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Study of biofilm production and antimicrobial resistance pattern of the bacterial isolates from invasive devices

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Abstract:

Background: Biofilms are a great threat to invasive devices causing slow persistent infections. Biofilms interfere in antibiotic therapy, undergo gene transformation to become highly virulent, develop quorum sensing and protect bacteria from host immunity. **Objectives:** 1. To isolate and identify the bacteria from invasive devices and to study their antibiotic susceptibility pattern. 2. Phenotypic detection of biofilm production of the bacteria by three different methods. **Materials and Methods:** A total of 229 samples like peripheral venous catheter tips, urine from Foley's catheter, central venous catheter tips and endotracheal tube aspirates were collected from January 2011 to December 2011 and processed. Organisms were isolated and antimicrobial susceptibility was done as per CLSI guidelines. Biofilm detection was done by the three different methods a) Tube method by Christensen in 1982 b) Congo red agar method by Freeman in 1989 c) Tissue culture plate method by Christensen, 1985. **Results:** A total of 55 bacterial isolates were obtained from 229 samples. Out of 55 bacterial isolates, 4(7%) were positive by all three methods. *Methicillin resistant Staphylococcus aureus* (10%) and 10% extended spectrum betalactamase producing *Klebsiella* species produced biofilms, where as sensitive strains did not produce. **Conclusion:** Routine and advanced studies of the biofilm production will help in making better usage of the invasive devices without any critical complications.

Key words: Biofilm; Colonization; Drug resistance; Invasive devices; Slime layer.

Introduction

Biofilm is an aggregate of microorganisms in which cells are stuck to each other on to a surface and produce matrix of extracellular polymeric substance. Biofilm formation is regulated by the expression of polysaccharide intracellular adhesion molecule, which mediates cell to cell adhesion and is a product of icaADBC genes.

Biofilm formation is a developmental process in which bacteria undergo a regulated lifestyle, switch from a nomadic unicellular state to a sedentary multicellular state, where subsequent growth results in structured communities and cellular differentiation [1]**.** The terms sessile and planktonic have evolved to describe surface-bound and free floating microorganisms respectively. The surface of interest to which sessile organisms are attached can be either abiotic (inert materials) and biotic (living tissues or cells). The bacteria communicate with each other in biofilms by chemical signals facilitating an interactive and coordinating activity. Bacteria prefer a community based surface bound sedentary life style to a nomadic existence. This tendency was recognized early in the 20th century as a habitual characteristic of aquatic bacterial populations [2]**.**

In a study it was observed that colonization of Armstrong-style silicone, fluoroplastic, ionized modified silicone and silver oxide-coated Armstrong-style silicone tubes by *Pseudomonas aeruginosa***,** *Staphylococcus aureus***,** and *Staphylococcus epidermidis* in trypticase soy broth [3]. They found that all three organisms developed biofilms on these tubes. Catheter-tip colonization was defined as a positive semi-quantitative culture of an intravascular catheter segment (>15 colony-forming units) and was synonymous with local colonization of the catheter. Catheter-related bloodstream infection was defined as isolation of the same strain from the catheter segment, a hub, or infusate and from one or more peripheral blood cultures, as proven by restriction-fragment subtyping [4,5]. Biofilms of various medical devices have been studied extensively over the last 20 years, much of the published research used very basic tools, such as viable culture techniques and scanning electron microscopy, to characterize the microbial diversity and visualize the biofilms. For certain devices, such as urinary catheters and contact lenses, research has also elucidated the susceptibility of various materials to bacterial adhesion and biofilm formation.

Catheters are colonized by single species initially, such as *Staphylococcus epidermidis***,** *Enterococcus faecalis***,** *Escherichia coli*, or *Proteus mirabilis***.** As the catheter remains in place, the number and diversity of organisms increase, mixed communities develop, containing organisms such as *Providencia stuartii***,** *Pseudomonas aeruginosa***,** *Proteus mirabilis***,** and *Klebsiella pneumoniae*. Other organisms isolated from urinary catheter biofilms include *Morganella morganii***,** *Acinetobacter calcoaceticus*, and *Enterobacter aerogenes* [6].

The present study attempts to isolate bacteria forming biofilms from invasive devices in a tertiary care hospital. The main objective of this study is to isolate the organisms from various invasive devices, phenotypic detection of biofilm production and also their drug resistance pattern.

