).

The present study was carried out to detect biofilm production in CoNS strains isolated from various clinical samples and to correlate this virulence marker with methicillin resistance.

Materials and Methods

A total of 100 CoNS isolates from various clinical specimens like pus (47), urine (32) and blood (21), were included in the study. The specimens were inoculated into blood agar and incubated at 37^{o}C for 24 hours. The isolates were identified as CoNS based on microscopy and standard biochemical reactions.

Detection of biofilm

Biofilm formation was studied using tube adherence and the microtitre plate method.

Tube adherence method

This is a qualitative method for detection of biofilm described by Christensen *et al.* (1982) with few modifications.

A loop ful of test organisms was inoculated in 10 mL of nutrient broth with 1% glucose in test tubes. The tubes were incubated at 37^{o}C for 24 h. After incubation, tubes were decanted and washed with normal saline and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains.

Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong (Figure 1).

Microtitre plate method

The quantitative method of adherence to polystyrene plates (TCP) proposed by Christensen *et al.* (1985) was also used in the present study, with modifications. This method is considered the gold-standard method for biofilm detection (Mathur *et al.*, 2006) Organisms isolated from fresh agar plates were inoculated in 10 mL of nutrient broth with 1% glucose. Broths were incubated at 37^{o} C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37^{o} C for 24 h. After incubation, contents of the wells were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) three times. The wells were stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying.

Optical density (OD) of stained adherent biofilm was obtained by using ELISA reader at wavelength 570 nm. The results were interpreted as follows >0.24-strong, 0.12-0.24- weak and <0.12– negative (Figure 2).

Antibiotic susceptibility testing

For the susceptibility test, isolates were suspended in nutrient broth and the suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. The antibiotic susceptibility test was performed with the agar disk diffusion method. Isolates were categorized as susceptible, moderately susceptible, and resistant, based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (2012) Penicillin (10 IU), methicillin (5 µg), gentamycin (10 µg), ampicillin (10µg), and vancomycin (30 µg) were used for antimicrobial susceptibility tests. All isolates were tested for their antiobiotic susceptibility pattern to first line drugs like Erythromycin (E), Ciprofloxacin (Cip), Amoxycillin (Am), Cotrimoxazole (Co) and Cefoxitin (Cn) and higher antibiotics like Vancomycin (Va), Amoxycillin/clavulanic acid (Amc), Tigecycline (Tgc), Teicoplanin (Te) and Linezolid (Lz).

Results and Discussion

Biofilm detection

Among the 100 CoNs isolates, 92 showed biofilm formation by both the methods.

In the tube method, out of the 74 biofilm forming isolates, 42 showed strong biofilms, 34 showed moderate biofilms (Table 1). By the plate method, 45 isolates were shown to be strong biofilm producers and 30 were shown to be moderate biofilm producers (Table 2).

Antibiogram

Among the first line antibiotics tested, 47 isolates were susceptible to erythromycin, cotrimoxazole, 56 to ciprofloxacin and 31 isolates were susceptible to amoxycillin. On screening for methicillin resistance using Cn, 55 isolates were found to be methicillin resistant. Among the second line antibiotics, all isolates were found to be susceptible to Vancomycin, Linezolid and Tigecycline, 72 isolates were sensitive to Teicoplanin and 31 to Amoxyclav (Table 3).

Table.1 Biofilm formation in tube method

TUBE METHOD		
3+	2+	1+
42	34	24

Table.2 Biofilm formation in plate method

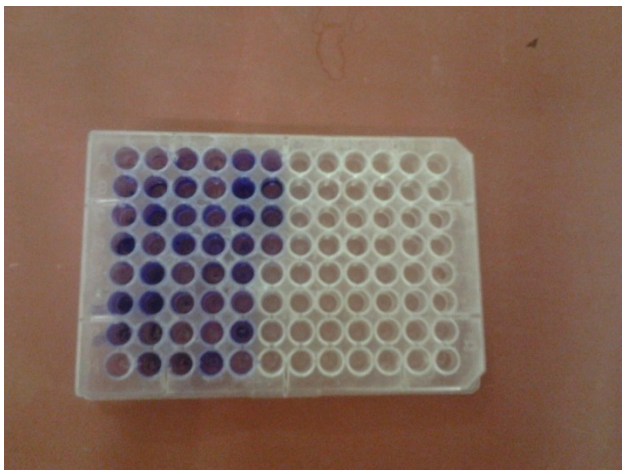
PLATE METHOD		
3+	2+	1+
45	30	2 5

Table.3 Antibiotic susceptibility testing

Antibiotic	Sensitive(S)	Resistant(R)	Antibiotic	Sensitive(S)	Resistant(R)
E	47	53	Va	100(100%)	-
Am	31	69	Amc	31	69
Cot	47	53	Te	72	28
Cip	56	44	Lz	100(100%)	-
Cn	**45**	**55**	Tgc	100(100%)	-

Figure.1 Biofilm in tube method

Figure.2 Biofilm in plate method



Biofilm facilitates infections by compromising the immune system of the patient and contributing to the failure of antibiotic therapy, which may result in recurrent infections and the emergence of multi resistant pathogens.

Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold (Stewart and Costerson, 2001). According to a publication by the National Institutes of Health (Research on microbial biofilm, 2012), more than 80% of all infections involve biofilms.

There are various methods to detect biofilm production. These include the Tube method (TM) (Christensen *et al.,* 1982), Tissue Culture Plate (TCP) (Christensen *et al.,* 1985), Congo Red Agar method (CRA) (Freeman *et al.,* 1989), bio-luminescent assay (Donlan *et al.,* 2001) piezoelectric sensors (Aparna and Yadav, 2008) and fluorescent microscopic examination (Zufferey *et al.,* 1988).

In the present study, Among the 52 CoNs isolates, 45 (90.38%) showed biofilm formation by both the methods.

In the tube method, out of the 45 biofilm forming isolates, 28 (53.84%) showed strong biofilms, 19 (36.53%) showed moderate biofilms. By the plate method, 30 (66.66%) isolates were shown to be strong biofilm producers and 17 (37.77%) were shown to be moderate biofilm producers. The results show fair correlation between both the methods. Our study results also correlate well with Ruzicka *et al.* (2004) who have reported biofilm detection in 53.7% isolates by tube method. However a higher percentage of 75 % biofilm producers in uropathogens was reported by Baqai *et al.* (2008).

According to Morales *et al.* (2004) and Cunha *et al.* (2006) the test provides reliable results for biofilm detection in CNS and is adequate for routine use.

Antimicrobial susceptibility tests are important for deciding the appropriate management. In the present study, results of antibiotic susceptibility tests showed multi-drug resistance. Variability in sensitivity and resistance patterns was noted. Maximum

resistance was observed against Amoxycillin and Amoxyclav (69%) followed by Cefoxitin, (55%), Erythromycin and Cotrimoxazole (53%), Ciprofloxacin (44%), and Teicoplanin (28%). All isolates investigated in this study were susceptible to Vancomycin, Linezolid and Tigecycline. The susceptibility to vancomycin in the investigated strains emphasized its importance. Davenport *et al.* (1986) had mentioned a link between the production of slime and the resistance of infection. Diaz-Mitoma *et al.* (1987) also found an association between antibiotic failure and slime production. In the present study, in slime producing strains, high resistance was determined against all antibiotics tested.

This study shows a definite association between biofilm production and antibiotic resistance. Hence the phenotypic detection of biofilm in CoNS should be emphasized routinely. Finally, a combination of effective detection and good infection control practices play a vital role in the battle against virulent multidrug resistant CoNS.

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