

Original Research Article

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Comparison of Antimicrobial Susceptibility Pattern of Biofilm Producing and Nonbiofilm Producing *Staphylococci* Isolated from Various Clinical Samples of Indoor Patients

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ABSTRACT

Keywords

Biofilm detection, Staphylococcus, tissue culture plate (TCP), Congo red agar (CRA), Tube method (TM).

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Biofilms are group of microorganisms encased in an exopolymeric coat. They have been associated with a variety of persistent infections that respond poorly to conventional antibiotics. To evaluate three different methods for detection of biofilm formation in staphylococci. For detection of biofilm formation, 120 clinical isolates of staphylococcus spp. were screened by Congo red agar (CRA) methods, Tube methods (TM) and Tissue culture plate (TCP) methods. Out of 120 *Staphylococcus* spp., 72 were coagulase positive staphylococci (CPS) and 48 were coagulase negative staphylococci (CNS). 59.72% of CPS and 58.33% of CNS were slime producers. 71 isolates were detected as slime producer by TCP method, 56 by TM and 34 by CRA method. High resistances to conventional antibiotics were shown by biofilm producers. The TCP method was found to be most sensitive, accurate and reproducible screening method for detection of biofilm formation by staphylococci and has advantage of being a quantitative model to study the adherence of staphylococci on bio-medical devices.

Introduction

A biofilm is a complex aggregate of microorganisms in which cells adhere to a surface to each other (micro colony). These adherent cells are embedded within a self produced matrix of extracellular polymeric substances (EPS)/slime, which is made up of proteins and polysaccharides. Biofilm are universal, occurring in aquatic and industrial water systems as well as large number of environments and medical devices relevant for public health (Bhardwaj *et al.*, 2012).

Microorganism growing in a biofilm are highly resistant to antimicrobial agents by one or more microorganisms. Biofilm associated microorganisms have been shown to be associated with several human diseases, and to colonize a wide variety of medical device (Chandler *et al.*, 1997). Chronic infections due to biofilm remain a major challenge for the medical profession and are of great economic relevance because traditional antibiotic therapy is usually not sufficient to eradicate these infection.

Biofilm producing *Staphylococci* frequently colonize catheters and medical devices and may cause foreign body related infections. They easily get attached to polymer surfaces (Deyal *et al.*, 2012; Gautam *et al.*, 2007; Kandler *et al.*, 1986). Crampton *et al.*, showed that like *S. epidermidis*, *S. aureus* also has *ica* locus encoding the function of intracellular adhesion and biofilm formation (Kloos *et al.*, 1985). According to a recent public announcement from National Institute of Health, more than 60% of all infections are caused by biofilm (Kyrikou *et al.*, 2007). Biofilm organisms have an inherent resistance to antibiotics, disinfectants and germicides.

Biofilms have been found to be involved in a wide variety of microbial infections in the body. Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle ear infections, formation of dental plaque, gingivitis, coating contact lenses and less common but more lethal processes such as endocarditis infections in cystic fibrosis and infection of permanent indwelling devices such as joint prostheses and heart valves (Leck, 1999). Most recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds (Leck, 1999; Muller *et al.*, 1993). In recent years, implanted medical devices have been crucial in advancement of patient care and management of serious medical conditions.

Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are certain factors which influence biofilm formation (Sambrook *et al.*, 1989).

Pathogenic Mechanisms (Sheppard, 2008)

Different pathogenic mechanisms of the biofilms have been proposed. These include:

1. Biofilm allow attachment to a solid surface;
2. Division of labor" increases metabolic efficiency of the community;
3. Evade host defenses such as phagocytosis;
4. Obtain a high density of microorganisms; Exchange genes that can result in more virulent strains of microorganisms;
5. Produce a large concentration of toxins;
6. Protect from antimicrobial agents; Detachment of microbial aggregates transmits microorganisms to other sites.

The biofilm form of each bacterial type is less sensitive to antimicrobials. Severe mechanisms have been proposed to explain this phenomenon.

1. Environmental gradients within the biofilm may result in varying antibiotic concentrations reaching the individual target bacterium, thus protecting some cells within the colony.
2. Varying chemical and pH gradients may affect antimicrobial action.
3. Properties of the biofilm matrix may prevent antibiotic penetration.
4. Within the colony, non-dividing or metabolically quiescent cells (termed persistors) can regenerate the biofilm.

