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Biofilm Formation and Siderophore Production by Pseudomonas Aeruginosa Isolated from Wounds Infection

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***Abstract*— The study was aimed to determine the efficiency of P .aeruginosa for biofilm formation and siderophore production, and to assess the existence of correlation between siderophore production and biofilm formation. Ten P .aeruginosa were isolated from different wounds infection and identified based on morphological, biochemical and fatty acid methyl esterase analysis. Biofilm formation capability of isolates was studied by growing them on 96-well microtiter plate containing trypticase soy broth at 37oC for 24h. It is found that all strain of P. aeruginosa have the ability to form biofilm. However the strains PA01, PA02 and PA03 were shown strong biofilm formation. Siderophore production by isolate were study on CAS agar plate, after incubation the individual colony of isolates shown orange colored zone around the colony, reveals that all are positive in siderophore production. Further siderophore production were estimated and it's found that, out of ten isolates, the strains PA01, PA02 and PA03 of P .aeruginosa were produced maximum siderophore i.e. 66, 68, and 65%.**

***Index Terms*— Biofilm Formation, Siderophore Production, P. Aeruginosa, Wounds Infection.**

I. INTRODUCTION

Pseudomonas aeruginosa, an extremely versatile opportunistic pathogen and responsible for a number of infections of eyes, burns, wounds, respiratory and urinary tract [1, 2]. In 2001 it has been observe that bacteria colonizing human chronic wounds exist as biofilm [3].

Kirketerp-Moller et al. evaluated specimen wound of 22 patients suspected of P. aeruginosa colonization [4]. They showed that P. aeruginosa existed as biofilm rather than single cells in these wounds. Biofilm formation of Pseudomonas aeruginosa play vital role in the pathogenesis of infection and protect bacteria from host defenses and antimicrobial medications by creating a barrier of exopolysaccharides that is difficult for the immune system and antibiotics to penetrate. Also it contributes to various diseases that are characterized by a bacterial infection and chronic inflammation, such as periodontal disease, cystic fibrosis, chronic acne, osteomyelitis and dental plaque formation [5]. Biofilm formation is initiated when bacterial cells attach and adhere to the surface of implants or host tissues. Formation of biofilm requires coordinated chemical signaling between cells known as quarm sensing. The other important factor allowing colonization of the host is the efficient uptake of iron by the bacterium. Singh et al, (2002), evaluated that by sequestering iron with chelator lactoferrin block the ability of P. aeruginosa to form large multicellular biofilm structure [6]. In mammalian host iron is not freely available since it is either present in the heme molecules found in hemoproteins (hemoglobin, cytochromes) or strongly chelated by extracellular proteins (transferrin and lactoferrin) [7]. To utilize such complexes as iron sources, bacteria generally possess sophisticated mechanism; they produce high affinity iron chelators called siderophore. By considering this fact, it is relevant to investigate first time the existence of correlation between biofilm formation and siderophore production by P. aeruginosa.

In this study ten Pseudomonas strain were isolated from different wounds infection and were identified as P. aeruginosa. All strain was studied for siderophore production and biofilm formation. Based on strength of biofilm formation and amount of siderophore production the correlation was determined. This study was important for determine the role of siderophore production in biofilm development.

II. MATERIALS AND METHODS

A. Isolation of Pseudomonas aeruginosa

The bacterial samples were collected from ten individuals independent of their age, sex and dietary habits by sterile cotton swabs from all septic wounds. Swabs were transported into 2 ml trypticase soya bean broth and incubated aerobically at 37oC for 18 h. One loopfull from each sample was streaked on citramide agar; the plates where then incubated at 30oC for 24 h a well isolated colony were selected for identification and characterization. The identification of P. aeruginosa was carried out using a combination of colonial morphology, Gram stain characteristics, motility tests, pigmentation, oxidation-fermentation tests, Catalase and oxidizer activity tests and pyocyanin production [8].

