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Formation of Biofilms at Surgical Site Infection Caused by *Pseudomonas aeruginosa* and Their Clinical Significance

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* is one of the primary opportunistic pathogens that causes a variety of nosocomial infections.[1] The ability of *P. aeruginosa* to form biofilms in a lot of environments renders antibiotic treatment unproductive and promotes persistent infectious diseases.[2] This is a Descriptive and Prospective study to identify formation of biofilms on Surgical sites and to evaluate its effects and significance.
Materials & Methods: All swabs from surgical sites were collected from the period February 2021 to January 2022 and were subjected to Standard Microbiological procedures like Gram staining and various biochemical reactions to identify *Pseudomonas aeruginosa*. Antibiotic Susceptibility Test was performed by Kirby-Bauer Disk Diffusion Method. Further, biofilm production was investigated from the isolated *Pseudomonas aeruginosa* by Congo Red Agar method and Tissue Culture Plate method. Conclusion was derived by taking Co-morbidities into consideration too.
Results: Out of 500 samples, 475 samples were sterile and 5 samples showed growth of *Pseudomonas aeruginosa*. These 5 samples when tested for biofilm production by Congo Red Agar method, 2 of them were identified as biofilm producers while by Spectrophotometric method (Tissue Culture Plate method) 3 isolates were identified as biofilm producers (2 strong and 1 weak).
Conclusion: Hence, we can conclude that 60% of *Pseudomonas aeruginosa* can form biofilms at Surgical Site Infection. The biofilm producers also show increased resistance to antibiotics by almost 80%, causing a persistent infection and delay the wound healing of surgical site specially in patients with co- morbidities.

Keywords: *Pseudomonas aeruginosa*, Biofilm, Pus, Surgical site Infections, Kirby-Bauer Disk Diffusion Method.

INTRODUCTION

Pseudomonas aeruginosa is considered as opportunistic pathogen, as serious infection often occurs during existing diseases or conditions–most notably cystic fibrosis and traumatic burns. It generally affects the immunocompromised host but can also infect the immunocompetent host as in hot tub folliculitis. Treatment of *P. aeruginosa* infections can be difficult due to its natural resistance to antibiotics. When more advanced antibiotic drug regimens are

needed, adverse effects may result.[3]

Biofilms have great importance for public health, because biofilm-associated microorganisms show signs of dramatically decreased vulnerability to antimicrobial agents. Evidence of the incidence of biofilms on medical devices has come from studies in which the devices either were examined upon removal from the patients or were tested in animal or laboratory systems.[4]

P. aeruginosa is the third most frequently isolated bacteria in Gram-negative bacilli bacteremia, accounting for 4267 community-acquired or nosocomial bacteremia. Antibiotics are used in most current therapeutic techniques. In addition to its intrinsic resistance to a wide range of antibiotics, *P. aeruginosa* has a unique potential to develop resistance mechanisms that, in the worst-case scenario, can result in a therapeutic dead lock. The large number of infection sites and high mortality rate indicate that this environmental disease has a strong capacity for adaptation. In *P. aeruginosa*, numerous virulence factors have been identified. Many studies are currently focused on quorum sensing, a bacterial communication system that regulates the majority of *Pseudomonas* virulence factors. Inhibition of quorum sensing could be a novel strategy of treating *P.aeruginosa* infections if its "language" is understood.[5]

To date, all the quorum-sensing mechanisms which have been described in detail had been studied in the context of planktonic cultures. This is comprehensible as it simplifies the signaling process. In shaken liquid culture, all microorganisms are presumed to be physiologically similar, are generating signal molecules at the equal rate, and are uncovered to the equal concentration of signal molecule.[6] In general, biofilm cells come up with much higher local cell densities than free-floating, planktonic cell populations. An apparent outcome of this is the increased levels of metabolic by-products, secondary metabolites and different secreted or excreted microbial elements that biofilm cells encounter. Of unique interest are intercellular signaling or quorum-sensing molecules. Because biofilms normally include aggregates of cells, one may argue that they constitute an environmentally relevant context for quorum sensing. For a few species, there is proof that quorum sensing is essential for the development and/or dissolution of biofilm communities.[6]

P. aeruginosa produces variety of virulence factors including lipopolysaccharide, exotoxin A, leucocidin, extracellular slime, proteases and phospholipases. Exotoxins cause destruction of tissues, protein synthesis inhibition and cell activity inhibition. Enterotoxins interrupt normal gastrointestinal activity resulting in diarrhea. Leucocidin inhibits neutrophil and lymphocyte functions. Elastase cleaves immunoglobulins and complement components. Alginate is capsular polysaccharide that permits infecting bacteria to stick to lung somatic cell surfaces and form biofilms which successively, protect bacteria from antibiotics and body's system. *P. aeruginosa* was first reported in human infections in 1862 by Luke who observed

Rod shaped particles in blue green pus of some infections.[7]

Aim: To determine the Formation of Biofilms at Surgical Site Infection caused by Pseudomonas aeruginosa and their Clinical Significance.

