

Biofilm Formation and Detection in Multi-drug Resistant Staphylococcus

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Abstract

Background: Biofilm formation is an ultra thin slimy layer produced by certain bacteria that imparts very significant resistance against variety of antibiotics. Biofilm can be detected by tube and microtiter plate assay

Objectives: To detect biofilm formation in Staphylococci isolated from various clinical samples received from hospitalized patients of Lahore General Hospital, Lahore and to compare biofilm detection by the Tube method and Microtitre plate assay

Study design: Experimental study

Place and Duration of Study: Microbiology laboratory, Post Graduate Medical Institute (PGMI), Lahore from December 2012 to June 2014

Material and Method: All specimens obtained from LGH were processed, Staphylococci species were isolated by routine microbiological and biochemical tests. Antibiotic sensitivity pattern was found out by modified Kirby-bauer disc diffusion method and multi-drug resistant Staphylococci species were selected for further processing. Staphylococcal isolates along with the controls were subjected to biofilm formation by two methods, Tube Method (qualitative) and Microtitre Plate Assay (quantitative).

Results: Biofilm formation was more commonly observed in the microorganisms with multi-drug resistance.; Penicillin, 92 (97.87%); Cefoxitin, 56 (59.57%); Erythromycin, 64 (68.08%); Clindamycin, 51 (54.25%); Fluroquinolones, 38 (40.42%); Doxycyclin, 47 (50.00%); Linezolid, 6 (6.38%); Trimethoprim-Sulphmethaxazole, 77 (81.91); Gentamicin, 36 (38.29%) Staphylococci species were resistant. Among the Staphylococcal isolates, resistant 88.29% Penicillin, 47.87% Cefoxitin, 72.32% Trimethoprim-Sulphmethaxazole, 61.70% Erythromycin, 48.93% Clindamycin, 35.10% Fluroquinolones, 43.61% Doxycyclin, 5.31% Linezolid, 31.91% Gentamicin depicted biofilm formation by the Tube Method. While among the Staphylococcal isolates resistant, 96.81% Penicillin, 56.38% Cefoxitin, 81.91% Trimethoprim-Sulphmethaxazole, 67.02% Erythromycin, 53.19% Clindamycin, 40.42% Fluroquinolones, 47.87% Doxycyclin, 6.38% Linezolid, 37.23% Gentamicin depicted biofilm formation by the Microtitre plate Assay.

Conclusion: Multiple drug resistant staphylococci are more prone to form biofilm and Microtitre plate assay is effective at determining biofilm as compared to Tube method.

Keywords: Multi drug resistant (MDR), biofilm formation, Staphylococci species.

Introduction

Multidrug resistance (MDR) microorganisms are the major culprits for many acute and chronic infections. Infection caused by such drug resistant pathogens leads to nosocomial outbreaks and sporadic infections.^{1,2} Increasing antimicrobial resistance to the available antibiotics is an important threat to public

health. It not only reduces effectiveness of treatment and prolongs the period of illness but also increases the burden on health resources of country and increases mortality and morbidity on health care costs on the global scale.³

Methodology

The present study was experimental study. Clinical samples were collected from the patients admitted in Lahore General Hospital (LGH), were transported to Microbiology laboratory in Pathology Department of PGMI, Lahore for culture and sensitivity testing. After

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preliminary identification by Colonial morphology, Gram staining, catalase test, coagulase test, Novobiocin sensitivity and DNase test, Antibiotic sensitivity pattern was found out by modified Kirby-bauer disc diffusion method and ninety four multidrug resistant Staphylococcal isolates along with the controls were subjected to biofilm formation by two methods, Tube Method (qualitative) and Microtitre Plate Assay (quantitative).

Tube method^{4,5,6,7}

Biofilm formation by individual test and control organisms was detected qualitatively by Tube Method. Tryptone soya broth (Oxiod) was prepared in the sterilized flasks. A loopful of test organism was inoculated in 10ml of tryptone soy broth in each conical bottom tube (Microbiologics, United State of America). The test tubes were incubated at 37 °C for 24 hours. After 24 hours of incubation, tubes were decanted and washed with phosphate buffer saline of pH 7.3 (Bio Basic Canada Inc). The tubes were dried in inverted position and stained with 0.1% crystal violet (Merck, Germany). Excess stain was washed with deionized water and dried in inverted position. In positive biofilm producers, a visible stained biofilm was seen in the form of coating on the wall and bottom of the tube. The results were scored visually as a- Non adherent / Absent= 0, b-Weakly adherent = +, c- Moderately adherent= ++, d- Strongly adherent = +++.

