

Original Research Article

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Prevalence of biofilm production by *Staphylococcus* species isolated from patients on indwelling medical devices/implants

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ABSTRACT

Keywords

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The increased use of indwelling medical devices has had considerable impact on the role of *Staphylococci* in clinical medicine, their major pathogenic factor being ability to form biofilm on polymeric surfaces. Bacteria growing within a biofilm lose their sensitivity to antibiotics quickly. Thus biofilms result in persistent infections that cannot be resolved with standard antibiotic treatments. The present study was done to know the ability for biofilm production and the antibiotic susceptibility among different species identified from 250 clinical isolates of *Staphylococci*. Three standard methods of Tissue culture plate, Tube method and Congo Red Agar method were used for detection of biofilm production. Biofilm production was detected in 172 (68.80%) of 250 staphylococcal isolates. *Staphylococcus aureus* accounted for 88 (51.16%) and *S. epidermidis* for 65 (37.79%) followed by *S. haemolyticus* which accounted for 19 (11.05%) biofilm producing *Staphylococcal* isolates. Persistent infection due to biofilm formation is certainly a new and additional burden to clinicians. Identification of biofilm production and its relation with antimicrobial resistance might help to elucidate the impact of *Staphylococci* in diagnosis of various infections.

Introduction

Biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to a surface, embedded within a self-secreted extracellular protective and adhesive matrix of a polymeric substance. Biofilm Extracellular polymeric substance (EPS), which is also referred to as slime (although not everything described as slime is a biofilm), is a polymeric conglomeration generally composed of extracellular DNA,

proteins, and polysaccharides.

Biofilms are widely distributed in nature. Biofilms can form in different situations in human body, such as attachment on a surface, or after exposure to sub lethal doses of antibiotics. Such surfaces may be provided by artificial devices such as prosthetic valves, breast implants, intravenous and urinary catheters,

intrauterine contraceptive devices or in dermatological situations like with fillers, and cheek implants.

Various microorganisms responsible for biofilm formation include *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, *Coagulase negative Staphylococci*, *Enterobacter cloacae*, *Enterococci*, and *Staphylococcus aureus*.

Staphylococci are most often associated with chronic infections of implanted medical devices. The use of indwelling medical devices is important in the treatment of critically and chronically ill patients, however bacterial colonization of implanted foreign material can cause major medical sequel. The increased use of indwelling medical devices has had considerable impact on the role of *Staphylococci* in clinical medicine, their major pathogenic factor being ability to form biofilm on polymeric surfaces.

S.aureus has an extraordinary repertoire of virulence factors that allows it to survive extreme conditions within the human host. *S. aureus* has been generally recognized to survive well both inside and outside of host cells. In the extracellular milieu, *S. aureus* must overcome opsonization by complement and antibodies, which directly or indirectly leads to killing of *S. aureus*. Virulence mechanisms of clinical significance include biofilm formation which allows *S. aureus* to persist on plastics and resist host defenses or antibiotics, and small colony variants which help *S. aureus* survive in a metabolically inactive state under harsh conditions. The capacity to adhere to polymer surfaces and consequent biofilm production are the main virulence factors of Coagulase negative *Staphylococcus* (CoNS). The biofilm protects CoNS against the action of

antibiotics administered for the treatment of these infections and also against the patient's immune system.

The main objectives of this study includes isolation of *Staphylococci* from various clinical samples. Characterisation and identification of isolated *Staphylococci* to species level. Detection of biofilm formation by various standard methods. Antibiotic sensitivity testing of biofilm producing strains by Kirby Bauer disc diffusion technique.

The purpose of our study was to detect the prevalence of biofilm production in various strains of *Staphylococcus* isolated from patients on indwelling medical devices and to study their antibiotic susceptibility pattern.

Materials and Methods

The present study comprised of 250 clinical isolates of *Staphylococci* isolated from various samples of patients with indwelling medical device admitted in hospitals attached to Government Medical College, Amritsar, Punjab, India. The various specimens collected were inoculated onto blood agar and MacConkey agar plates and incubated aerobically at 37⁰C for 18-24 hours. Culture isolates were identified on the basis of colony, morphological, staining characteristics and various biochemical reactions⁹ as per CLSI standards. Various biochemical tests namely Coagulase, Novobiocin sensitivity, Urease, acid from d-Mannose, PYR and Ornithine decarboxylase test were performed to identify *Staphylococcus* isolates to species level. Detection of biofilm formation was done by various standard methods described as:

Tissue Culture Plate (TCP) method: Strains isolated from fresh agar plates were inoculated in Brain Heart Infusion (BHI)

broth with 2% sucrose. Broth was incubated for 24 hours at 37°C. The broth culture was diluted 1 in 100 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates were filled with 0.2 ml diluted cultures. The tissue culture plates were incubated for 18 hours to 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with phosphate buffer saline (PBS pH 7.2). Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1%). Excess stain was rinsed off by thorough washing with deionized water and plates were dried. Optical density (OD) of stained adherent bacteria were determined with a micro ELISA reader. These OD values were considered as an index of bacteria adhering to surface and forming biofilm. OD values greater than 0.240 were taken as Strong biofilm producer. OD values less than 0.120 as non-biofilm producer and those b/w 0.120 – 0.240 were taken as moderate biofilm producers.