B. FAME analysis

Fatty acid methyl ester (FAME) analysis for identification of Pseudomonas spp was carried out as per methods described by MIDI (Newark, DE, USA) on Agilent 6890N Network GC system. The strain were grown on Trypticase Soy Broth Agar and their fatty acid was are extracted by a procedure which consists of saponification in dilute sodium

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hydroxide/methanol solution followed by derivatization with dilute hydrochloric acid/methanol solution to give the respective methyl esters (FAMEs). The FAMEs are then extracted from the aqueous phase by the use of an organic solvent and the resulting extract is analyzed by GC

C. Biofilm formation

Biofilm forming ability of Pseudomonas aeruginosa strains were studied by modified method of O'Toole, 2011[9]. All the isolates were grown in trypticase soy broth (TSB) with 0.25% sucrose and incubated for overnight at 37°C temperature. 200µl diluted overnight culture was transferred in to 96-well microtiter plate and incubated at 37oC for 24 h and the broth without culture was used as control. After incubation period the content of each well was gently removed by slightly tapping the plates. The wells were then washed three times with phosphate buffer saline (PBS pH 7.3) to remove free-floating planktonic bacteria. Plates were air dried for 45 min and each well was stained with 150µl of 0.1% (w/v) crystal violet solution for 10 min. After staining, the plates were washed with sterile distilled water three times to remove excess crystal violet. At this point, biofilm were visible as purple rings formed on the side of each well. To measure the strength of biofilm 200µl of 95% ethanol where added in each wells to destaine the crystal violet. Further 100µl destaine solution from each well was transferred to a new microtiter plate and the level (OD) of the crystal violet present in the destaining solution was measured at 595nm. The mean OD value obtained from control was deducted from the all test OD values. The strain was classified based on OD values as per [10], Table 1.

Table 1 Biofilm formation classification of P. aeruginosa

Mean OD value	**Adherence**	**Biofilm Formation**
< 0.35	Weak	Weak
0.35 – 0.83	Moderate	Moderate
> 0.83	Strong	Strong

D. Detection of Siderophore Production

Siderophore production by different strain of Pseudomonas aeruginosa was tested by chromo azural S (CAS) assay [11]. The strains were spread over citramide agar and incubated for 48h at 30oC.After incubation a thin layer of CAS reagent in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24h at 30oC formation of yellow orange zone around the colonies indicates siderophore production.

Quantitative Estimation of Siderophore

To determine the amount of siderophore produced by isolated Pseudomonas aeruginosa strain were grown on succinate medium [12], containing of gm/l K2HPO4 6.0, KH2PO4 3.0, MgSO4 0.2, (NH4)2SO4 1.0 and succinic acid 4.0, pH 7.0. It was incubated for 24-30h at 28oC with constant shaking at 120 rpm on rotator shaking incubator. After incubation the fermented broth were centrifuge at 10,000 rpm in cooling centrifuge at 4oC for 10 minute and cell free supernatant was then mixed with 0.5 ml CAS solution and 10ul shuttling solution (Sulfosalicyclic acid). The color obtained was determined using the spectrophotometer at Absorbance 630 nm after 20 min of incubation with blank (Succinate medium). The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula [(Ar - As)/Ar] x 100, where Ar is the A630nm of reference sample (medium + CAS assay solution + shuttle solution) and As is the A630nm of the sample (supernatant + CAS assay solution + shuttle solution) [13,]

III. RESULTS AND DISCUSSION

Total ten Pseudomonas aeruginosa strains were isolated from different wounded patients and have assigned name PA01 to PA10. They were identified as Pseudomonas aeruginosa based on morphological, biochemical characteristic shown in Table 2.