Microtitre plate assay^{5,7,8}

A loopful of Staphylococci isolates was inoculated in 10ml of tryptone soya. Broth was incubated for 24 hours at 37 °C. The liquid culture was diluted in 1:100 ratio with fresh medium. Ninety six well flat bottom polystyrene microtitre plate with lid (Coster, Corning, New York 14831, United State of America) was used and each well was filled with 200µL of diluted culture. The positive and negative control organisms were also incubated, diluted and added to microtitre plate. The three sets of test and control organisms in microtitre plates were incubated for 24 hours at 37°C.

The next day, contents of each well were aspirated and individual wells were washed three times with 250 µl of sterile physiological saline after shaking vigorously. This removed free floating bacteria. Fixation of biofilm forming bacteria was done by 200 µl of 99% methanol

per well for 15 min, emptied and left to air dry and stained by 2% crystal violet for 5 min. Excess stain was removed by placing the plates under running water and then plates were dried.

After air dry, dye bound to adherent cells were solubilized with 160 µl of 33% (v/v) glacial acetic acid (Merck, Germany) per well. Optical density (OD) of adherent stained biofilm was obtained by ELISA reader (Labsystems, Model 362, Serial number 2701071, Finland) at wavelength 490 nm after addition of glacial acetic acid.

All the test organisms were classified into following categories, depending upon the optical density of the test organism (OD) and the optical density of the negative control (OD_c).

Non adherent	OD ≤ OD _c
Weakly adherent	OD _c < OD ≤ 2 OD _c
Moderately adherent	2 OD _c < OD ≤ 4 OD _c
Strongly adherent	4 OD _c < OD

Results

Staphylococcal isolates demonstrated high resistance to many groups of antimicrobial drugs. The antimicrobial resistance was 97.87% to Penicillin, 57.44% for Cefoxitin, 68.08% for Erythromycin, 54.25% for Clindamycin, 40.42% for Ciprofloxacin, 50.0% for Doxycyclin, 6.38% for Linezolid, 81.91% for Trimethoprim - Sulphmethaxazole, 38.29% for Gentamicin and 26.59% for Amikacin.

Increased antimicrobial resistance has an impact of increase formation of biofilm. Among the Staphylococcal isolates, resistance to β lactam antibiotics, Penicillin and Cefoxitin was 97.87% and 57.44% while depiction of biofilm formation by the Tube Method was 88.29% and 47.87% and by the Microtitre Plate Assay was 96.81% and 56.38% respectively. Macrolide (Erythromycin) and Lincosamide (Clindamycin) resistance was 68.08% and 54.25% while 61.70% and 48.93% depicted biofilm formation by the Tube Method and 67.02% and 53.19% by the Microtitre Plate Assay respectively. Flouroquinolones (Ciprofloxacin) and Aminoglycoside (Gentamicin) resistance was 40.42% and 38.29% while 35.10% and 31.91% depicted biofilm formation by the Tube Method and 40.42% and 37.23% by the Microtitre Plate Assay respectively. Ox-

azolidinone (Linezolid) resistance was 6.38% while 5.31% depicted biofilm formation by the Tube Method and 6.38% by the Microtitre Plate Assay.

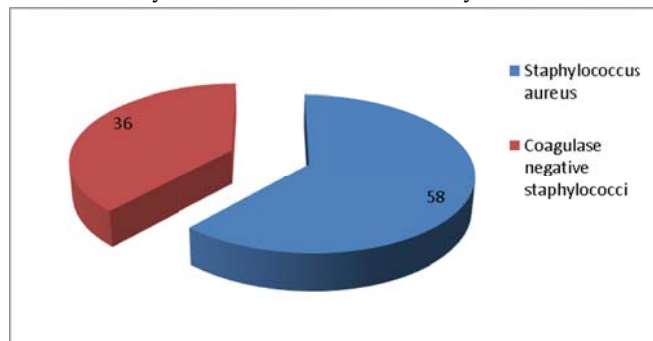


Figure1: Species distribution of Staphylococci (n=94)

Table I: Relation of Multidrug resistance with bio-film formation

Multidrug Resistance	No of iso-lates	Biofilm Formation	
		Tube Method	Microtitre Plate assay
SA	58	52	56
CoNS	36	29	35
Total	94	81	91

Discussion

Biofilm forming microorganisms are extremely resistant to multiple antimicrobial drugs.⁹⁻¹² Biofilm forming microorganisms are 100 – 1000 times more resistant to antimicrobial drugs as compared to the planktonic microorganisms.¹³⁻¹⁵

In the present study, Staphylococcal isolates had demonstrated high resistance to many groups of antimicrobial drugs. The antimicrobial resistance was 97.87% to Penicillin, 57.44% for Cefoxitin, 68.08% for Erythromycin, 54.25% for Clindamycin, 40.42% for Flouroquinolones, 50.0% for Doxycyclin, 6.38% for Linezolid, 81.91% for Trimethoprim – Sulphmethaxazole and 38.29% for Gentamicin respectively.

