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Biofilm formation and Antibiotic susceptibility pattern among *Staphylococcus aureus* in a tertiary care hospital in Kanchipuram: An Evaluation of screening methods for biofilm formation.

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Abstract: The ability of *Staphylococcus aureus* to form biofilms is of significant clinical interest, as biofilm development impacts the efficacy of antimicrobial therapy and the subsequent outcome of an infection. The present study is undertaken to detect the biofilm production and to determine the antibiotic susceptibility pattern among the *Staphylococcus aureus* isolates. A total of 100 Staphylococcus aureus isolated for the first time from pus, blood, catheter, IV cannulas were included in the study. Biofilm detection was done by tube method and Microtitre plate method. Antibiotic susceptibility was done by Kirby bauer disc diffusion method. Methicillin resistance was detected by Cefoxitin disc diffusion method. By tube method and Microtitre plate method 26% and 46% of the isolates were identified as biofilm producers. By Microtitre plate method, BHI broth (Brain heart infusion broth) and BHI broth with sucrose was used and the difference in the biofilm forming ability was compared. When BHI broth with sucrose was used 69% showed biofilm formation whereas when tested with BHI broth, only 46% were identified as biofilm producers. Good sensitivity was observed for Amikacin (88%) and cefotaxime (82%). MRSA (Methicillin resistant *Staphylococcus aureus*) was detected among 19% of the isolates. Among the biofilm producers if there are drug resistant bacteria like MRSA the problem becomes challenging and requires combination of several antibiotics. Hence Screening for biofilm production by bacterial isolates should be performed. Infection control program should address the effective execution of disinfection procedures.

Key words: Biofilm formation, Staphylococcus aureus, Microtitre and Tube method

Introduction

Biofilms are bacterial population that are enclosed in a matrix of extracellular polymeric substances. Biofilm displays an altered phenotype when compared to the planktonic cells such as growth rate and resistance to antimicrobial agents. Bacteria in biofilm have been reported to be more resistant to antibiotics than planktonic cells.[1]Staphylococci are commonly associated with infections such as urinary tract infection, wound infection and infection of medical devices. Biofilm production is one of its important virulence factor. The ability of *Staphylococcus aureus*, to form biofilms is of significant clinical interest, as biofilm development impacts the efficacy of antimicrobial therapy and the subsequent outcome of an infection. [2] Polysaccharide intracellular adhesion (PIA) regulates the production of biofilm among *Staphylococcus aureus*.[3]The bacterial cells within biofilm require more than 100 times the Minimum inhibitory concentration of antibiotic required compared to free floating cells.[4] Biofilm producing bacteria are also implicated in the transfer of drug resistance within the bacteria. Hence the present study is undertaken to detect the biofilm production and to determine the antibiotic susceptibility pattern among the *Staphylococcus aureus* isolates.

Objectives:

1. To isolate *Staphylococcus aureus* from clinical samples such as pus, urine, blood, catheter, I.V cannulas etc.
2. To detect biofilm production by Tube method and Microtitre plate method.
3. To perform Antibiotic susceptibility testing by Kirby Bauer disc diffusion method
4. To detect MRSA (Methicillin resistant *Staphylococcus aureus*) by cefoxitin disc diffusion method.

Materials and Methods

This observational Study was conducted in the Department of Microbiology. The study was approved by the institutional Ethical Committee. A total of 100 *Staphylococcus aureus* isolated for the first time from urine, pus, blood, catheter, IV cannulas were included in the study. All the isolates were identified by colony morphology, Gram staining, catalase test, coagulase test to differentiate *Staphylococcus aureus* from *Coagulase negative Staphylococci* as per standard microbiologic techniques.

