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Research Article

Evaluation of Biofilm Production and Antibiotic Resistance Pattern Between Biofilm Producing and Non-biofilm Producing Isolates of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is a common cause of nosocomial infection and biofilm is one of its important virulence factors. Biofilm is a group of microorganisms enclosed in a self-produced exopolysaccharide matrix. *S. aureus* biofilms have been extensively involved in several chronic untreatable and medical implants related infections. The treatment of biofilm infections is laborious and challenging. On this account, the study was undertaken to detect the biofilm production capacity and antibiotic susceptibility pattern in *Staphylococcus aureus* derived from various clinical samples. A total 100 *S. aureus* strains were isolated from various clinical samples and tested for production of biofilm using three methods namely Congo Red Agar Plate method (CRA), Microtiter Plate Method (MTP) and Tube Method (TM). Antibiotic susceptibility comparison between biofilm producers and non-producers were also performed by modified Kirby Bauer's disc diffusion method. The results showed better correlation between MTP and TM when compared to CRA method. Of the 100 *S. aureus* strains 6% were strong biofilm producers, 11% were moderate, 47% were weak positive, and 36% were negative by MTP while 56% were strong positive, 26% were moderate positive and 18% were negative for biofilm by TM. The CRA method showed that only 8% were strong positive, 3% were moderate and rest all were negative for biofilm production. None of the *S. aureus* isolates was resistant to glycopeptides (Vancomycin). The resistance exhibited by *S. aureus* to penicillin, ciprofloxacin and erythromycin resistant was 100%, 70% and 62% respectively. The antibiotic resistant rate (biofilm producers v/s non producers) for penicillin (64% v/s 36%), erythromycin (38% v/s 24%), and ciprofloxacin (48% v/s 22%) revealed that biofilm producers were more resistant to all the tested antibiotics.

Keywords: *Staphylococcus aureus*; Biofilm; Microtiter Plate Method; Tube Method; Congo Red Agar Plate Method; Antibiotic Susceptibility Testing

Introduction

Staphylococcus aureus is an important pathogen, frequently isolated from nosocomial infections, causing great problems in medical field as well as in food industry. The multi drug environment in a hospital can provide a setting for emergence and reinforcement of antibiotic resistance in bacteria coupled with risk of cross contamination and nosocomial infection. *S. aureus* is frequently associated with indwelling device infections by forming biofilm on metal or polymeric prosthetic surface [1]. In a recent study, 77% of medico-technical samples and surfaces analyzed in hospital settings were contaminated with Staphylococcus spp and 37% of the isolates showed biofilm formation capacity [2]. Biofilm act as the one of the key virulence factor for *S. aureus*. The bacterial strain that are embedded in biofilm are immensely difficult to eradicate

with conventional antibiotics and inherently resistant to host immune mechanisms. As a result the treatment of many chronic *S. aureus* biofilm related infections, (like endocarditis, osteomyelitis and indwelling medical device infections) is challenging [3]. Hence the management of biofilm related *S. aureus* infection often requires surgical removal of infected tissue or implanted devices [4]. This study aims to detect the biofilm production and compare the antibiotic susceptibility pattern between biofilm producers and non-biofilm producers in *S. aureus*.

Materials and Methods

S. aureus isolation from clinical samples

Isolates of 100 *S. aureus* from different clinical samples such as blood, pus, sputum, urine etc, submitted to the microbiology laboratory of St. Johns Medical College, Bengaluru were cultured and characterized. All *S. aureus* isolates were identified by Gram stain, colony characters on the culture plates, catalase test (to differentiate *S. aureus* from *Streptococcus* species) and tube coagulase test (to differentiate *S. aureus* from coagulase negative Staphylococci).

Biofilm detection was done by using Microtiter Plate Method (MTP), Tube Method (TM) and Congo Red Agar method (CRA). ATCC *S. aureus* 25923 (American Type Culture Collection) was used as negative control [5].