Table 2: Antimicrobial resistance pattern in Staphylococcal species (n=94)

Drugs	Resistance pattern (%)
Penicillin	97.87
Trimethoprim-sulphmethaxazole	81.91
Erythromycin	68.08
Cefoxitin	57.44
Clindamycin	54.25
Doxycyclin	50
Floroquinolones	40.42
Gentamicin	38.29
Linezolid	6.38

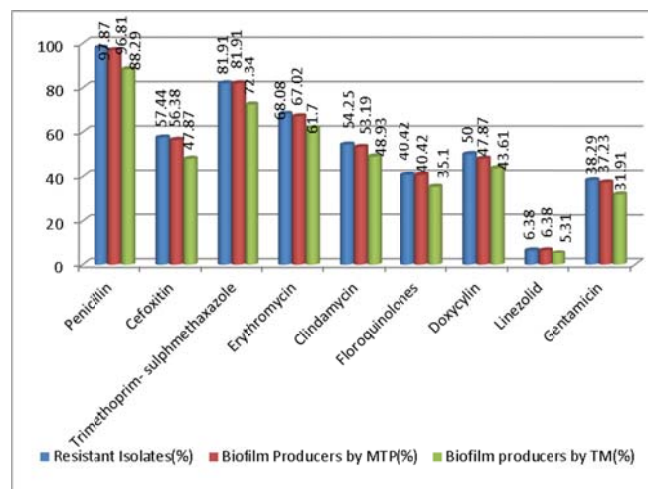


Figure 2: Relation of biofilm formation with individual antimicrobial drug by Tube Method and Microtitre plate assay (n=94)

Methicillin resistant *Staphylococcus aureus* were 31 (32.97%). Biofilm detection in the Methicillin resistant *Staphylococcus aureus* by the tube method was 29 (30.85%) while all the isolates of Methicillin resistant *Staphylococcus aureus* form biofilm by the microtitre plate assay. Methicillin resistant Coagulase negative

Staphylococci were 23 (24.46%). Biofilm detection by the both methods was 22 (23.40%).

According to a study carried out in Department of Urology, Graduate School of Medicine and Dentistry, Okayama, Japan on biofilm formation by Methicillin resistant *Staphylococcus aureus*, out of 109 samples of the Methicillin resistant *Staphylococcus aureus*, 95.4% isolates were detected to form biofilm.¹⁶ The increased formation of biofilm in Methicillin resistant *Staphylococcus aureus* was in accordance to our study.

Similar type of study was performed by Mirzaee and his colleagues in 2014 at Iran. Mirzaee *et al.* determined biofilm formation and the effect of *icaABCD* genes in Methicillin resistant *Staphylococcus aureus* isolates. All of the sixty three isolates of Methicillin resistant *Staphylococcus aureus* were found to form biofilm. These findings were also consistent with our study findings.¹⁷

CharanKaur and Khare conducted similar type of study in 2013 at the Department of Microbiology, Pune, Maharashtra, India about biofilm formation and antimicrobial susceptibility pattern of Methicillin resistant *Staphylococcus aureus* isolates. Out of total 231 isolates, 182 (78.8%) of the Methicillin resistant *Staphylococcus aureus* were found to form biofilm.¹⁸

In the present study, increased formation of biofilm was detected in the Methicillin resistant Coagulase negative Staphylococci. Similar type of study was conducted by Sharvari and Chitra in 2012 at the Department of Microbiology, Mahatma Gandhi Mission's Medical College, Navi Mumbai, Maharashtra, India, on biofilm detection in Staphylococci. According to Sharvari and Chitra, increased formation of biofilm was demonstrated in the Methicillin resistant coagulase negative Staphylococci as compared to Methicillin sensitive coagulase negative Staphylococci. The findings of the above mentioned study were in accordance with our study finding.¹⁹

While another study was carried out in Rome, Italy on the methicillin resistance, biofilm formation and resistance offered to Benzalkonium chloride. According to the authors findings, minimal bactericidal concentrations (MBC) were higher for the Methicillin resistant *Staphylococcus aureus* embedded in the biofilm as compared to minimal inhibitory concentration

(MIC) that does not increase in case of Methicillin resistant *Staphylococcus aureus*. Furthermore, the author stated that there was no correlation of Methicillin resistant *Staphylococcus aureus* with the ability of the microorganism to form biofilm. These findings were in contrast to the findings of the present study.²⁰

In the light of above mentioned discussion, it is interpreted that biofilm formation play a significant role in the resistance offered by the Staphylococci. It is the need of the hour that research like ours should be performed to determine various new mechanisms of drug resistance and most important microorganisms affected by them. Moreover the researches like this will open the horizons for discovery of new antimicrobial drugs that can limit and minimize the misery and pain suffered by the mankind.

Conclusion

Multiple drug resistant staphylococci are more prone to form biofilm and Microtitre Plate assay is effective at determining biofilm as compared with Tube Method.

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