Antibiotic susceptibility testing will be performed by Kirby bauer disc diffusion method for Amoxycillin (20µgm), Erythromycin (15µgm), Gentamicin (10µgm), Amikacin (30µgm), Cephalexin (30µgm), Cefotaxime (30µgm),

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Ceftazidime (30µgm), Ciprofloxacin (5µgm), Vancomycin (30µgm) and Linezolid (30µgm) as per CLSI guidelines (Clinical and laboratory standard institute). [5]

Detection of MRSA by Cefoxitin disc diffusion test:
The test was performed with 30 µg of Cefoxitin placed on Mueller Hinton agar plate. The zone of inhibition was determined after 24hours incubation at 37°c. Zone size is interpreted based on CLSI guidelines as ≥22 as sensitive and ≤ 21 as resistant. [5, 6]

Biofilm production was detected by Tube method and Microtitre plate method.
Microtitre plate method: The isolates were inoculated into BHI broth (Brain heart infusion broth) and incubated at 37° c for 24 hours. 96 well micro titre plates were used for detection of biofilm formation. Into each well 200 µl of brain heart infusion broth was added. 2µl of each sample was added to the wells and incubated at 37° c for 24 hours. After 24 hours the contents of the wells were discarded and removed by tapping the plate. Then each well was washed four times with 200 µl PBS. Then 100µl of 0.1% crystal violet was added to each well to stain and kept for 15 minutes, and washed repeatedly with sterile distilled water. ATCC *Pseudomonas aeruginosa* 27853 was used as positive control. The plates were allowed to dry and read at 570 nm using ELISA plate reader. The same procedure was repeated with Brain heart infusion broth with 2% sucrose. [3]

The reading values are interpreted as follows
Sample OD >0.12- strong biofilm producers
Sample OD values between 0.06 -0.12- moderate to weak biofilm producers
Sample OD< 0.06 -Non biofilm producers.

Tube method: Tubes containing 2ml of brain heart infusion broth was inoculated with a loopful of culture and incubated for 24 hours at 37° C. The culture tubes were then decanted and washed with Phosphate buffer saline and dried. The dried tubes were stained with crystal violet. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. [7]

Results

A total of 100 staphylococcal isolates were obtained and biofilm production was studied by Microtitre plate method and Tube method. By tube method 26% were identified as biofilm producers. By Microtitre plate method 46% were identified as biofilm producers. The distribution of moderate and strong biofilm producers is depicted in Table 1. By Microtitre plate method 4% of the isolates were identified as biofilm producers whereas by tube method 12% were strong biofilm producers. By Microtitre plate method, BHI broth and BHI broth with sucrose was used and the difference in the biofilm forming ability was compared. When BHI broth with sucrose was used 69% showed biofilm formation whereas when tested with BHI broth, only 46% were identified as biofilm producers. The sensitivity and specificity of tube method of biofilm detection was 8.7% and 59.26% respectively. (Table 3) Antibiotic susceptibility was performed by disc diffusion method. Methicillin resistance was detected by cefoxitin disc diffusion method and 19% of the isolates were identified as MRSA. (Table 4) Among the MRSA, Erythromycin and Amoxicillin showed the lowest sensitivity of 2 (10.5%). Amikacin showed a sensitivity of 13 (68.4%). All the isolates were 19 (100%) sensitive to Vancomycin and Linezolid. MDR (resistance to three or more groups of antibiotic) was observed in 12 (63.1%) of MRSA. Ciprofloxacin showed a resistance of 8 (16%) among the MRSA isolates (Ciprofloxacin Resistant MRSA CR – MRSA).

Out of the 81 (81%) MSSA, 76 (93.8%) and 75 (92.5%) were susceptible to cefotaxime and Amikacin respectively. Amoxycillin and ciprofloxacin showed a susceptibility of 37 (45.6%) and 42 (51.8%) respectively. Erythromycin showed a susceptibility of 62 (76.5%). All the isolates were susceptible to Vancomycin and linezolid. Among the eight ciprofloxacin resistant MRSA isolates, 5 (62.5%) were biofilm producers. (Table 5)

Table 1: Distribution of biofilm producers by Tube method and Microtitre plate method

	Tube method	Microtitre plate method
Non biofilm producers	74 (74%)	54 (54%)
Moderate biofilm producers	14 (14%)	42 (42%)
Strong biofilm producers	12 (%)	4 (4%)

Table 2: Distribution of biofilm producers in different media (Brain heart infusion broth with and without the addition of sucrose by Microtitre plate method)