Tube Method (TM)⁵: Brain heart infusion media with 2% sucrose was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lining the wall and bottom of the tube was present.

Congo Red Agar (CRA) Method: Congo red was prepared as concentrated aqueous solution and autoclaved at 121°C for 15 minutes, separately from other medium

constituents. Agar was cooled to 55°C and then congo red was added. Plates were inoculated and incubated aerobically for 24-48 hours at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of dry crystalline colonial morphology indicated an intermediate result.

Susceptibility testing against CLSI recommended antibiotics was performed by Kirby Bauer disk diffusion method in these biofilm producing isolates.

Results and Discussion

The present study was conducted on 250 isolates of *Staphylococcus* species isolated from various clinical specimens received from hospitalized patients with an indwelling medical device in the department of Microbiology, Govt. Medical College Amritsar.

Maximum isolates were from pus 107 (42.80%) followed by urine 73 (29.20%), vaginal swabs 24 (9.60%), blood 15 (6%) and majority of isolates were of *Staphylococcus aureus* 139 (55.60%) followed by *Staphylococcus epidermidis* 75 (30.00%) (Table I). Among 111 coagulase negative *Staphylococcus spp.* majority of the isolates were of *S. epidermidis* 75 (30%) followed by *S. hemolyticus* 30 (12%) and *S. saprophyticus* 6 (2.4%) (Table II).

All Staphylococcal isolates were tested for biofilm formation by three methods (TCP, TM and CRA). 172 (68.80%) isolates were detected to be biofilm producers (Table III) by TCP method whereas TM detected biofilm production in 150 isolates (60%) and only 78 (31.20%) isolates were detected positive for biofilm production by CRA method. Incidence of biofilm production

was studied in all the four species of *Staphylococcus* isolated in our study. Out of 75 *S. epidermidis* isolates 65 (86.67%) were found to be positive for biofilm production. This was followed by 88 *S. aureus* isolates among 139 (63.76%) and 19 *S. hemolyticus* isolates (63.33%) found as biofilm

producers. None of the *S. saprophyticus* (0%) showed biofilm production (Table IV). Clinical isolates of biofilm producing *Staphylococci* showed maximum sensitivity to Linezolid (97.7%) and Vancomycin (95.5%) followed by Amikacin (87.5%) (Table V).

Table.1 Species Distribution of Staphylococci

| Species | Cases | Percentage (%) |
|-------------------------------------|-------|----------------|
| <i>Staphylococcus aureus</i> | 139 | 55.60 |
| <i>Staphylococcus epidermidis</i> | 75 | 30.00 |
| <i>Staphylococcus haemolyticus</i> | 30 | 12.00 |
| <i>Staphylococcus saprophyticus</i> | 06 | 2.40 |
| Total | 250 | 100.00 |

$\chi^2 = 13.662$; $df = 3$; $p = 0.003$; Significant

Table.2 Species Distribution amongst Coagulase Negative Staphylococci

| CoNS | Cases | Percentage (%) |
|-------------------------------------|-------|----------------|
| <i>Staphylococcus epidermidis</i> | 75 | 67.57 |
| <i>Staphylococcus haemolyticus</i> | 30 | 27.02 |
| <i>Staphylococcus saprophyticus</i> | 06 | 05.41 |
| Total CoNS | 111 | 100.00 |

$\chi^2 = 10.692$; $df = 2$; $p = 0.005$; Significant

Table.3 Distribution of Detection of Biofilm Production by the Three Methods in Isolates from Patients with Indwellin Medical Device

| Biofilm production | Detection method | | | | | |
|--------------------|------------------|--------|----------|--------|-----------|--------|
| | TCP (n/%) | | TM (n/%) | | CRA (n/%) | |
| HIGH* | 70 | 28.00% | 51 | 20.40% | 28 | 11.20% |
| MODERATE** | 102 | 40.80% | 99 | 39.60% | 50 | 20.00% |
| NON/WEAK*** | 78 | 31.20% | 100 | 40.00% | 172 | 70.40% |