Table 2. Morphology and biochemical tests of *P. aeruginosa*

Morphology and biochemical tests	Results
Grams Staining	+ ve
Bluish green colour colony on citramide agar	+ ve
Oxidase	+ ve
Catalase	+ ve
Growth on Nacl (25%)	+ ve
Urease	-ve
Indole	-ve
Methyl red	-ve
Vogues Prosker	- ve
Gelatin Hydrolysis	+ ve
Manitol	+ ve

Biofilm formation by Pseudomonas aeruginosa was studied on polystyrene, 96 well-flat bottom tissue culture plates at 37oC for 24 h. It was found that all ten isolates shown significant growth on tissue culture plates and the growth was visualized by adding 0.1% crystal violet to the well, the adherent biofilm acquire purple colour reveals that all isolate having ability to form biofilm, but the intensity of purple colure and the OD of destaine solution of biofilm varying according to isolates shown in Figure 1. The strains PA01, 02, 03 were shown strong biofilm, while the strain PA04, 06, 10 shown moderate and strain PA05, 07, 08, 09 showed weak biofilm formation Table 3. The ability of P. aeruginosa to form biofilm in wound has confirmed on the result of Harrison Balestra et al, 2003 [14], who reported that biofilm formation by certain pathogens such as P. aeruginosa can sometimes be rapid and the presence of such organisms in wounds could lead to the development of biofilms within a period of 24 to 48 hours after colonization. However the difference in biofilm formation shown by above strain of P. aeruginosa reveals that biofilm-forming capabilities of bacteria depend on multiple factors including the attachment surface, presence of other bacteria, temperature, availability

of nutrients, amino acid and iron [15,16]. Burmolle et al. (2006) [17], and Fux et al. (2005) [18], demonstrate that the biofilm's strengths are found in its heterogenicity (different protein expression), interspecies cooperation and intercellular matrix structure.

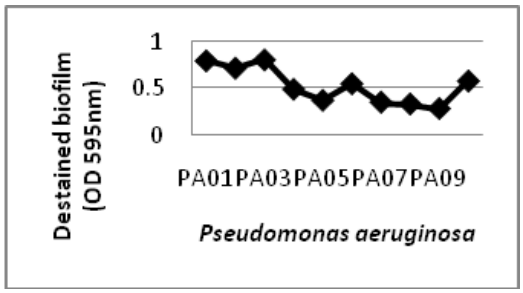

Fig 1: Crystal violet OD of biofilm destaine produced by Pseudomonas aeruginosa

Table 3: Strength of biofilm produced by Pseudomonas aeruginosa

Strains of *P. aeruginosa*	Category of biofilm formation
PA01	+++
PA02	+++
PA03	+++
PA04	++
PA05	+
PA06	++
PA07	+
PA08	+
PA09	+
PA10	++

+++ Strong, ++ Moderate, + Weak

Siderophore production by different P. aeruginosa strains were detected by growing them individually on citramide agar, after spreading layer of CAS reagent and incubation each colony has developed yellow to orange colour zone on CAS agar plate. The development of orange colour zone around the colony reveals that all isolate were positive for siderophore production. Similar finding have been reported by Ali and Vidhale, (2011) [13]. However the change in color from blue to orange around the colony is due to removal of Fe++ iron from dye by siderophore. Cox and Adams, 1985 [19] and Cox and Graham, 1979 [20] reported that in iron-limiting conditions P. aeruginosa strain produced two types of siderophore i.e. pyoverdin and pyochelin for high-affinity iron uptake. In order to estimate the amount of siderophore produced by different isolates, a CAS liquid assay has performed. The data presented in Figure 2, showed that the amount of siderophore produced by P. aeruginosa varies with strain and it was highest in three strains PA01, PA02 and PA03. The other strain has shown moderate to low siderophore production. These indicate that the strain produce highest percent of siderophore growing in the region of biofilm were limited iron is available and whose siderophore producing gene is well expressed which produce more and more siderophore for iron acquisition; while other strain embedded in biofilm seems that there siderophore producing gene were not well expressed or they may be siderophore deficient. According to the generally accepted definition of siderophore are ferric-specific microbial iron chelator compounds whose biosynthesis is regulated by the availability of iron in the surrounding medium [21]. Further the strong biofilm and highest siderophore producing strain were subjected to fatty acid methyl ester analysis MIDI (Newark, DE, USA) on Agilent 6890N Network GC system. The chromatogram of fatty acid analysis obtained is more descriptive and elaborative Figure 3. It corresponds to the gas chromatographic profile of P. aeruginosa in the Sherlock TSBA Library version 3.9 (Microbial ID, MIDI Inc.).