	Brain heart infusion broth	Brain heart infusion broth with sucrose 2 %
Non biofilm producers	4 (4%)	18 (%)
Moderate biofilm producers	42 (42%)	51 (%)
Strong biofilm producers	54 (54%)	31 (%)

Table 3: Sensitivity, Specificity, Positive predictive value(PPV) and Negative predictive value(NPV) of Tube method for biofilm detection

Sensitivity	Specificity	PPV	NPV
8.7%	59.26%	15.38%	43.24%

Table 4: Antibiotic susceptibility pattern

S.No	Antibiotic disc	Staphylococcus aureus N= 100	MSSA N=81	MRSA N=19
1	Amoxycillin	39 (39%)	37 (45.6%)	2 (10.5%)
2	Erythromycin	64 (64%)	62 (76.5%)	2 (10.5%)
3	Gentamicin	76 (76%)	72 (88.8%)	4 (21%)
4	Amikacin	88 (88%)	75 (92.5%)	13 (68.4%)
5	Cephalexin	67 (67%)	61 (75.3%)	6 (31.5%)

6	Cefotaxime	82 (82%)	76 (93.8%)	6 (31.5%)
7	Ceftazidime	80 (80%)	74 (91.35%)	6 (31.5%)
8	Ciprofloxacin	50 (50%)	42 (51.8%)	8 (42.1%)
9	Vancomycin	100 (100%)	81 (100%)	19 (100%)
10	Linezolid	100 (100%)	81 (100%)	19 (100%)
11	Pipericillin tazobactum	100 (100%)	81 (100%)	19 (100%)

MRSA- Methicillin Resistant *Staphylococcus aureus*
MSSA- Methicillin Sensitive *Staphylococcus aureus*

Table 5: Antibiotic susceptibility pattern of MRSA isolates for Anti MRSA drugs with reference to Biofilm Producers Vs non biofilm producers

Antibiotic Disc	Biofilm Producer	Non Biofilm Producer
Ciprofloxacin n=8 CR MRSA	5 (62.5%)	3 (37.5%)
Ofloxacin N=10	6 (60%)	4 (40%)
Vancomycin N=19	9 (47.3%)	10 (52.6%)
Linezolid N=19	7 (36.8%)	12 (63.1%)

CR MRSA- Ciprofloxacin resistant MRSA

Discussion

The ability of *Staphylococcus aureus* to form biofilm helps the bacterium to resist host immune response and is considered responsible for chronic or persistent infection. Biofilm forming *Staphylococcus aureus* are implicated in life threatening infection associated with IV catheters, artificial heart valves and prosthetic joints with a propensity for delayed healing of wound. Bacterial cells in biofilm exhibit intrinsic resistance to antibiotics due to certain mechanism conferred by changes in the biofilm environment such as altered oxygen and carbon di oxide gradient and inactivation of antimicrobial agents by exopolysaccharide. [8]

In the present study biofilm production by tube method was 26%. Out of the 26 isolates strong biofilm production was seen in 12% and moderate biofilm production was seen in 14% of the isolates. Mathur *et al.,* (2006) has reported 11.8% as strong biofilm producer similar to the present study. [7] Taj *et al.,* (2012) and Ansari *et al.,* (2013) has reported 23.2% and 63.4% as biofilm producers respectively. [8, 9] The sensitivity and specificity of the tube method by comparison with Microtitre plate method was 8.7% and 59.26% respectively. Mathur *et al.,* have recorded the sensitivity and specificity of tube method as 73.6% and 92.6% respectively. Though tube method is easy to perform, in the present study the sensitivity and specificity are very low, with a higher rates of false positive results due to observer variation.

By Microtitre plate method with brain heart infusion broth, 46% were identified as biofilm producers and 4 isolates were strong biofilm producers (8.69%). With Brain heart infusion broth with sucrose, biofilm production was detected among 69% of the isolates and 18 (26 %) were strong biofilm producer. Indrawattana *et al.,* (2013) and Gamalfadh *et al.,* (2009) has documented 83.3% and 72.83% as biofilm producers respectively. [10, 11] Mathur *et al.,* has documented 57.8% as biofilm producers by Microtitre plate method. The percentage of biofilm producers were high among BHI broth with sucrose indicating that biofilm production can be advanced by altering the conditions such as the PH, temperature and sugar concentration. Biofilm formation was detected among more isolates when sucrose was added to BHI broth, indicating an association between growth condition such as addition of sucrose and biofilm formation in staphylococci. [7]

Microtitre plate method gives a better result in screening the isolates for biofilm production because in this method the adherence of the bacterial isolates to the wall of the Microtitre plate is quantitatively measured as optical density.