Materials and Methods

In the present study, 229 invasive devices such as central venous catheter tips from cardiology and nephrology departments, peripheral line tips, endotracheal tube aspirates, urine from indwelling Foley's catheter from intensive care units of various departments were collected for a period of one year from January 2011 to December 2011.

Culture Method:

Catheter cultures were performed by the Semiquantitative method and by a modified quantitative method [4,5]. Each catheter segment was transferred to the surface of a 90mm blood agar plate and rolled back and forth across the surface at least four times, after this, the catheter lumen is flushed with 2ml of trypticase soy broth, 0.1ml of broth was streaked onto blood agar plates. Thus the internal surface of each catheter segment was cultured by the quantitative method and the external surface was cultured by the semiquantitative technique. Then the colonies were counted after 24-38 hrs of incubation.

Primary isolation was done on three routine media such as are blood agar, Mac Conkey agar, & Chocolate agar. Presumptive identification is inferred by gram staining of the smear from the colonies obtained on primary plating media. Further identification is followed by performing the biochemical tests under sterile conditions and in a proper method with controls being satisfactory [7]. Antimicrobial susceptibility testing is done by Kirby Bauer Disc Diffusion Method by using commercial antibioltic dics (Himedia) [8].

Detection of biofilm production

Out of 55 isolates, 11(20%) were *Coagulase Negative Staphylococcus*, 10(18%) were *Staphylococcus aureus* and *klebsiella species* each, 08(15%) were *Escherichia coli, Pseudomonas species* were 06(11%), *Citrobacter species and Acinetobacter species* were 03(05%) each and 01(02%) each of *Proteus species, Morgenella species, Micrococcus species and Enterococcus species*.

The isolates were tested by three phenotypic methods for biofilm detection.

1. Tube method by Christensen GD [9,10]
2. Tissue culture plate method by Christensen GD [9,10]
3. Congo red agar method by Freeman DJ [11]

These three methods were standardized with *Staphylococcus epidermidis* ATCC 35984, which is the known biofilm producer and *Staphylococcus epidermidis* ATCC 12228 non-slime producer as controls [12].

Tube Method (TM)

Trypticase soy broth with 1% glucose (TSBglu) media (10mL) was inoculated with loopful of bacterial colonies from culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with phosphate buffer saline (pH 7.3) and the 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. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube.

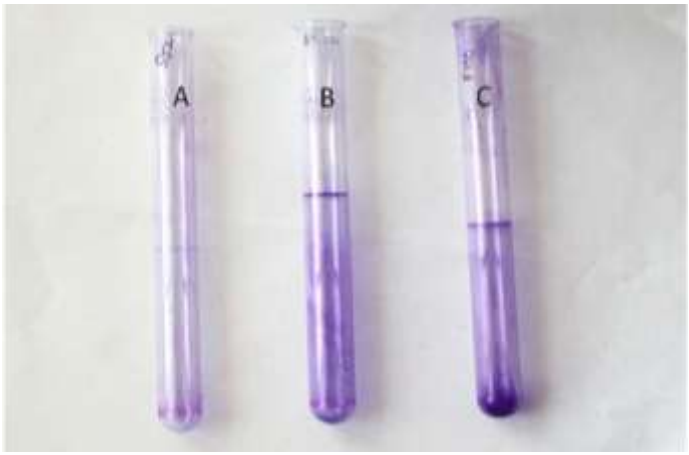


Figure 1: Tube method A. No visible film. B & C Visible film lining the wall and bottom of tube.

Congo red agar Method (CRA)

Congo red was prepared as concentrated aqueous solution separately from other constituents of media, and autoclaved at 121°C for 15 minutes, and then added to the agar which is cooled at 55°C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

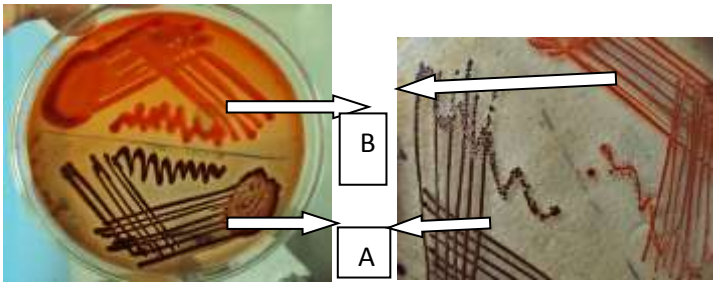


Figure 2: Congo red agar A. Black colonies indicating slime production B. Pink colonies indicating no slime production