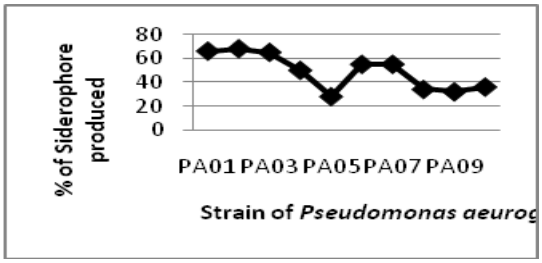

Fig 2: Percent of siderophore produced by different strain of Pseudomonas aeruginosa

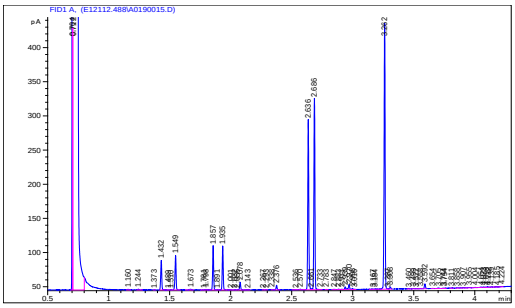

Fig 3: Chromatogram of *P. aeruginosa* fatty acid obtained from Gas Chromatography.

III. CONCLUSION

Iron is essential for almost all living organisms, including bacteria. Acquiring of iron by pathogenic bacteria was absolutely essential for their growth and biofilm formation. In human host the iron is usually bound to protein and not easily available for bacteria, to utilize such complex iron they synthesize siderophore. In above investigation we found that the strain P. aeruginosa PA01, 02, 03 were produce maximum siderophore which seems that they were growing in iron limited condition and to full-fill iron requirement they produced maximum siderophore. Thus the supply of sufficient iron by siderophore insists the strain to form strong biofilm. While the strain produce low to moderate amount of siderophore shown weak biofilm formation. This reveals that low amount of siderophore insufficient to transport optimum iron for growth and biofilm development under iron restricted condition. In Pseudomonas aeruginosa, biofilm formation requires iron [6], and siderophore deficient mutants show a pleiotropic reduction in biofilm-forming ability [22]. Hence it was concluded that iron limitation reduced biofilm formation by siderophore- deficient P. aeruginosa and this supports the

link between siderophores, iron and biofilm. Also Griffin et al., 2004 [23] reported that siderophore deficiency decreases iron-limited monoculture growth. Further the highest siderophore producing and strong biofilm forming strain were identified as P. aeruginosa by fatty acid methyl ester (FAME) analysis.

REFERENCES

[1] E.V. Wagner, B.H. Iglewski, "P. aeruginosa Biofilms in CF Infection", Clinic Rev Allerg Immunol. 2008, 35, 124-34.

[2] H. Zhu, R. Bandara, T.C. Conibear, S.J. Thuruthyil, S.A. Rice, "Pseudomonas aeruginosa with lasI quorum sensing deficiency during corneal infection", Invest Ophthalmol vis Sci. 2004, 45, 1897-1903.

[3] H. Akiyama, S. Morizane, O. Yamasaki, T. Oono, K. Iwatsuki, "Assessment of Streptococcus pyogenes microcolony formation in infected skin by confocal laser scanning microscopy", J Dermatol Sci. 2003, 32, 193-199.

[4] K. Kirketerp-Moller, P.O. Jensen, M. Fazli ,K.G. Madsen, J. Pedersen, C. Moser, T. Tolker-Nielsen, N. Hoiby, M. Givskov, T. Bjarnsholt, "Distribution, organization, and ecology of bacteriain chronic wounds", J Clin Microbiol. 2008, 46, 2717-2722.

[5] Hall-Stoodley, P. Stoodley, "Evolving concepts in biofilm infections", Cell Microbiol. 2009, 11, 1034-43.