Both *Staphylococcus epidermidis* and *Staphylococcus aureus* are important causes of infections associated with catheters and other medical devices. It has recently been shown that not only *S. epidermidis* but also *S. aureus* can produce slime and carries the *ica* operon responsible for slime production. In the operon, coexpression of *icaA* and

icaD is required for full slime synthesis (Sivan, 2011).

We plan to compare the antimicrobial susceptibility of biofilm producers and nonbiofilm producers *Staphylococci* isolated from our setup, and to find out their antimicrobial susceptibility pattern. This will help our clinicians in prescribing appropriate antibiotic against chronic infection for patients having indwelling devices, chronic rhino sinusitis and non healing wound(burn) who have chances of infections by biofilm producing organism.

The present study was undertaken to detect the prevalence of biofilm producers and nonbiofilm producers *Staphylococci* isolated from clinical samples in laboratory at the Department of Microbiology, S.M.S. Medical college and attached Hospitals, Jaipur, Rajasthan comparing three different methods, viz. tissue culture plate (TCP) method, tube method (TM) and Congo red agar (CRA) method and to assess and compare the antimicrobial susceptibility pattern of biofilm producing and nonbiofilm producing staphylococci.

Materials and Methods

The present study was conducted in the Department of Microbiology, SMS Medical College and Attached Hospitals, Jaipur (Rajasthan), over a period of one year From October 2011 to September 2012. A total of 120 non-repetitive clinical isolates of *Staphylococci* obtained from various clinical samples were included in study. Samples were received from patients admitted in the various wards and intensive care units (ICUs) of the hospital during this period. Detailed relevant history such as age, sex, primary disease and associated predisposing diseases was obtained from patients. All the specimen were inoculated on appropriate culture media like blood agar, Mac Conkey

agar and incubated for 24 hour at 37°C. After incubation organism were identified by standard microbiological procedures gram stain appearance, colonial morphology, catalase test, coagulase test (Tamura *et al.*, 2007). Reference strains of *Staphylococcus epidermidis* ATCC 35984 (high slime producer), ATCC35983 (moderate slime producer) and ATCC 12228 (nonslime producer) were also included in this study. Detection of biofilm production of 40 *Staphylococci spp.* was done by following three methods. 1. Tissue culture plate (TCP) method 2. Tube method (TM) 3. Congo red agar (CRA) method.

Tissue Culture Plate: Method 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The broth was incubated at 37°C for 24 hours. The culture was further diluted 1:100 with fresh medium. 96 wells flat bottom tissue culture plates were filled with 0.2 ml of diluted cultures individually. Only sterile broth was served as blank. Similarly control organisms were also diluted and incubated. All three controls and blanks were put in the tissue culture plates. The culture plates were incubated at 37°C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria.

Classification of bacterial adherence by TCP Method

Mean OD values	Adherence	Biofilm formation
<0.120	None	None/weak
0.120-0.240	Moderate	Moderate
≥0.240	Strong	High

Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA autoreader at wave length 570 nm. Experiment was performed in triplicate and repeated thrice. Average of OD values of sterile medium were calculated and subtracted from all test values (Tamura *et al.*, 2007).

Tube Method: 10 ml Trypticase soy broth with 1% glucose was inoculated with a loopful 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 (pH 7.3). The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position. In positive biofilm formation, a visible stained film was seen lining the wall and bottom of the tube. Experiments were done in triplicate for 3 times and read as absent, weak, moderate and strong.

CongoRed Method - 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.

Antibiotic sensitivity test was done on Muller-Hinton agar (MHA) using following

antibiotic discs- amoxicillin-clavulanic acid (20/10 mg), Clindamycin (2 µg), oxacillin (1µg), ciprofloxacin(5µg), erythromycin (15µg), ticarcillin-Clavulanic acid (75/10µg) gentamicin (10µg), doxycycline (30µg), linezolid (30µg), vancomycin (30µg), Antibiotics discs were procured from HiMedia Laboratories.

ATCC *Staphylococcus aureus* 25922 was used as control. Antibiotic sensitivity test was done as per Kirbybauer disc diffusion method.

Results and Discussion

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices and causing nosocomial infection (Sivan, 2011). The different mechanisms, by which biofilm producing organism cause disease are detachment of the cells from medical device biofilm causing bloodstream and urinary tract infection, endotoxin formation, resistance to host immune system and generation of resistance through plasmid exchange.