Microtiter plate method

Biofilm detection by MTP was performed as described by Eiichi Ando, Koichi Monden., *et al*. 2004. *S. aureus* isolates were grown over night at 37^{0}C in brain heart infusion broth supplemented with 2% glucose and 2% sucrose. The culture was diluted 1:100 in medium, and 150 µl of this cell suspension was added to each well of sterile 96 well polystyrene microtiter plates. After 48hr at 37^{0}C wells were gently washed three times with 300 µl distilled water without shaking, dried in an inverted position, and stained with 300 µl of 2% safranine solution for 45 minutes. After staining, plates were washed three times with distilled water. Quantitative analysis of biofilm production was performed by adding 200 µl of ethanol acetic acid (95:5 vol/ vol) to each well to dissolve the remaining stain from well. The optical density was then recorded at 492nm with 630nm reference filter using ELISA reader [6].

We considered the ODs 0.012 to be negative (mean value of all negative controls included in the tests), and those with OD > 0.012 to be positive. All the strains were tested in triplicate in 3 experiments and the average value for each sample was calculated (Table 1).

Mean OD value	Adherence	Biofilm formation
<0.04	Non	Non
0.04-0.08	Weak	Weak
0.09-0.11	Moderate	Moderate
>0.11	Strong	Strong

Table 1: Classification based on OD values obtained from *S. aureus* spp by Microtiter Plate method.

Congo red agar method

Freeman., *et al.* had described a method of screening biofilm formation by *S. aureus* isolates; which requires the use of a specially prepared solid medium brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo Red. The medium is composed of BHI (37 gms/L), sucrose (50 gms/L), agar no. 1 (10 gms/L) and congo red stain (0.8 gms/L). Congo red was prepared as concentrated aqueous solution and autoclaved at 121^{0}C for 15 minutes, separately from other medium constituents and was then added when agar cooled to 55^{0}C. Plates were inoculated and incubated aerobically for 24 to 48 hours at 37^{0}C [7].

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 colonies with the absence of dry crystalline colony morphology indicated an intermediate result. The experiment was performed in triplicate and repeated three times.

Tube method

A quantitative assessment of biofilm formation was determined as previously described by Christensen., *et al*. 10 ml trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture. Broths were incubated at 37^{0}C for 24 hours. After incubation the cultures were decanted and washed with phosphate buffer saline (pH 7.3). The tubes were dried and stained with 0.1% crystal violet for 30 minute at room temperature. After that stain was discarded and washed with deionized water. Tubes were dried in inverted position at room temperature.

In positive biofilm formation, a visible stained film was seen lining the wall and bottom of the tube. Experiments were done in triplicate and read the result as absent, weak, moderate, and strong positive [8].

Antibiotic susceptibility testing by modified Kirby Bauer disc diffusion method

Antibiotic susceptibility testing of the isolates was performed by modified Kirby-Bauer disc diffusion method. The antibiotic concentration of disc used and zone size interpretation was in accordance with clinical laboratory standards institute (CLSI) and compared both biofilm producers and non-producers [9]. First line antibiotics used are Gentamicin - 10 mcg, Tetracycline - 30 mcg, Co-trimaxazole - 25 mcg, Pencillin - 10 mcg, Erythromycin - 15 mcg and Ciprofloxacin - 5 mcg. Second line antibiotics tested were Amikacin - 30 mcg, Netillin - 30 mcg, Chloramphenicol - 30 mcg, Rifampicin - 5 mcg, Cefoxitin - 30 mcg and Vancomycin - 30 mcg.

Results

S. aureus isolation from clinical samples

Out of the 100 *S. aureus* isolated from various clinical materials, 72% were from pus, 7% were from blood, 8% were from sputum, 1% were from throat swab, 1% were from urine and the rest 11% were from other miscellaneous samples (Figure 1). Among these, 82% were slime or biofilm producers and 18% were non slime producers.

