



Antibiotic Susceptibility and Biofilm Formation of Clinical Isolates of Pseudomonas Species from Wounds Specimens

Mercy Ugbede Enemali¹, Danung Istifanus Yilkahan², Tamizhazhagan V^{3*}

^{1,2}Department of Natural and applied science ATBU bauchi

^{3*}Department of Zoology, Syed Ammal Arts and Science College, Ramanathapuram

Corresponding Email: ^{3*}tamilzoon@gmail.com

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Abstract: *A hallmark of Pseudomonas aeruginosa is its ability to establish biofilm based infections that are difficult to eradicate. Biofilms are less susceptible to host inflammatory and immune response and have higher antibiotic tolerance that frees living planktonic cells. The aim of the study is to investigate biofilm forming capacity and the antibiotic susceptibility profile of Pseudomonas aeruginosa strains isolated from clinical wound specimen. A total number of 60 wound specimens will be submitted to the bacteriology laboratory of Abubakar Tafawa Balewa University Teaching Hospital for investigation, and will be screened for Pseudomonas aeruginosa and strains will be identified on the basis of cultural characteristics, Gram staining, biochemical tests such as citrate, urease, indole, fermentation of sugar using triple sugar agar. The biofilm forming capacity of the strains will be tested using the test tube method after the strains are standardized to approximately standard inoculated into a cooked meat broth. The growth rate of Pseudomonas aeruginosa clinical strains after 48 hours incubation will be measured by taking the absorbance using Densi-Check. The strain growth rate will be checked. Biofilm formation at the liquid interface (pllicle) will be observed as a ring and this will be qualitatively scored from the first to the last strain. The cultures will be decanted gently and rinsed twice with sterile distilled water and steined with 0.1% w/v safranin, the stain will be decolorized using 100% alcohol, and the absorbance for strains will be measured at 590nm. The strain with the most and least biofilm formation will be recorded with the absorbance rates. The clinical significance of the Pseudomonas aeruginosa biofilm forming capacity and resistance to antibiotics which could result to none healing, delayed healing, foul smell of wound infection will be checked for the experiment.*

Keywords: *Pseudomonas Aeruginosa, Gram Staining, Distilled Water, Wound Infection.*



1. INTRODUCTION

The onset of wound infection depends on the integrity and protective function of the skin (Anupurba *et al.*, 2010). Infection is the outcome of the dynamic interaction that takes place between a host, potential pathogen and the environment (Mehta *et al.*, 2007). It occurs when the host's defense strategies are successfully evaded by microorganism and results in deleterious changes in the host (Lambert *et al.*, 2002). Complex interactions that are not yet fully understood precede the development of infection.

The widespread use of antibiotics, together with the length of time over which they have been available has led to major problems of resistant pathogens in wound infections, thus, contributing to morbidity and mortality. It has been shown that wound infection, is universal and the bacterial type varies with geographical location and resident flora of the skin (Anupurba *et al.*, 2013). *Pseudomonas* is a genus of Gram negative, aerobic Gammaproteobacteria, belonging to the family Pseudomonadaceae and containing 191 validly described species. They are Gram negative rod shaped and polar flagellated bacteria with some sporulating species.

A biofilm is any group of microorganism in which cells stick to each other and often these cells adhere to a surface. These adherent cells are frequently embedded within a self-produced matrix of extra cellular polymeric substance. Microscopic evaluation of specimens from chronic wound indicates presence of biofilm. Bacteria escape from biofilm that do not produce polysaccharide and are easily attacked by immune system cells. Inactivation of antibiotic takes place when bound to biofilm matrix. *Pseudomonas* has alginate exopolysaccharide which is anionic in nature. *Pseudomonas* has a strong tendency to form biofilm which has been found to be partially responsible for chronic infection.

Antibiotics are used for the control of bacterial infections in humans but biofilm have a high resistance to antibiotics. Biofilm, if allowed to be developed forms an aggregate cell or colony (colonies) which are increasingly resistant to antibiotics. *Pseudomonas* species have a strong tendency to develop biofilm and such biofilm has been found to be partially responsible for chronic infections. According to the National Institute of Health (NIH) about 65% of all microbial infections and 80% of all chronic infections are associated with biofilm. Hence there is a need to conduct studies to profile biofilm formation from *Pseudomonas* isolate so as to generate data that would help clinicians to choose correct antibiotics for treatment and therapeutic strategies are mandatory.

2. MATERIALS AND METHOD

Sample collection

Samples will be collected from 60 patients with complaints of discharge, pain, swelling, foul smell, delayed and none healing wound infection. The wound sample were collected with the use of a sterile cotton swab, the linear surface of the infected wound will be swabbed gently and then the swab will be transported to the laboratory on a transport medium to the microbiology department of the Abubakar Tafawa Balewa University Teaching Hospital Bauchi.



Isolation and Identification

The isolates that will be collected will be inoculated on Nutrient Agar and McConkey Agar aerobically at 37°C for 24 hours; it will then be sub cultured for discrete colonies. Discrete colonies that are lactose negative on McConkey Agar.