In the present study, we isolated 120 strains of Staphylococci from samples of urinary catheter tips(40), wound swabs(40) and paranasal sinus mucosa of chronic sinusitis(40), received from different wards and ICUs of SMS Medical College & Attached Hospitals, Jaipur, over a period of one year. Biofilm detection was done by three different methods i.e. Tissue Culture Plate method, Tube method and Congo Red Agar method. We compared the results of different methods, along with determination of antimicrobial susceptibility pattern of the isolates.

In our study, out of 120 staphylococci, we isolated 72 (60%) and 48 (40%) CPS and CNS respectively. Similarly, Gjødsbol *et al.*

in their study found *S. aureus* as the most common isolate. Bose *et al.* reported that out of 179 staphylococcus spp. 68 (38%) were CPS and 111 (62%) were CNS. Hassan *et al.* reported that out of 45 isolates 18(40%) were CPS and 27 (60%) were CNS. This difference in rate of isolation might be due to difference in type of sample taken for study and sample size.

In our study, out of 72 CPS isolates, 43 (59.88%) and out of 48 CNS isolates, 28 (58.33%) were biofilm producers. Near similar results were obtained by different authors. Mathur *et al.*, reported 53.81% biofilm producers out of 152 CNS isolates. Knobloch *et al.*, reported 57.1% biofilm producers out of 128 *S.aureus*. However, different incidence of biofilm producers was found by different authors ranging from 48% to 81%.

In the current study, three different methods for detection of biofilm formation were used and their results were compared to find out the most appropriate method for demonstrating biofilm. Out of the 120 isolates, the TCP method detected biofilm in 71 isolates (59.16%), TM method detected biofilm in 56 isolates (46.66%) and CRA method detected biofilm in 34 isolates (28.33%). The present study showed the TCP method to be most sensitive for the

biofilm detection, followed by the TM and CRA method.

Other authors have also reported TCP as the most sensitive method for biofilm detection. According to Mathur *et al.*, 82 (53.94%) were biofilm producers by TCP method, 63 (41.4%) by TM and 8 (5.2%) by CRA method.

Knobloch *et al.*, reported 3.8%, 40.08% and 57.1% biofilm formation by CRA, TM and TCP method respectively.

Bose *et al.*, reported 97 (54.19%) were biofilm producers by TCP method, 76 (42.46%) by TM method and 11 (6.15%) by CRA method.

Among 110 isolates, tested by Hassan *et al.*, the TCP method showed biofilm in 70 isolates (63.6%), tube method in 54 (49%) and CRA method in 11 (10%) isolates.

Our study shows TCP is the better screening test for biofilm production than CRA and TM. The test is easy to perform and assess both qualitatively and quantitatively. In our study, positivity rate of CRA method was higher than observed by other workers, e.g. Mathur *et al.* Who has reported 5.26% biofilm producers by CRA method.

Table.1 Distribution of *Staphylococci* in the different isolates(n=120)

Sources	Coagulase positive <i>Staphylococci</i>	Coagulase negative <i>Staphylococci</i>
Wound swabs(n=40)	28	12
Urinary catheter tips(n=40)	14	26
Paranasal sinus mucosa(n=40)	30	10
Total(n=120)	72	48

Table. 1 Shows Depicts the distribution of Staphylococci in different isolates into coagulase positive and coagulase negative Staphylococci. Out of 120 Staphylococci, 72 (60%) were coagulase positive and 48 (40%) coagulase negative Staphylococci. Maximum number of CPS i.e.30 out of 40 were from PNS mucosal isolates and maximum number of CNS i.e. 26 out of 40 were from Urinary catheter tips isolates.

Table.2 Grading of biofilm formation in Total Isolates by the three different methods (n=120)

Biofilm formation	TCP %	TM %	CRA %
High	27(22.5%)	22(18.33%)	11(9.16%)
Moderate	44(36.66%)	34(28.33%)	23(19.16%)
Weak/None	49(40.83%)	64(53.33%)	86(71.66%)

Table 2 shows the grading of bacterial biofilm formation in total staphylococcal isolates by three different methods i.e. TCP, TM, CRA method into high, moderate and weak/none biofilm producers as depicted in figure 7. Out of three methods TCP method detected strong biofilm production in maximum number of isolates 22.5%, whereas detection of strong biofilm production by TM and CRA methods was seen 18.33% and 9.16% respectively. The TCP method had also detected more moderate biofilm producing bacteria 36.66% as compared to other methods i.e. 28.33% and 19.16% by the TM and CRA methods respectively.

