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Microbiology

DETECTION OF BIOFILM BY THREE DIFFERENT METHODS IN VIRULENT STRAINS OF CLINICALLY SIGNIFICANT COAGULASE NEGATIVE STAPHYLOCOCCI

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ABSTRACT Coagulase Negative Staphylococci(CoNS) are ubiquitious micro organisms. CoNS previously dismissed as contaminants are now emerging as important potential pathogens. The pathogenicity of this clinically significant CoNS strains is due to the production of biofilm (slime). The slime permits these microorganisms to adhere on implants and defend themselves from host immune system and antimicrobial therapy.

Ninty clinically significant CoNS isolates were included in this study. Isolates were speciated by standard microbiological methods . Biofilm detection was done by Micro titre plate method , Tube Method, and Congo red Agar method. . Of the 90 isolates tested by MTP method for biofilm formation, 13(14%) were strong biofilm producers and 34 (37.7%) were moderate biofilm producers.

When the three methods were compared Micro titre plate method was most reliable and easy method so it can be used as a General screening method for detection of Biofilm producing Bacteria

KEYWORDS : CoNS, Biofilm, Microtitre plate method, Tube Method, Congo Red Agar Method

Introduction

Historically, due to their ubiquitous nature, the coagulase Nagative staphylococci have been regarded as saprophytes with little pathogenic potential. However , recently CoNS have emerged as significant pathogens, particularly in infections associated with medical devices and in immuno compromised patients.One of the major problems facing the clinical laboratory is in distinguishing clinically significant pathogenic strains of CoNS from any contaminant strains [2,7]

Biofilms

CoNS, especially S. epidermidis, frequently infect biomedical implants, transcutaneous devices such as intravascular and peritoneal catheter [10] CSFshunts [1] and prosthetic cardiac valves [1,10] The pathogenesis of biomedical implant infections caused by S. epidermidis can be separated into two distinct phases:

I) Primary bacterial adherence to the surface of the device and
II) the production of a stable biofilm matrix over the surface of the device

It is now well documented that biofilms are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy and removal of infected device becomes necessary [5,6].

A number of tests are available to detect slime production by Staphylococci. The methods include Micro tittre plate (MTP) method [3,11] Tube method (TM) [3,13] Congo red agar (CRA) [14] bioluminescent assay [15] and light or fluorescence microscopic examination [8,9] However, these methods often suffer from severe analytical limitations.

The adherence of CoNS to smooth surfaces was assayed by measuring the optical densities of stained bacterial films adherent to the floors of micro titre plates. The optical densities serve as a quantitative model for the study of the adherence of CoNS to medical devices.

MATERIALS AND METHODS

This study was conducted in Coimbatore medical college hospital, Coimbatore for a period of 1 year .A total of 90 clinically significant CoNS Strains were isolated from deep wounds, blood samples, pus, urine samples, body fluids, and IV catheter tips by conventional cultural methods.

The strains collected were initially identified by colony morphology, Gram staining, catalase, slide and tube coagulase .The isolates were speciated by using standard biochemical tests according to the reference method.

Detection of biofilm production:

Early biofilm formation detection might help in treatment because in long standing cases they may be very damaging and may produce immune complex squeal. There are three methods for detection of biofilm: Micro Titre plate method, Tube Method and Congo Red Agar method.

1.Micro Titre Plate Method [3,11]

Overnight culture of the isolate from nutrient agar plate is inoculated into Trypticase soy broth (TSB with 1% glucose prepared in Different dilutions (1:20, 1:40, 1:80, and 1:100) and loaded into 96 wells of flat bottom microtitre plate. Plates are covered and incubated at 37°c for 24 hours in aerobic condition, the well are then decanted and washed three times with Phosphate buffer saline (PBS). After washing, fixed with methanol for 15 minutes. Then the wells are decanted and stained with crystal violet for 20 minutes. The wells are again decanted and washed with distilled water. Finally 33% glacial acetic acid is added to the wells to extract the stain and adherence of the stained cells to the wells. Optical density of each well is measured at 490 nm using an automated ELISA plate reader

2.Tube method [3,13]

10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopfull of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37 c for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline pH7.3.The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position and observed for biofilm formation. Biofilm Production 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 biofilm formation was scored as 0-absent, 1-weak, 2-moderate, 3-strong.