Objectives:

- To detect Pseudomonas aeruginosa in patients with Surgical Site Infections.
- To detect formation of Biofilms by Pseudomonas aeruginosa isolated from patients with Surgical Site Infections.
- To compare between two different phenotypic methods for detection of biofilms.
- To study Clinical features of biofilm formation in patients with Surgical Site Infections caused by Pseudomonas aeruginosa.
- To compare the effects of different antibiotics on isolates of Pseudomonas

aeruginosa producing biofilms with those not producing Biofilms.

MATERIALS AND METHODS[8]

Study Period: February 2021 to January 2022

Place of Study: MGM Medical College and Hospital, Kamothe, Navi Mumbai

Type of Study: Descriptive and Prospective study

Inclusion Criteria: All *P. aeruginosa* isolates obtained from pus swabs taken from surgical sites.

Exclusion criteria: Pregnant women, patients below 18 years of age and HIV positive patients.

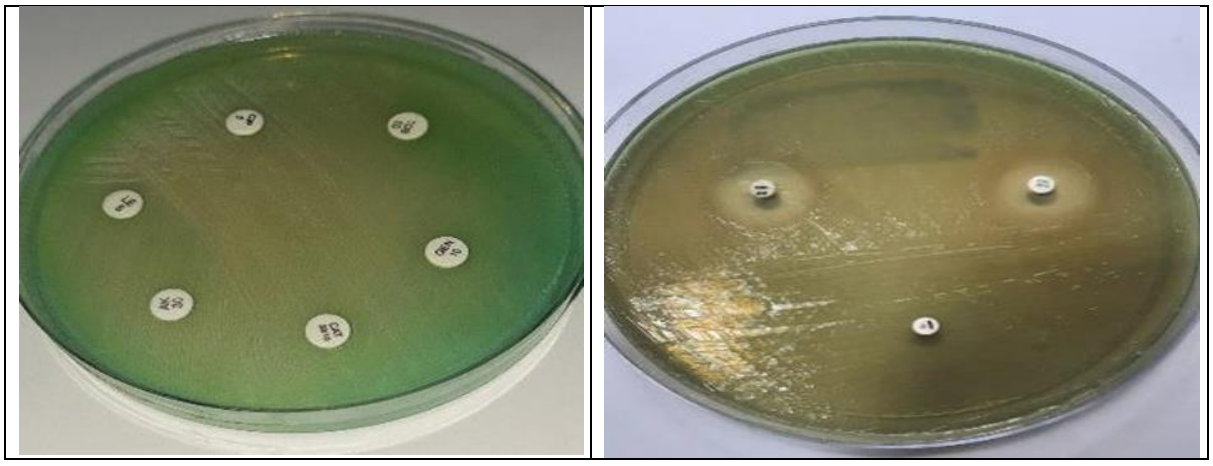
Sample Size: All pus swabs from Surgical Sites of patients from February 2021 to January 2022

Sample Processing: All samples were received from patients with surgical sites admitted to the MGM Medical College and Hospital, Kamothe, Navi Mumbai. The samples were subjected to Standard Microbiological Procedures (Gram staining, Culture for colonial morphology and biochemical test) and *Pseudomonas aeruginosa* was identified from them.[8]

A total of 500 pus swabs were taken from surgical sites in a year and were identified and studied by standard microbiological procedures from February 2021 to January 2022 in Microbiology Department of MGM Medical College and Hospital, Kamothe, Navi Mumbai.

Nature of sample: Pus swab

Antibiotic Susceptibility Test by Kirby-Bauer disk diffusion method was performed according to CLSI guidelines. Measurement of zone diameters was done by Examining the plates after overnight incubation. With the use of sliding calipers, a ruler, or a template, the zones of complete growth inhibition around each of the disks are carefully measured to within the nearest diameter of the disk. An interpretation (Susceptible, Moderately susceptible, Intermediate, Resistant) is provided by reference to published guidelines.[9]



Biofilm production testing

These isolates were tested for biofilm production by two methods:

CONGO RED AGAR METHOD[10]

Freeman et. al. have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA). CRA plates were inoculated with test organisms (*P. aeruginosa*) and incubated at 37ºC for 24h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.