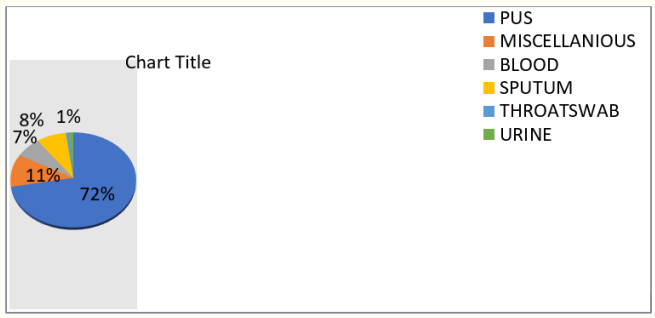

Figure 1: Clinical samples for isolation of *S. aureus.*

Microtiter Plate method

In Microtiter Plate method (Figure 2), among the total number of 100 isolates tested for biofilm formation, 6 were strong biofilm producers, 11 were moderate, 47 were weak and 36 were non biofilm producers.

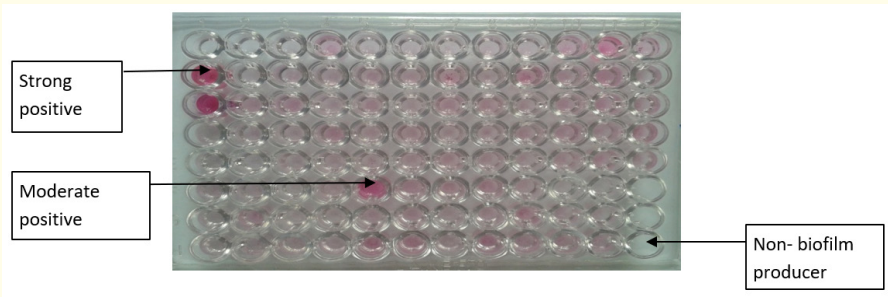

Figure 2: Screening of biofilm production by Microtiter Plate method.

Tube method

In tube method (Figure 3) 56 isolates were strong positive 26 were moderate biofilm producers, 18 were non biofilm producers. In TM, 26 were found to be false positive and 5 false negative.

Congo red agar method

By CRA method only 11 isolate were identified as biofilm producers. Findings of CRA method did not correlate well with the MTP method.

Antibiotic resistance

The antibiotic susceptibility test results of the slime producers and non-slime producing *S. aureus* strains collected from various clinical samples are shown in figure 4.

High resistance pattern was observed among biofilm producers in comparison with non-biofilm producers. Maximum resistance was noted against penicillin, followed by ciprofloxacin, erythro-

mycin, cefoxitin, cotrimaxazole, whereas all the strains were sensitive to vancomycin. Resistance to rifampicin, chloramphenicol and netillin were showed only by biofilm producers. Out of the 8% amikacin resistant isolates 6% were biofilm producers while only 2% were non biofilm producers.

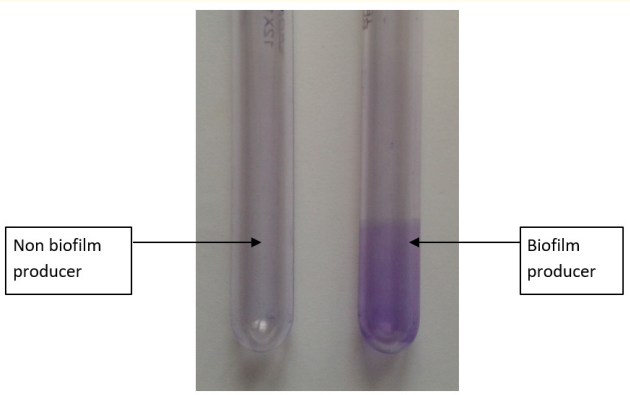

Figure 3: Screening of biofilm production by Tube method.