Among the 100 isolates 19 % were identified as MRSA by cefoxitin disc diffusion method. Indian studies have documented the prevalence of MRSA as 20-74%. [12] Uday Shankar *et al.,* (1997) and Paul *et al.,* (2007) has reported MRSA rates as 24% and 29% respectively similar to the present study. [13, 14]

Among the MRSA isolates, Erythromycin and Amoxicillin showed lowest sensitivity of 2 (10.5%). Amikacin showed a sensitivity of 13 (68.4%). All the isolates were 19 (100%) sensitive to Vancomycin and Linezolid. MDR (resistance to three or more groups of antibiotic) was observed in 12 (63.1%) of MRSA. Study by Sanchez *et al.,* (2013) has showed erythromycin and gentamicin sensitivity as 18% and 4% respectively among the MRSA isolates. [15] In our study ciprofloxacin and ofloxacin showed a sensitivity of 42.1% which was similar to ohadian moghadam *et al.,* (2014). [16] Sanchez *et al.,* showed ciprofloxacin sensitivity as 88%.

Out of the 81 (81%) MSSA, 76 (93.8%) and 75 (92.5%) were susceptible to cefotaxime and Amikacin respectively. Amoxycillin and ciprofloxacin showed a susceptibility of 37 (45.6%) and 42 (51.8%) respectively. Erythromycin showed a susceptibility of 62 (76.5%). All the isolates were susceptible to vancomycin and linezolid. Study by Sanchez *et al*., (2013) showed Erythromycin and ciprofloxacin susceptibility as 89% and 99% respectively among the MSSA isolates. Paul *et al.,* (2007) showed susceptibility to Ciprofloxacin and Gentamicin as 84% and 66.6% respectively. In our study highest sensitivity was seen for Amikacin followed by cefotaxime.

Among the MRSA isolates 13 (68.42%) were biofilm producers. Karen smith *et al.,* (2008) has documented biofilm production to be 53.8% among the MRSA.[2] Gogoi *et al.,*(2015) has documented 50% of MRSA as biofilm producers.

[17] MRSA are multidrug resistant isolates and along with biofilm producing phenotype becomes a greater therapeutic challenge. Dadri kaur *et al.,* (2014) has documented 70 (82.3%) as biofilm producers among the MRSA. [18] In contrast to the present study Mariuz grunholc (2007) has documented that biofilm production was high among MSSA (66-69%) than in MRSA (45-47%). [19]

Ciprofloxacin resistance among the MRSA isolates in the present study was 42.1%. Among the ciprofloxacin resistant MRSA isolates 5 (62.5%) were biofilm producers. Studies have reported resistance to ciprofloxacin among MRSA ranging from 39% to 68%. [20] In a study by Neeta D Gade *et al.,* (2013) ciprofloxacin resistant MRSA was 6.5%. [20] Agarwal *et al.,* (2013) have reported 53.7% of the biofilm producing MRSA isolates as Ciprofloxacin resistant MRSA. [4]

Biofilm producing bacterial isolates are recalcitrant to antibiotic therapy leading to chronic infection. Among the biofilm producers if there are drug resistant bacteria like MRSA the problem becomes challenging and requires combination of several antibiotics. The most efficient means of combating biofilm related infections is to prevent the occurrence of infection in the first place by following aseptic precautions combined with prophylactic administration of antibiotics.

Biofilm mediated infection in a hospital set up have a adverse impact on patients health and place an massive burden on the health care resources. Hence Screening for biofilm production by bacterial isolates should be performed. Infection control program should address the effective execution of disinfection procedures. Rational antibiotic prescription based on the susceptibility pattern and Changing the antibiotic recommendation periodically could help in limiting the multidrug resistant organism.

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