SPECTROPHOTOMETRIC METHOD[10]

This method was used to demonstrate the production of slime by *P. aeruginosa* as per the method described by Christensen et al.

18 hours cultures were standardized by McFarland's Standards. 200 micro liters of standardized cultures were added to the flat bottom wells of sterilized polystyrene plate and incubated for 18 hrs at 37°C. Following incubation, the contents of the plate were gently aspirated. The plates were washed with sterile phosphate - buffered saline four times at ph. 7.2. Slime and Adherent organisms were fixed overnight with Bouins fixative. The fixative was removed by washing the wells three to four times with 50% ethanol. The wells were stained with Huckers crystal violet and excess stains were removed by washing the plate with distilled water. Then the plates were dried. The optical densities of the stained adherent films were read by an Elisa reader (MULTISCANMS) at a wavelength of 630 nm. The measurements were repeated in duplicates and the mean Optical Density (OD) was calculated. OD value greater than 0.1 were considered positive for slime production.

RESULTS

Out of 500 samples, 475 samples showed no growth of any bacteria.

Remaining 25 showed growth of bacteria.

Out of these 25 samples, 5 showed growth of *Pseudomonas aeruginosa* and 20 showed growth of other Gram-negative organisms like *E. coli* and *Klebsiella.*

Biofilm production by spectrophotometric method was more sensitive than Congo Red Agar method.

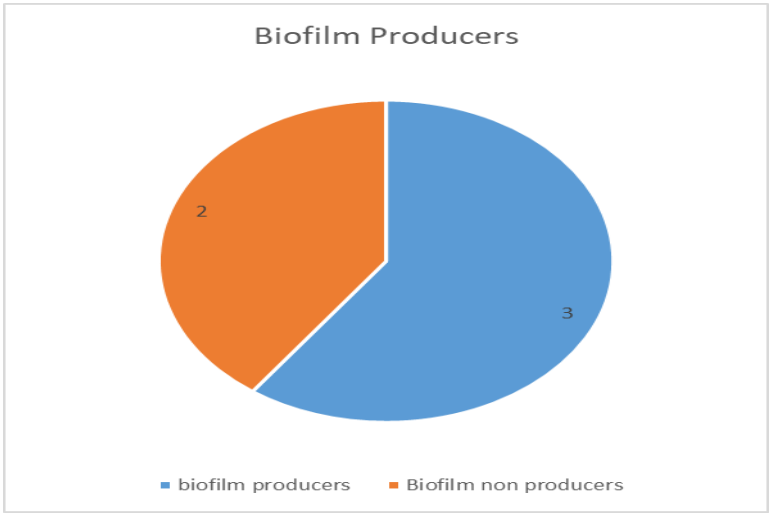
The 5 samples when tested for biofilm production by Congo Red Agar method identified 2 of them as biofilm producers while Spectrophotometric Method (Tissue Culture Plate method) concluded 3 isolates as biofilm producers (2 strong and 1 weak).

RESULT AND OBSERVATION

Incidence of *Pseudomonas aeruginosa* in pus swabs form surgical site.

Swabs	No.	Percentage
No. of swabs collected	100	100%
No. of sterile swabs	75	75%
No. of organisms isolated other than *Pseudomonas aeruginosa*	20	20%
No. of *Pseudomonas aeruginosa* isolated	5	5%

Pie chart 1



Comparison of Biofilm production by two phenotypic methods

***Pseudomonas aeruginosa* Isolates**	**Biofilm produced by Congo Red Agar Method**	**Biofilm produced by Tissue Culture Plate Method**
Isolate no. 1	No	No
Isolate no. 2	No	No
Isolate no. 3	No	Yes Weak producer
Isolate no. 4	Yes	Yes Strong producer
Isolate no. 5	Yes	Yes Strong producer

Clinical features of the patients with SSI caused by *Pseudomonas aeruginosa*

Clinical Features	**Isolate no. 1**	**Isolate no. 2**	**Isolate no. 3**	**Isolate no. 4**	**Isolate no. 5**
Surgical site/ Surgery type	Left Humerus fracture	Post Ileostomy	Below knee Amputation	Diabetic foot Ulcer	Traumatic Amputation
Efficacy of antibiotics	75%	66.66%	25%	8.3%	16.6%
Effects of co-morbidity	No Comorbidity	High Blood Pressure	Chronic Kidney Disease	Diabetes	Dengue