Preservation

Strains will be identified and then preserved on nutrient agar slant and will be preserved in the refrigerator at about 4°C for further use

Biofilm formation test

Cooked meat broth will be prepared for 24 hours, 3ml of the cooked meat broth will be transferred into a clean sterile test tube. A loop full of *Pseudomonas* colonies will be emulsified into the broth and incubated for 48 hours

Antibiotics and Susceptibility Test

Kirby-Bauer Disc diffusion method will be used to test the susceptibility of the *Pseudomonas* isolate to different antimicrobial agent. The test will be done on Mueller-Hinton agar using 0.5 McFarland standards for gram negative organism. Discrete colonies of *Pseudomonas* specie will be picked and emulsified on 3ml normal saline and inoculums; density will be checked using Densi-Check. Spread plate will be used to obtain a confluence with the inoculated plate will be kept on the bench for 15minutes to allow all moisture absorb and a Gram negative antibiotics discs will be placed on the culture without moving the disc after it is placed. The plate will be incubated for 24 hours at 37°C aerobically. The result will be measured with the aid of a metre rule in two different directions across each inhibition zone as per clinical laboratory standards (CLSI, 2011). The isolate will thus be designated susceptible or resistant.

3. RESULTS AND DISCUSSION

A total of 60 samples will be collected form patients with wound infections attending the Abubakar Tafawa Balewa University, Bauchi. Table 1: shows the biochemical test that will be carried out on the isolate Table 2: shows the Densi-Check (McFarland) standard of the inoculums Table 3: shows the resistance and susceptibility pattern of the organism on the subjected antibiotics Table 4: shows the qualitative analysis of biofilm, formation Table 5: shows the qualitative analysis absorbance of biofilm formation

Table 1: Biochemical Test

	Indole	Citrate	Urea	TSI	
Specie				Slope	Butt
PS1	–	+	+	R	R
PS2	–	+	+	R	R
PS3	–	+	+	R	R
PS4	–	+	+	R	R
PS5	–	+	+	R	R
PS6	–	+	+	R	R
PS7	–	+	+	R	R



PS8	-	+	+	R	R
PS9	-	+	+	R	R

KEY: R-Red. PS-*Pseudomonas* specie

Table 2: Density Check of the Inoculums (McFarland Standard)

Number of Sample					Average
PS1					0.52
PS2					0.47
PS3					0.50
PS4					0.47
PS5					0.52
PS6					0.48
PS7					0.47
PS8					0.52
PS9					0.51

Key: PS- *Pseudomonas* specie

Table 3: Resistance ad Susceptibility of Antibiotics on the Organism

ANTIBIOTICS	CSLI BREAK POINT	No OF ISOLATE TESTED	SUSCEPTIBILITY n= (%)	RESISTANCE n= (%)
Augmenting	<16 >	9	0 (0)	9 (100)
Gentamycin	≤ 12 >	9	4 (44.4)	5 (55.5)
Perfloxacin	≤15 >	9	3 (33.3)	6 (66.6)
Orfloxacin	≤ 14 >	9	4 (44.4)	5 (55.5)
Streptomycin	≤ 11 >	9	8 (88.8)	1 (11.1)
Seprtrin	≤ 15 >	9	0 (0)	9 (100)
Chloramphenicol	≤ 17 >	9	0 (0)	9 (100)
Sparfloxacin	≤ 15 >	9	2.(22.2)	7 (77.7)
Ciprofloxacin	≤ 15 >	9	9 (100)	0 (0)
Amoxicilin	≤ 15 >	9	2 (22.2)	7 (77.8)

Table 4: Qualitative Analysis of Biofilm Formation

Number of Sample					Biofilm Formation
PS1					+3
PS2					+2
PS3					+2
PS4					+2
PS5					+3
PS6					+2
PS7					+3



PS8					+3
PS9					+3

Key: PS- *Pseudomonas* specie

Table 5: Qualitative Analysis of the absorbance of *Pseudomonas* strain

PS	ABSORBANCE AT 590 nm	AT	ABSORBANCE AT 590 nm	MEAN AND STANDARD DEVIATION
1	22		19	20.5±2.12
2	23		22	22.5± 0.71
3	24		23	23.5± 0.71
4	22		20	21± 1.41
5	23		23	23± 0.00
6	22		20	21± 1.41
7	20		23	21.5± 2.12
8	21		22	21.5± 0.71
9	23		22	22.5± 0.71