Tissue Culture Plate Method (TCP)

Isolates from fresh agar plates were inoculated on trypticase soy broth with 1% glucose (TSBglu) media and incubated for 18 hours at 37°C and then diluted 1in100 with fresh medium. Individual wells of sterile polystyrene, 96 well-flat bottom tissue culture plates (Tarson Kolkata, India) were filled with 0.2 ml aliquots of the diluted cultures and only broth served as control to check sterility and non-specific binding of media.

The tissue culture plates were incubated for 18 hours and 24 hours at 37°C. After incubation, the content from 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) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent cells usually form biofilm and wells were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria were determined with a micro ELISA auto reader (model PR 601, Qualigens) at wavelength of 570 nm (OD570 nm). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values.

Interpretation of Results:

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

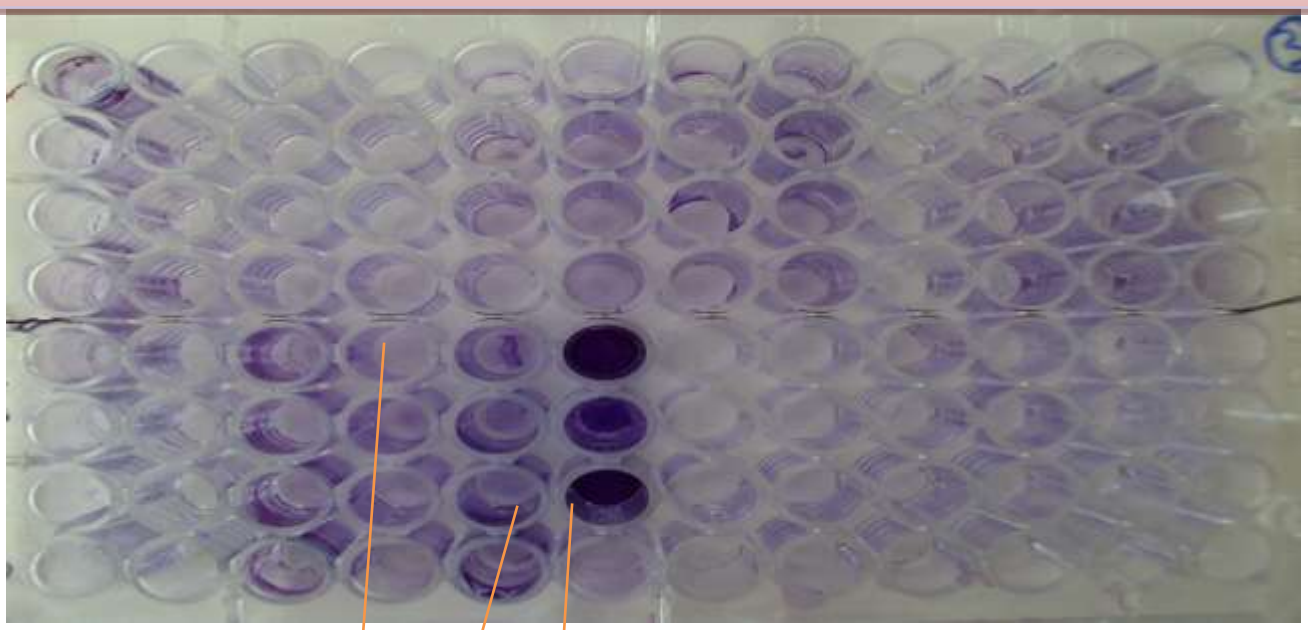
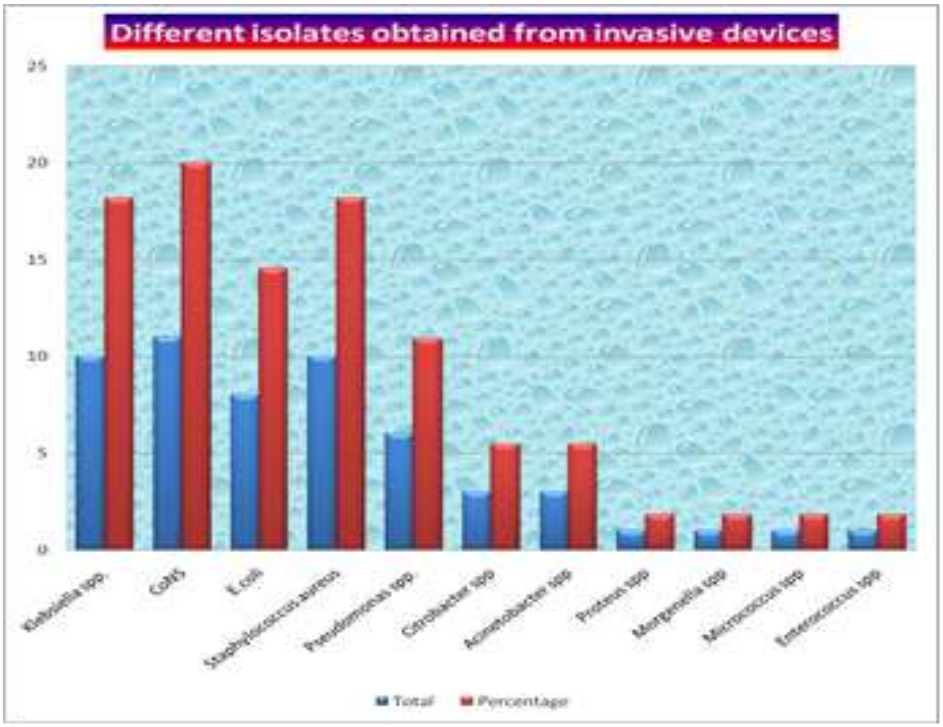