Antibiotic Susceptibility pattern seen in the 5 isolates of *Pseudomonas aeruginosa* first line

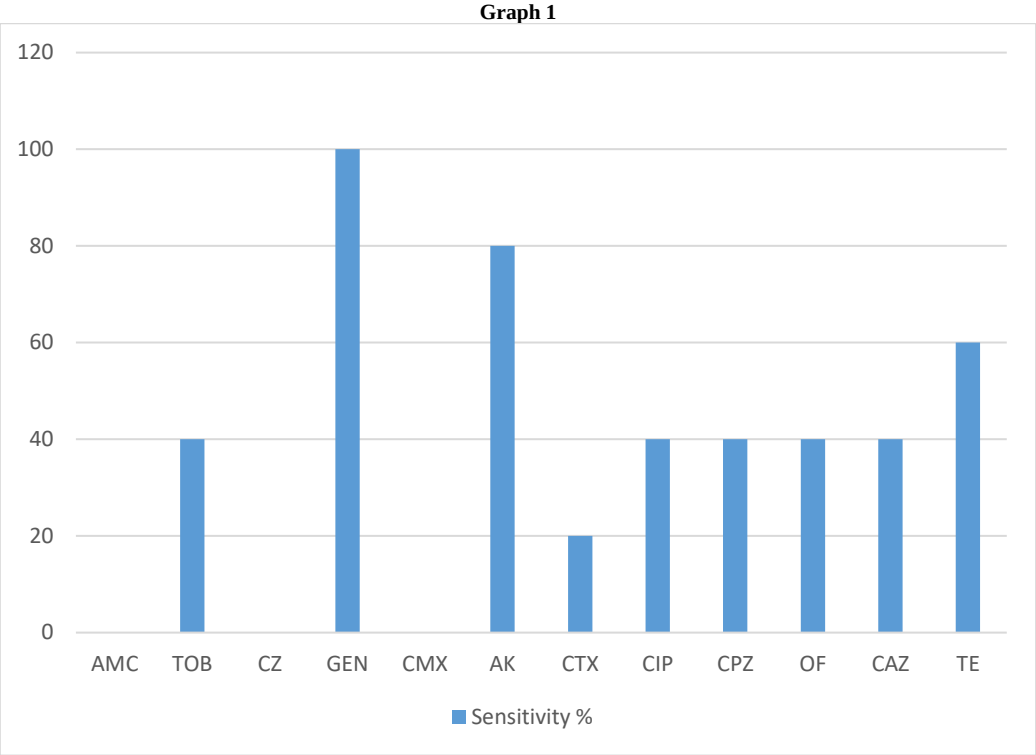
Antibiotics	**Symbol**	**Conc.**	**Isolate no. 1**	**Isolate no. 2**	**Isolate no. 3**	**Isolate no. 4**	**Isolate no. 5**	**% sensitivity**
Augmentin	AMC	20/10 mcg	R	R	R	R	R	00
Tobramycin	TOB	10 mcg	S	S	R	R	R	40
Cefazolin	CZ	30 mcg	R	R	R	R	R	00
Gentamicin	GEN	10 mcg	S	S	S	S	S	100
Cefuroxime	CXM	30mcg	R	R	R	R	R	00
Amikacin	AK	30 mcg	S	S	S	R	S	80
Cefotaxime	CTX	30 mcg	S	R	R	R	R	20
Ciprofloxacin	CIP	05 mcg	S	S	R	R	R	40
Cefoperazone	CPZ	30 mcg	S	S	R	R	R	40
Ofloxacin	OF	05 mcg	S	S	R	R	R	40
Ceftazidime	CAZ	30 mcg	S	S	R	R	R	40
Tetracycline	TE	30 mcg	S	S	S	R	R	60

Antibiotic Susceptibility pattern seen in the 5 isolates of *Pseudomonas aeruginosa* 2nd Line

Antibiotics	**Symbol**	**Conc.**	**No. of sensitive isolates**
Augmentin	AMC	20/10 mcg	0/5
Tobramycin	TOB	10 mcg	2/5
Cefazolin	CZ	30 mcg	0/5
Gentamicin	GEN	10 mcg	5/5
Cefuroxime	CXM	30 mcg	0/5
Amikacin	AK	30 mcg	4/5
Cefotaxime	CTX	30 mcg	1/5
Ciprofloxacin	CIP	05 mcg	2/5
Cefoperazone	CPZ	30 mcg	2/5
Ofloxacin	OF	05 mcg	2/5
Ceftazidime	CAZ	30 mcg	2/5
Tetracycline	TE	30 mcg	3/5

%Antibiotic Susceptibility in 5 isolates of *Pseudomonas aeruginosa*

Graph 1



DISCUSSION

Pseudomonas aeruginosa, our test organism was isolated from 5 samples out of 500 samples collected.