Table.3 Biofilm production of Staphylococci with regards to source of isolation

Source	CPS		CNS		Total
	BP	NBP	BP	NBP	
Wound swabs	15	13	06	06	40
Urinary Catheter tips	07	07	15	11	40
PNS	21	09	07	03	40
Total	43	29	28	20	120

CPS = Coagulase positive Staphylococci CNS = Coagulase negative Staphylococci
 BP = Biofilm producer NBP = Non Biofilm producer

Table 3 shows that maximum number of biofilm producing Staphylococci i.e. 28 out of 40, were from PNS mucosal isolates. Among 28, 21 were coagulase positive and 07 coagulase negative Staphylococci. In wound swab isolates, 21 were biofilm producers, in which 15 were coagulase positive and 6 coagulase negative Staphylococci. In urinary catheter tip isolates, 22 were biofilm producer, in which 15 were coagulase negative and 7 coagulase positive Staphylococci.

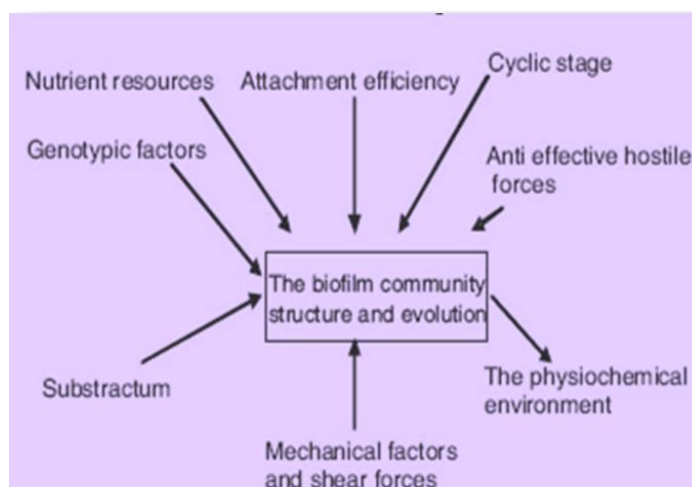
Table.4 Antibiotic Resistance Pattern (in %) of biofilm forming (BF) and non biofilm forming (NBF) Staphylococci in Total isolates (n=120)

Antibiotics	Resistance in % of BF* isolates (n=71)		Resistance in % of NBF† isolates(n=49)		p value
	n	%	N	%	
Amoxi clav	51	71.83	22	44.89	0.005
Ciprofloxacin	49	69.01	20	48.80	0.004
Clindamycin	57	80.28	29	59.18	0.021
Erythromycin					0.062
Gentamicin	52	73.23	27	55.10	0.043
Oxacillin	49	69.01	24	48.97	0.862
Vancomycin	67	94.36	45	91.83	NA
Doxycycline	0	0	0	0	0.892
Linezolid	47	66.19	31	63.26	NA
Ticarillin/Clavulinic acid	0	0	0	0	0.053
	71	100	45	91.83	

Biofilm Forming NA → Not Applicable

Table 4 shows the comparison of the resistance pattern of biofilm forming and Nonbiofilm forming Staphylococci in total 120 Staphylococcal isolates. This shows that the biofilm producers are more resistant to the various antibiotics as compared to the non-biofilm producers. The BF bacteria showed 69.01% resistance to ciprofloxacin as compared to NBF (40.80%) bacteria. There was similar resistance pattern between both the groups in case of oxacillin, doxycycline and ticarcillin/clavulinic acid . Vancomycin and linezolid are 100% effective in both the groups. The difference in the resistance pattern of these drugs was statistically significant ($p < 0.05$).

Fig.1 Environmental and cultural characteristic which affect the selection of biofilms multispecies



In this study antibiotic sensitivity pattern of various biofilm producers and non-producer *Staphylococci spp.* was studied. The significant and clinically relevant observation was that the high resistance

shown by biofilm producers to conventional antibiotics than non-biofilm producers. This observation was supported by other studies also. All strains were sensitive to linezolid and Vancomycin.

In conclusion, bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infection. The increasing use of catheters, artificial implants and antimicrobials as well as high numbers of immunocompromised patients are major causes for concern over biofilm infections. These infections are characterized particularly by high resistance to antimicrobials and formation of persistent foci that may complicate therapy and lead to chronic infections. Therefore, detection of biofilm formation is of high relevance to the clinician and his/her approach to the treatment.

Recommendations

Use of Tissue Culture Plate (TCP) method for accurate detection of biofilm producers.

Vancomycin and linezolid as drugs of choice to treat Staphylococcal biofilm formation in suspected patients, as these drugs are effective, relatively safe and can be used in patients of all ages.

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