Figure 3: Tissue culture plate method A. No colour B & C. Violet colour indicating biofilm production.

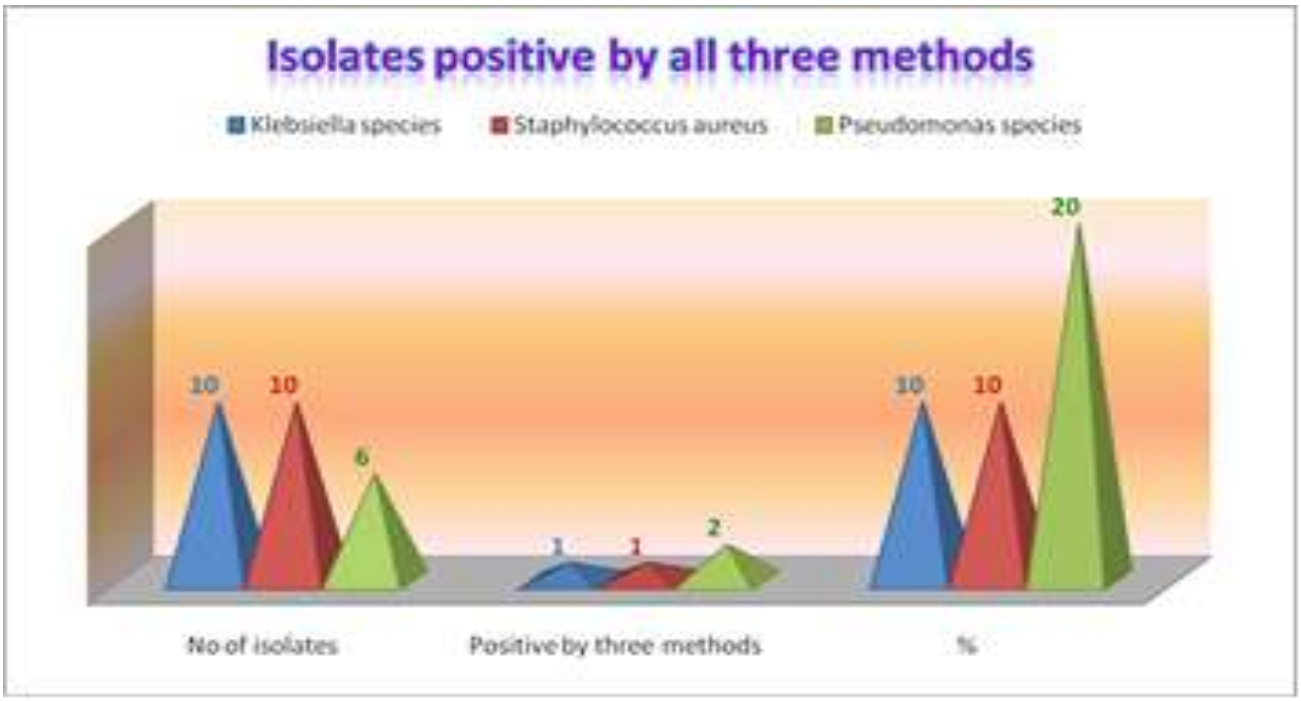
Results

Graph no 1: Showing different isolates grown from various invasive devices



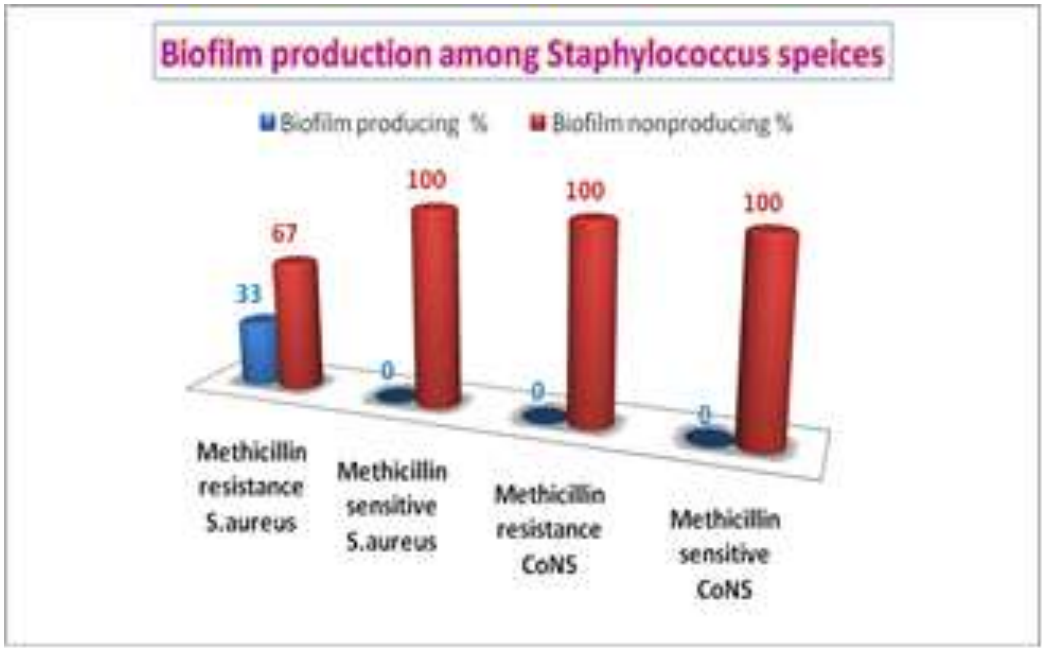
Out of 55 isolates, 11(20%) were *Coagulase Negative Staphylococcus*, 10(18%) were *Staphylococcus aureus* and *Klebsiella species* each, 08(15%) were *Escherichia coli, Pseudomonas species* 06(11%), *Citrobacter species and Acinetobacter species* were 03(05%) each and 01(02%) each were *Proteus species, Morgenella species, Micrococcus species and Enterococcus species*.