*OD > 0.240

**OD b/w 0.120 – 0.240

***OD < 0.120

For TCP, $\chi^2 = 3.859$; $df = 1$; $p = 0.048$; Significant

For CRA, $\chi^2 = 70.682$; $df = 1$; $p < 0.001$; Highly Significant

Table.4 Biofilm Production by Different *Staphylococcal* Species

| Isolate | Biofilm Producers | Non-Biofilm producers | Percentage (%) |
|--|-------------------|-----------------------|----------------|
| <i>Staphylococcus aureus</i> n=139 | 88 | 51 | 63.31 |
| <i>Staphylococcus epidermidis</i> n=75 | 65 | 10 | 86.67 |
| <i>Staphylococcus haemolyticus</i> n=30 | 19 | 11 | 63.33 |
| <i>Staphylococcus saprophyticus</i> n=06 | 00 | 06 | 00.00 |
| Total n=250 | 172 | 78 | 68.80 |

$\chi^2 = 19.518$; $df = 3$; $p < 0.001$; Highly Significant

Table.5 Antibiotic Sensitivity Pattern in Biofilm Producing *Staphylococcus* Isolates

| Isolate | AK | G | A | CF | Cs | E* | N** | NR** | Cx | Va | LZ |
|--|--------------|--------------|--------------|--------------|------------------|------------------|------------------|-------------------|------------------|------------------|------------------|
| | n/% | n/% | n/% | n/% | n/% | n/% | n/% | n/% | n/% | n/% | n/% |
| <i>S.aureus</i> n=88 | 77/ 87.50 | 25/ 28.41 | 11/ 12.50 | 15/ 17.05 | 13/ 14.7 7 | 17/ 25.7 5 | 14/ 63.6 3 | 10/ 45.45 | 23/ 26.1 4 | 84/ 95.4 5 | 86/ 97.7 2 |
| <i>S.epiderm</i> <i>idis</i> n=65 | 53/ 81.54 | 18/ 27.70 | 13/ 14.77 | 12/ 13.64 | 09/ 10.2 3 | 13/ 31.7 1 | 10/ 41.6 7 | 09/ 37.50 | 21/ 32.3 1 | 62/ 95.3 8 | 61/ 93.8 4 |
| <i>S.haemol</i> <i>yticus</i> n=19 | 14/ 73.68 | 08/ 42.11 | 01/ 5.26 | 06/ 5.26 | 03/ 15.7 8 | 02/ 13.3 3 | 03/ 75.0 0 | 04/ 100.0 0 | 08/ 42.1 1 | 16/ 84.2 1 | 18/ 94.7 3 |

AK-Amikacin, G-Gentamicin, A-Ampicillin, CF-Ciprofloxacin, Cs-Cephalexin, E-Erythromycin, N-Nitrofurantoin, NR-Norfloxacin, Cx-Cefoxitin, Va-Vancomycin, LZ-Linezolid

* not applied for urine samples

** was applied for urine sample only

Figure.1 Congo Red Agar Plate with Biofilm Producing and Non-Biofilm Producing Strains



Figure.2 Tube Method



Figure.3 Tissue Culture Plate Method



Biofilm has been described as a barrier, which is produced by microorganisms to survive and protect themselves against various environments. Biofilm production is an important mechanism that allows microbes to escape host defenses and antimicrobial therapy. Biofilm formation on indwelling medical devices and implants such as catheters, mechanical heart valves, pacemakers, prosthetic joints, and contact lenses pose a critical medical problem. *Staphylococcus sp.* is a common cause of nosocomial and environmental infection. It has been thought that testing for biofilm formation could be a useful marker for the pathogenicity of *Staphylococci*. *Staphylococcus* is known for its ability to produce biofilm which is considered to be an important virulence factor as well as one of the mechanisms for antimicrobial

resistance. Identification of biofilm production and its relation with antimicrobial resistance might help to elucidate the impact of *Staphylococci* in diagnosis of various infections.

The maximum number of *Staphylococcus* isolates were from pus i.e., 107 (42.80%) followed by urine (29.20%), vaginal swabs (9.60%) and blood (6.00%) in patients with indwelling medical device. The findings are similar to the study conducted by Munesh Kumar Gupta et al Rajadurai et al with maximum isolates from clinical specimens of pus followed by blood and urine. *Staphylococcus aureus* was the most common species isolated i.e. 139(55.20%). The role of *S. aureus* as a human pathogen is widely known fact supporting our finding as its main causative role in infections. This is

consistent with the study done by MullaSummaiya A and Jethwani Urmi N on ventilator associated pneumonia in which *S. aureus* was the leading Gram positive organism isolated. However Iorio N.L et al in their study isolated lesser percentage of *Staphylococcus aureus* strains. *Staphylococcus epidermidis* was the second most common species followed by *Staphylococcus haemolyticus* isolated in the present study. This difference among CoNS species was statistically significant ($p = 0.005$). This was in concordance with the study done by Iorio N.L et al who found *S. epidermidis* as the most common species isolated in their study.