[6] P.K. Singh, M.R. Parsek, E.P. Greenberg, & M.J. Welsh, "A component of innate immunity prevents bacterial biofilm development", Nature. 2002, 417,552-555.

[7] C.N. Cornelissen, and P.F. Sparling, "Ironpiracy: acquisition of transferring-bound iron by bacterial pathogens", Mol. Microbial. 1994, 14, 843-850, doi: 10.1111/j.1365-2958.1994.tb01320.x

[8] M. Cheesbrough,. "Medical Laboratory Manual for Tropical Countries Vol. II Microbiology". Butterworth Heinemann Ltd. Linacre House, Jordan Hill Oxford OX2 8DP. 1993, 2, 64-265.

[9] G.A. O'Toole, "Microtiter Dish Biofilm Formation Assay", 2011, JoVE 47. http://www.jove.com/ details. php? id =2437, doi: 10.3791/2437.

[10] G.D. Christensen, W.A. Simpson, J.J. Younger, "Adherence of coagulase - negative Staphylococci to plastic tissue culture plates: a quantitative model for the adherence of Staphylococci to medical devices", Journal of Clinical Microbiology. 1985, 22, 996-1006.

[11] B.Schwyn, and J.B. Neilands, "Universal chemical assay for the detection and determination of siderophores", Anal. Biochem. 1987, 160, 47-58.

[12] J.M. Meyer, M.A. Abdallah, "The fluorescent pigment of Pseudomonas fluorescence: biosynthesis, purification and Physico-chemical properties", J. Gen. Microbiol. 1978, 107, 319-328.

[13] S.S. Ali and N.N Vidhale, "Evaluation of Siderophore Produced by Different Clinical Isolate Pseudomonas aeruginosa", International Journal of Microbiology Research. 2011, 3, 131- 135. http://dx.doi.org/10.9735/0975-5276.3.3.131-135

[14] C. Harrison-Balestra, A.L. Cazzaniga, S.C. Davis, and P.M. Mertz, "A wound-isolated Pseudomonas aeruginosa grows a biofilm in vitro within 10 hours and is visualized by light microscopy", Dermatologic Surgery. 2003, 29, 631 - 635.

[15] S.A. Rice, K.S. Koh, S.Y. Queck, M. Labbate, K.W. Lam, and S. Kjelleberg, "Biofilm formation and sloughing in Serratia marcescens are controlled by quorum sensing and nutrient cues", Journal of Bacteriology. 2005, 187, 3477 - 3485.

[16] A.Heydorn, B. Ersbøll, J. Kato,"Statistical Analysis of Pseudomonas aeruginosa biofilm development: Impact of mutations in genes involved in twitching motility, cell-to-cell signalling, and stationary-phase sigma factor expression", Applied and Environmental Microbiology. 2002, 68, 2008 - 2017.

[17] M. Burmolle, J.S. Webb, D. Rao, L.H. Hansen, "Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms", Appl Environ Microbiol. 2006,72, 3916-3923.

[18] C.A Fux, J.W. Costerton, P.S. Stewart, P. Stoodley, "Survival strategies of infectious biofilms". Trends Microbiol. 2005, 13, 34-40.

[19] C.D. Cox, and P. Adams, "Siderophore activity of pyoverdin for Pseudomonas aeruginosa", Infect. Immun. 1985, 48, 130-138.

[20] C.D. Cox, and R. Graham, "Isolation of an iron-binding compound from Pseudomonas aeruginosa", J. Bacteriol. 1979, 137, 357–364.

[21] N, Orsi, "The antimicrobial activity of lactoferrin: current status and perspectives", Biometals. 2004, 17,189-196.

[22] Banin, M.L.Vasil, E.P. Greenberg, "Iron and Pseudomonas aeruginosa biofilm formation", Proc Natl Acad Sci USA, 2005,102,11076-11081.

[23] A.S. Griffin, S.A. West, "Buckling,. Cooperation and competition in pathogenic bacteria", Nature. 2004, 430,1024–1027.