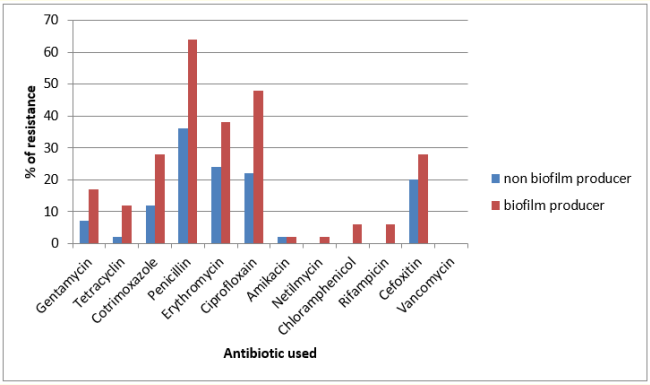

Figure 4: In vitro antibiotic susceptibility of slime producing and non-slime producing *S. aureus.*

Discussion

Pili, fimbriae, toxin and biofilm production are certain virulence factors that contribute to the pathogenicity of most bacteria and out of these biofilm production is the most aggressive, exhibiting resistance to anti-microbial agents. Bacterial biofilm also contributes to infection associated with various device and hospital acquired infections. Quarum sensing via the accessory gene regulator (agr) system has been assigned a control role in the pathogenesis of Staphylococci particularly *S. aureus.* Biofilm formation by such microorganism on indwelling devices leads to failure of such devices and also causes recurrent chronic and untreatable infections in patients. To control such infections it is important to detect biofilm producing microorganism and determine the antimicrobial activity of drugs against biofilm producing microorganism. We isolated 100 *S. aureus* spp from various clinical samples and tested their biofilm production capacity by three *in vitro* screening procedures namely Microtiter Plate method, Tube Method and Congo Red Agar method.

In modified Microtiter plate method [supplementation with 2% glucose and 2% sucrose and extended incubation for 48h] biofilm formation was observed in 64 isolates while non-biofilm producers were seen in 36 isolates. In Tube method, 82 isolates were biofilm producers while 18 isolates were non biofilm producers. The TM does not correlate well with MTP method but it was difficult to differentiate the weak biofilm producers with the negative ones. In CRA method, 11 strains were biofilm producers whereas 89 were non biofilm producers. There is no correlation between MP method and TM. Previous studies have also showed that the CRA method cannot be recommended for the accurate detection of biofilm formation by Staphylococcus isolates [8,10].

In a similar recent study of biofilm production involving 92 bacterial isolates from chronic wound infections, the TCP method revealed 78% as biofilm producers (and 21% as weak or non-biofilm producers), the TM method showed 37% as biofilm producers (and 63% as non-biofilm producers) while the CRA showed only 55% as biofilm (and remaining 45% as non-biofilm) producers [11]. Even though TM method did not give any false positive results, 41.3% isolates had given false negative results. For TM method, the specificity was 100% while sensitivity and accuracy 47% and 58% respectively. Whereas for CRA method, the sensitivity, sensitivity (57%), specificity (50%) and accuracy (57%, 50% and 55% respectively) were low compared to both TM and TCP method [11]. In another recent study of biofilm production in uropathogens using the three methods, TCP method detected 45% as biofilm producers, TM detected 40% while CRA revealed only 11% of isolates as biofilm producers. This study concluded that TCP was more sensitive than TM and CRA methods, for assessment of bio-

film producers and TM method, even though was difficult to distinguish between weak and non production of biofilm, correlated with TCP method [12]. We considered Modified MP method as gold standard for this study as various researches also proven this method is better than standard MP method trypticase soy broth without glucose [8].

In this study we compared antibiotic sensitivity pattern of biofilm producers and non-producers of *S. aureus*. The significant observation was that the high resistance shown by biofilm producers to conventional antibiotics than non-biofilm producers. This result was supported by other studies also [13,14]. Mostly all of the strong biofilm producers and most weak biofilm producers showed multi drug resistance while only few non-biofilm producers had multi drug resistance, confirming that the production of biofilms made the pathogen sensitive to a limited spectrum of antibiotics [15]. All strains were sensitive to vancomycin this is also concordant with other study also [16]. Our study showed that modified MTP method was a better screening method for biofilm production than TM and CRA method and antibiotic resistance was more in biofilm producing strains compared to non-producing strains. Individual antibiotics are generally ineffective against bacterial biofilms. To overcome such problems, combination of antibiotics is a possible alternative to treat Staphylococcal biofilm infections.