The biofilm formation has been documented as survival strategy of pathogens. It has been well documented that biofilms add to the virulence of the pathogen. 172/250 (68.80%) Staphylococcal isolates were found to be positive for biofilm production. It has been estimated that the frequency of infections caused by biofilms, especially in developed world, lies between 65% and 80% as per reports from centres for disease control and prevention (CDC) and National Institute of Health (NIH), respectively. Biofilm production was detected by the three standard methods i.e., Tissue Culture Plate (TCP), Tube Method (TM) and Congo Red Agar (CRA) methods.

Whereas TCP detected biofilm production in 68.80% of the clinical isolates, TM could detect it in 60%. The tube method correlated well with strong biofilm production but it was not able to detect weak production. These observations were similar to the one made by T Mathur in his study on an evaluation of the three screening methods. However a study by Adilson Oliveira found TM to be highly sensitive and highly specific for the detection of biofilm production. This can be due to observer

variation in detection of biofilm by TM. Moreover in their study, they compared the TM against PCR which detects the presence of *icaA* and *icaD* or *icaACD* genes.

The CRA method failed to correlate well with TCP and TM in our study. CRA detected biofilm production in only 78 of the 250 clinical isolates (31.20%). These observations were found to be highly significant ($p < 0.001$) against CRA. This was in concordance with the studies of T Mathur, Silva et al and YasmeenTaj et al. However these observations were entirely in disagreement with observations reported by Freeman DJ et al and Munish Kumar Gupta et al who found CRA as more sensitive method for biofilm detection. But according to findings of our present study CRA did not correlate well with TCP and TM. TCP method was found to be the most sensitive followed by TM.

The biofilm formation has been observed in the clinical isolates of *Staphylococcus aureus*. Maximum biofilm production was seen in *Staphylococcus aureus* (51.16%) and *S. epidermidis* (43.60%) followed by *S. haemolyticus* (11.05%). This difference was statistically found to be highly significant ($p < 0.001$). This observation is in concordance with T Mathur who observed biofilms in 53.9% Staphylococcal isolates. However *S. epidermidis* was the most frequently detected species (81%) by Adilson Oliveira in their study. But this difference was perhaps due to the fact that their study was concentrated on coagulase negative Staphylococci and he found this observation amongst CoNS. In our study *S. aureus* was the most common species identified. Amongst CoNS, in our study also *S. epidermidis* was the most common species isolated accounting for 67.57% biofilm producing CoNS. Similarly Elizabeth Fredheim et al found greater prevalence of

biofilm production by *S. haemolyticus* as their study included only *S. haemolyticus* strains.

Biofilms act as virulence factor for the pathogen. Greater resistance was observed to all antimicrobial drugs in the biofilm producing isolates. Maximum resistance was observed for ampicillin (85.46%) while resistant strains to cefoxitin were 66.86%. Vancomycin and Linezolid resistance was observed in 5.81% and 4.07% of the biofilm producing Staphylococcal isolates, respectively. The results were consistent with the study conducted by Nizami Duran that showed a high level of resistance among the staphylococcal isolates to most of the commonly used antimicrobials.

In conclusion, Biofilm production is one of the virulence factor of *Staphylococcus* and should be looked for along with coagulase production and other virulence factors. This trait is responsible for chronicity of infections and persistence of these organisms in infection sites and hospital environment. Biofilm forming capacity among *Staphylococci* in association with invasive devices is significant addition to the list of other contributory virulent factors like coagulase, toxin production and antibiotic resistance and it has accelerated the emergence of *Staphylococci* as a global human pathogen. This must be looked for along with other virulence factors. This may contribute to the increased hospital stay, patient's morbidity, mortality and increased health care cost associated with invasive devices.

Most common organism isolated among various isolates was *Staphylococcus aureus*(55.60%) followed by *Staphylococcus epidermidis* (30.00%). Most common species amongst coagulase negative Staphylococci was found to be *S.*

epidermidis 75/111 (67.57%).

Tissue culture plate (TCP) method was observed to be highly sensitive for detection of biofilm production with detection rate of 68.80%.

Biofilm production was detected in 172 out of 250 isolates i.e. 68.80%. Most common species amongst which biofilm production was detected was *Staphylococcus epidermidis* (86.67%).

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