3.Congo Red Agar method [3,14]

An alternative method for screening biofilm formation, as described byFreeman et al for Staphylococcal isolates.The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5gm/l), agar number 1 (10 gm/l) and Congo red dye (0.8 gm/l). Congo red stain was

prepared as concentrated aqueous solution and autoclaved at 121 C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55 C. Plates were inoculated with test organism and incubated at 37 C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; weak producers usually remained pink, though occasional darkening at the centre of colonies was observed.

RESULTS

In the present study, out of 90 CoNS isolates S. epidermidis was the major species that produced slime (67%), followed by S.haemolyticus (27%), S.saprophyticus (50%), and S.lugdunensis (50%).

Chart 1

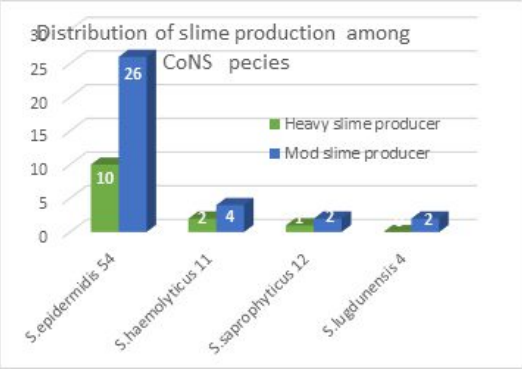
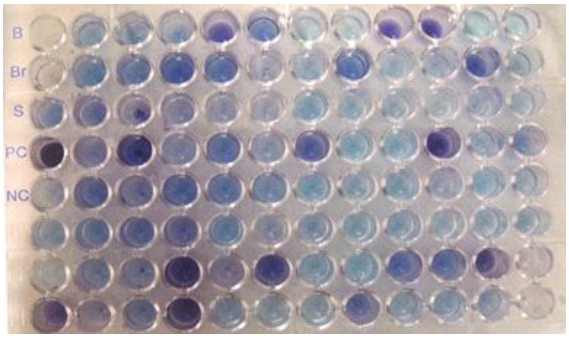


FIG 1. BIOFILM DETECTION BY MTP METHOD



Biofilm formation	MTP	TM	CRA
High (13)	13	12	0
Moclerate(34)	34	29	3
Nonbiofilm producers(43)	43	49	87

Forty seven out of 90 (52%) isolates produced slime by MTP method

Table 1 Comparison of biofilm formation by MTP, TM and CRA methods

Of the 90 isolates tested by MTP method for biofilm formation,13 (14%) were strong biofilm producers, and 34 (37.7%) were moderate biofilm producers. Results from the TM showed good correlation with those obtained with the MTP method. A total of 12 (11.8%) isolates were strong biofilm producers, while 29 (32%) were moderate biofilm producers. In contrast by CRA method though 1 (1.97%) isolate produced occasional blackening of colonies, it did not display the dry crystalline morphology. After 24-48 hours only 2 (2.2%) isolates produced red colonies with dry crystalline morphology.

Discussion

The most important colonizing and virulence factor ie biofilm production in CoNS was evaluated. Forty seven out of 90 (52%) isolates produced slime Comparable with results of Deighton et al(1988) [16]Kotilainen et al (1990[17] and Mathur et al(2006[3]

In the present study, out of 90 CoNS isolates S. epidermidis was the major species that produced slime (67%). Comparable pattern of slime production by S.epidermidis species was observed by Rathinam. K. et al (1993) [18] (62%), Kleeman et al (1993)[4] (64.5%) & Deighton et al [16] 1988 (53%)

When the biofilm production was compared by 3 different methods, MTP method was the most sensitive as it could detect all the slime producers, whereas TM correlated well with the MTP method for strong biofilm producing strains; weak slime producers were difficult to discriminate from biofilm-negative isolates. CRA does not correlate well with either of the above two methods. The MTP method was found to be the most accurate and has the advantage of being a quantitative model to study the adherence of staphylococci on biomedical devices, a finding that correlates very well with the reports by Christensen et al(1983)[4] and Mathur et al(2006)[3]

CONCLUSION

The study of CoNS isolated from various sources indicated 66% of isolates as clinically significant and S. epidermidis as the most frequently isolated species. The most important virulent factor biofilm production in CoNS was associated mostly in prosthetic device related conditions like orthopedic wounds, PDF, CSF and IV catheters and biofilm on implanted foreign material can cause major medical and economic sequel. It is necessary to predict the potential pathogenicity by biofilm demonstration in vitro which can be easily done by MTP method each clinical isolates, early in the course of infection, which will be helpful to provide appropriate antibiotic therapy and prevent implant removal.

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