Graph no 2: Isolates positive by all the three methods



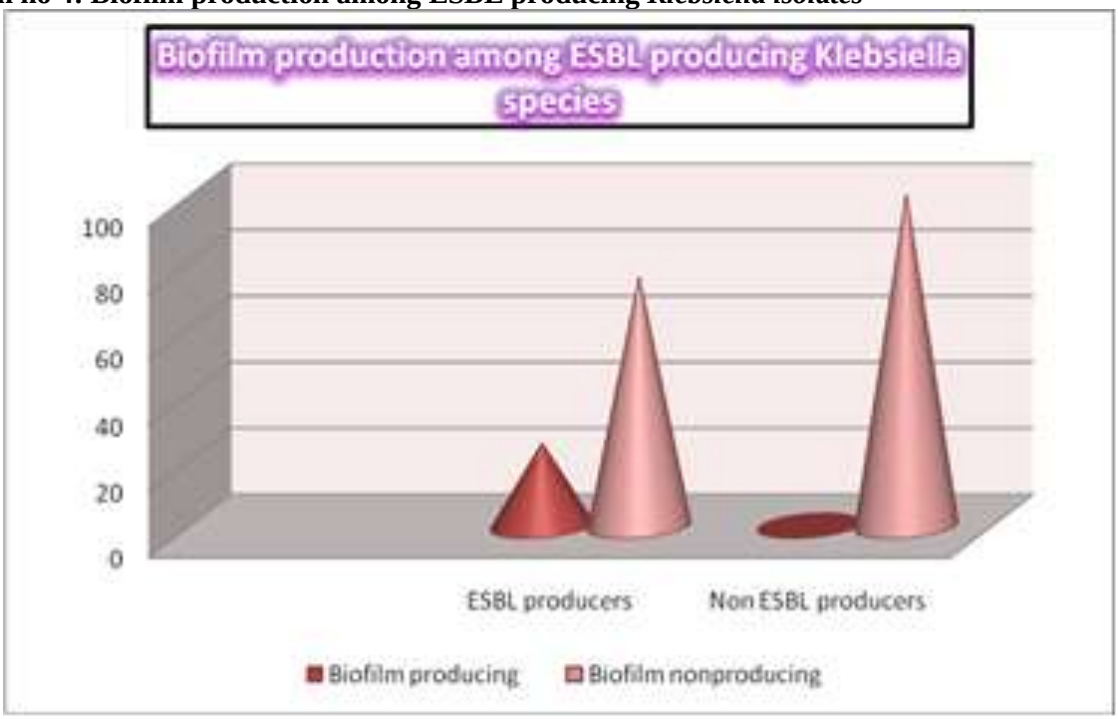
Among the 55 isolates obtained from various invasive devices, 4(7%) were positive by all three methods. Among the four, 2 were *Pseudomonas species*, 1 each of *Klebsiella species* and *Staphylococcus aureus.*

Graph no 3: Comparison of drug resistance and biofilm production by all three methods among *Staphylococcus species*



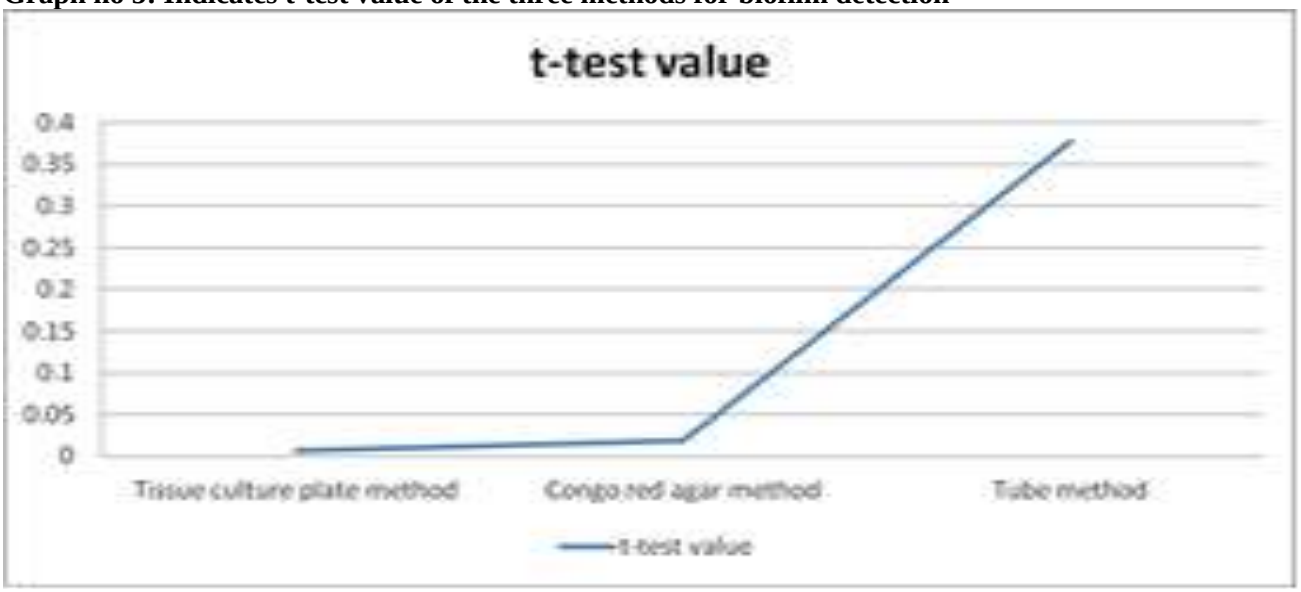
In this graph, among 21 *Staphylococcus species*, 10 were *Staphylcocccus aureus* and 11 were *Coagulase negative staphylococcus*. Among *Staphylococcus aureus* only 1 isolate which was methicillin resistant showed biofilm production by all three methods.