Conclusion

The modified MTP method is an accurate and reproducible screening method with the potential to serve as a reliable quantitative technique for determining the biofilm formation capacity by clinical isolates from clinical samples. TM and CRA methods are cheaper and easier technique compared to MTP method which can be modified to improve the accuracy and sensitivity of the test. Biofilm producing *S. aureus* exhibit more antibiotic resistance compared to no biofilm producers. Individual antibiotics are ineffective against bacterial biofilms. Combination of antibiotics is a possible alternative method for the treatment of such infections.

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Conflict of Interest

There is no conflict of interest.

Bibliography

1. Ji- Lu Sun., *et al*. "Growth properties of Staphylococcus aureus in biofilm formed on polystyrene plate". *African Journal of Microbiology Research* 6.13 (2012): 3284-3291.

2. Akim Socohou., *et al*. "Antibiotics Resistance and Biofilm Formation Capacity of Staphylococcus spp. Strains Isolated from Surfaces and Medicotechnical Materials". *International Journal of Microbiology* (2020): 6.

3. Sander Cores., *et al*. "Staphylococcus aureus biofilm formation at the physiologic glucose concentration depends on the S. aureus lineage". *BMC Microbiology* 9 (2009): 229.

4. Blais R Boles., *et al*. "Staphylococcal biofilm disassembly". *Trends in Microbiology* 19 (2011): 449-455.

5. Rania M Abdel Halim., *et al*. "Detection of Biofilm Producing Staphylococci among Different Clinical Isolates and Its Relation to Methicillin Susceptibility". *Open Access Macedonian Journal of Medical Sciences* 6.8 (2018): 1335-1341.

6. Eiichi Ando Koichi Monden., *et al*. "Biofilm Formation among Methicillin Resistant Staphylococcus aureus Isolates from Patients with Urinary Tract Infection". *Acta Medica Okayama* 58 (2004): 207-214.

7. Freeman DJ., *et al*. "New method for detecting slime production by coagulase negative staphylococci". *Journal of Clinical Pathology* 42 (1989): 872-874.

8. T Mathur., *et al*. "Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods". *Indian Journal of Medical Microbiology* 24 (2006): 25-29.

9. Performance Standard for Antimicrobial Susceptibility Testing, 15th International supplement testing M 100- S 15.Wayne (CLSI); Clinical Laboratory Standard Institute (CLSI) (2005).

10. Dr MK Lalitha. "Methods of Antimicrobial Susceptibility Testing". In: Manual on Antimicrobial Susceptibility Testing (2004): 7.

11. Harika K., *et al*. "Detection of Biofilm Production and Its Impact on Antibiotic Resistance Profile of Bacterial Isolates from Chronic Wound Infections". *Journal of Global Infectious Diseases* 12.3 (2020): 129-134.

12. Panda PS., *et al*. "Comparison of four different methods for detection of biofilm formation by uropathogens. *Indian Journal of Pathology and Microbiology* 59.2 (2016): 177-179.

13. Afreenish Hassan., *et al*. "Evaluation of different detection methods of biofilm formation in the clinical isolates". *The Brazilian Journal of Infectious Diseases* 15 (2011): 305-311.

14. Ramakrishna Pai., *et al*. "Biofilm: Comparison between the Staphylococcus aureus and coagulase negative staphylococcus species isolated from a rural medical college hospital in North Kerala, India". *International Journal of Current Microbiology and Applied Sciences* 3.1 (2014): 23-29.

15. Veronica Folliero., *et al*. "Evaluation of Antibiotic Resistance and Biofilm Production among Clinical Strain Isolated from Medical Devices". *International Journal of Microbiology* (2021): 11.

16. Samant Sharvari A and Pai Chitra G. "Evaluation of Different Detection Methods Of Biofilm Formation In Clinical Isolates Of Staphylococci". *International Journal of Pharma and Bio Sciences* 3.4 (2012): 724-733.