These 5 isolates were tested for biofilm production by Congo Red Agar Method from which 2 (40%) samples were positive for biofilm production.

These 5 isolates were also tested for biofilm production by Spectrophotometric method from which 3 (60%) samples were positive for biofilm production.

Out of these 3 biofilm producers, 2 were strong producers while 1 was weak producer of biofilm.

Hence, Congo Red Agar Method shows 20% less biofilm production and hence can be concluded as less sensitive method compared to tissue culture plate method.

Agar and Tube methods were 39.1%, 50.9% and their features were 78.3%, 79.7% respectively.[12]

The wound healing time was delayed by weeks in the cases where organism causing infection was a biofilm producer and hence suggests that biofilms might delay the wound healing time.

This correlates with the study carried out by Daniel G. Metcalf et. al. who reported Biofilm is associated with impaired epithelialization and granulation tissue formation, and promotes a low-grade inflammatory response that interferes with wound healing. Polymicrobial biofilms, which invariably exist in chronic wounds, have been shown to delay healing to a greater extent than single-species biofilms.

This study correlates with the Nosrati Net. al., who reported in tube method, 23% of the isolates formed strong and 59% formed weak biofilm. In Tissue Culture Plate method 40%, 22% and 28% of isolates formed strong, moderate and weak biofilm respectively and 10% were biofilm negative. According to the Congo Red Agar method only 4% of the isolates formed strong biofilm, 65% and 31% respectively had a weak biofilm and no biofilm respectively. The sensitivity and specificity of Congo Red

The isolates were also found to have an effect on persistency of infection as the biofilm producers caused more persistent infection with high risk of reoccurrence than non-biofilm producers.

This correlates with the study of Lichen who recorded Bacterial biofilms can be viewed as a specific type of persistent bacterial infection.[14]

The biofilm producers also increase the resistance of antibiotics by almost 80%.

This correlates with the study carried out by Asim I Shaikh et. al. Their study suggested that there is a relation between biofilm production and multidrug resistance in *P. aeruginosa* as this may be due to the delayed penetration of antimicrobial agents inside the bacterial cell.[15]

CONCLUSION

Conclusion

The present study included 100 pus samples from surgical sites and were subjected to standard microbiological procedures.

- Out of 100 samples, 75 samples had no organism grown or were sterile.
- Remaining 25% isolated 5% *Pseudomonas aeruginosa* and 20% other organisms like *E. coli* and Kelbsiella
- These 5 samples when tested for biofilm production by congo red agar method gave 2 samples as positive for biofilm production while 3 non-biofilm producers.
- These 5 samples when tested for biofilm production by spectrophotometric method (tissue culture plate method) concluded 3 isolates as biofilm producers (2 strong and 1 weak) and 2 isolates as non-biofilm producer.
- Biofilm production by spectrophotometric method was more sensitive than congo red agar method.
- Biofilm production caused delay in wound healing time of the surgical sites in patients.
- Biofilm production by *Pseudomonas aeruginosa* at Surgical Site Infection also causes the infection to be persistent and sometimes reoccurring.
- The study of antibiotic susceptibility pattern showed that biofilm producing *Pseudomonas aeruginosa* was more resistant to antibiotics than biofilm non-producing *Pseudomonas aeruginosa*.
- The 1st and 2nd isolate which were non biofilm producers were 75% and 66.66% sensitive to 1st line antibiotics respectively.
- The 3rd, 4th and 5th isolate which produced biofilms were found to be Multi drug resistant as they were resistant to more than 75% of the 1st line antibiotics.
- The 2nd line antibiotics were found to be sensitive to 3rd isolate while 4th and 5th were 70% and 60% sensitive to second line antibiotics respectively.
- Hence, we can conclude that 60% of *Pseudomonas aeruginosa* can form biofilms at Surgical site infection. The biofilm producers also increase the resistance of antibiotics by almost 90% most probably due to the difficulty in penetration of antimicrobial agents through the biofilm. Hence causing a persistent infection and delay the wound healing of surgical site specially in patients with comorbidities.

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