Graph no 4: Biofilm production among ESBL producing *Klebsiella isolates*



This graph shows that among the *Klebsiella species*, only extended spectrum betalactamase producers were showing biofilm production by all three methods.

Graph no 5: Indicates t-test value of the three methods for biofilm detection



Graph 5 indicates the significant method in detecting the biofilm positive samples by eliminating the biofilm negative isolates. Tissue culture plate method has the lowest t-test value of 0.006 and is followed by Congo red agar method which

has 0.019 and tube method is at last with the t-test value of 0.378. The method with the lowest t-test value is the highest significance.

Discussion

In a study by Donlan [13], the organisms most commonly isolated from central venous catheters are C*oagulase negative Staphylococcus, Staphylococcus aureus, Pseudomonas species, Klebsiella species, and Enterococcus species*. These are known to cause nosocomial infections, and may be the common cause of colonization in indwelling medical devices even responsible for biofilm production. These microorganisms survive in hospital environments despite unfavorable conditions such as desiccation, nutritional starvation, and antimicrobial treatment. It is hypothesized that their ability to persist in these environments, as well as their virulence, is a result of their capacity to colonize medical devices.

In our study, from endotracheal tube aspirates predominantly gram negative bacilli were isolated, among eleven isolates three of each were *Klebsiella species and Pseudomonas species* followed by two isolates of *Staphylococcus Species* and one each of *Coagulase Negative Staphylococcus, Escherichia coli and Acinetobacter Species.* In a study by Feldmann [14], it was documented that the interior of endotracheal tube of patients undergoing mechanical ventilation can rapidly be colonized with gram negative microorganisms which commonly appeared to survive within the biofilm layer. It appears that colonization of endotracheal tube may begin from as early as 12 hours and become most abundant by 96 hours. Colonization of endotracheal tube with microorganisms which causes nosocomial pneumonia appears to persist in many cases, despite successful treatment of the previous pneumonia.

Stickler [15,16] showed that the organisms commonly contaminating urinary catheter and developing biofilm are *Staphylococcus epidermidis, Enterococcus faecalis, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumonia* and other gram negative organisms. In our study, out of 55 isolates, 9 isolates were obtained from urinary catheter samples. The most common isolates were four *Escherichia coli* and *Staphylococcus species* each followed by one *Klebsiella species*.

The percentage distribution of biofilm producing organisms among various invasive devices is influenced by several factors such as number of patients, severity of the disease and may also vary according to tertiary care hospitals. In our study 27 (49%) CVP tips yielded growth from a total of 229 samples. In a tertiary care hospital, central venous catheters pose a greater risk of device related infection as any other indwelling medical devices. These catheters are inserted for administration of fluids, blood products, medications, nutritional solutions and hemodynamic monitoring.

In our study among the isolates with biofilm production detected by all the three methods ,were found to be highly resistant strains. Among the ten isolates of *Klebsiella* species, four were found to be ESBL producers and six were non ESBL producers. The organisms which formed biofilm are also ESBL producers. Among *Staphylococcus* species, only methicillin sensitive strains showed biofilm production.

In a study by Sangita Revdiwala [6], it showed that antimicrobial drug resistance profile of bacterial isolates producing biofilms are multiple drug resistance strains. Which included ESBLs, carbapenemase producers in gram negative organisms and methicillin resistance among the gram positive organisms. Resistant strains isolated in the hospital environment are responsible for contamination and colonization of different indwelling medical devices used for patients' management that complicated the course of treatment.

Conclusion

Specifically, a better understanding of the genes responsible for biofilm formation in bacterial infections will provide the biological framework necessary for studying biofilms. In addition, new methods for measuring local compositions of biofilm components will guide in appropriate application of soft matter physics principles. This makes the better usage of the invasive devices without any critical complications and helps in providing better treatment.

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