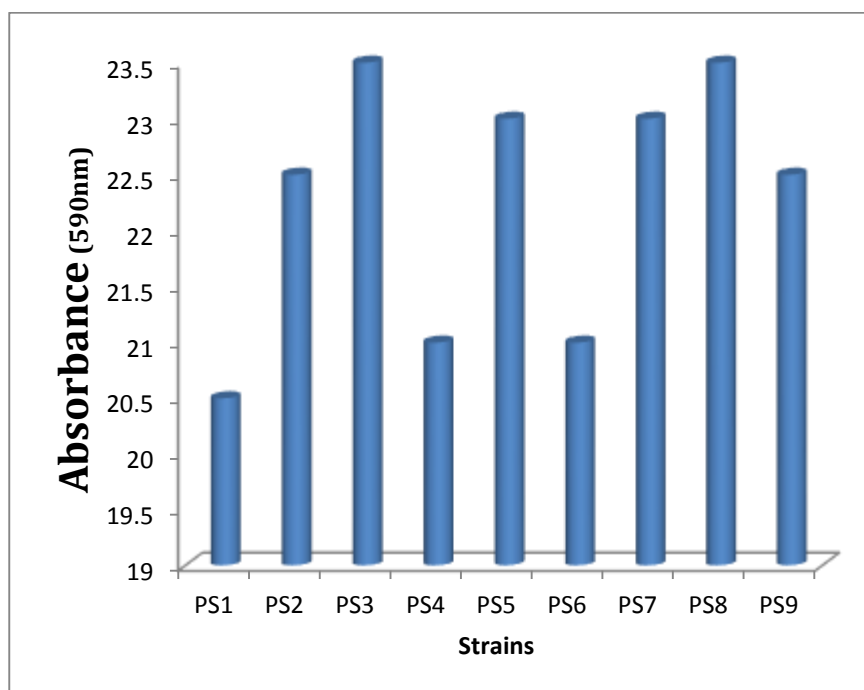


Figure 1: Quantitative Analysis (Absorbance) for Biofilm Formation

Pseudomonasaeruginosa is one of the most frequently found bacterial pathogens in patients with chronic infections, such as chronic wound and cystic fibrosis. The persistence of *Pseudomonas* in this infection is enabled by its ability to form biofilm. Standard antibiotic treatments, effective against bacteria living as single cells, are generally unsuccessful against biofilm. The presence and persistence of biofilms on chronic skin wounds can affect cellular (leukocytes, keratinocytes endothelial cells, and fibroblasts) function, the inflammatory



cellular response, cutaneous innate immune response, and the repair phase and may be slow to produce overt symptoms. Once established, however, biofilm infections often persist.

Neutrophils are among the first inflammatory cells to populate the initial wound site and contribute to wound healing by removing bacteria, foreign material, necrotic tissue, and releasing cytokines to promote revascularization and fibrosis. However, prolonged presence of neutrophils delays wound healing through the release of inflammatory factors, oxygen species, and proteinases (elastase and cathepsin G) that degrade extracellular matrix and key protein involved in the wound healing cascade causing collateral damage to neighboring healthy host tissue. Keratinocyte migration is reduced in the presence of neutrophils. A study of human chronic venous leg ulcers showed that *P. aeruginosa* containing wounds had significantly higher number of neutrophils compared with *S. aureus* containing wounds.

The roles of macrophages in wound healing are complicated. Although macrophages may not be required in embryonic or neonatal wound healing, there are important in mediating wound healing. Macrophages are dependent on the wound microenvironment in which they are located. Most in vivo investigation of macrophages response to biofilm are performed in foreign body associated infection, in which macrophages are key component (Marcia *et al.*, 2013). The research work will show that the strains are more susceptible to Ciprofloxacin and Streptomycin while the strains are less susceptible to Ofloxacin and Gentamycin. Most knowledge of medical biofilms was derived from studies of *Pseudomonasaeruginosa* and *Staphylococcus aureus*.

In 2008, Kirketerp-Moller *et al.*; when examining wound specimens from 22 patients using the PNA_Fish method, found *P.aeruginosa* within the tissue samples aggregated as microcolonies embedded in alginate, that is, biofilm. In the same year Bjarnsholt *et al.* analyzed sections of chronic wound using fluorescent In-Situ Hybridization techniques and identified distinct microcolonies, confirming the basal structure of bacterial biofilms. In 2010, Kennedy *et al* found evidence of biofilms in the ulcerate areas of burn wounds using light and electron microscopy techniques. Microbial nature of wound infections which also support biofilm presence in chronic venous leg ulcers, Fazli *et al.*, used PNA –FISH and Confocal laser scanning microscopy (CLSM) on biopsy samples to detect bacteria and large aggregates of cells. Utilizing inflammatory markers, the researchers concluded that *P. aeruginosa* biofilms lead to the influx of high numbers on neutrophils and biofilms may be one of the main factors leading to a persistent inflammatory response, with a resultant impairment of wound healing. A further study by Han diversity within chronic wounds epifluorescence microscopy revealed the presence of highly organized thick confluent biofilms and 47% of specimens had significant biofilm coverage arranged as aggregating colonies of varying size.

4. CONCLUSION

Overall, the data derived from human clinical studies make clear that biofilm have an important adverse effect on wound healing. Despite this, more fundamental scientific studies are required to understand what biofilm are doing to normal wound healing processes from cellular and immunological perspective. In particular, there is a need for more studies to understand why some wounds with biofilm growing in them heal and other do not. However, in conducting appropriate large randomized controlled trials on biofilms is possible to view biofilms when biopsies are analyzed microscopically.



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