Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 09, pp.38974-38977, September, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF BIOFILM FORMING *PSEUDOMONAS AERUGINOSA* FROM WOUNDS INFECTION

***Syed Sajeed Ali and Wakte, P. S.**

Department of Microbiology, D.S.M. College Parbhani- 401431 (MS) India

ARTICLE INFO

Article History:
Received 19th June, 2016
Received in revised form
26th July, 2016
Accepted 18th August, 2016
Published online 30th September, 2016

Key words:

Biofilm, Identification,
Pseudomonas aeruginosa,
Wounds infection.

ABSTRACT

Development of biofilm by *Pseudomonas aeruginosa* in wounds infection caused severe threat to patients and prevents the healing of wounds. To control such infection it's important to characterize the pathogenic biofilm forming bacteria of wounds infection. The present study was aimed to isolate and identify the biofilm forming *Pseudomonas aeruginosa* from wounds infection. 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 tube and microtiter plate methods. From the ten isolates the strain PA01, 02, 03 shown strong biofilm and strain PA04, 06, 10 moderate, while strain PA05, 07, 08, 09 showed weak biofilm formation on both methods except the strain PA10 shown strong biofilm on microtiter plate method.

Copyright © 2016, Syed Sajeed Ali and Wakte. *This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.*

Citation: Syed Sajeed Ali and Wakte, P. S. 2016. "Isolation and identification of Biofilm forming *Pseudomonas aeruginosa* from wounds infection" *International Journal of Current Research*, 8, (09), 38974-38977.

INTRODUCTION

Chronic wounds cause a significant burden to healthcare system as well as morbidity and mortality to mankind. It is an important and often unrecognized cause of disease and disability of the elderly population (Davies *et al*., 2007; Howell-Jones *et al*., 2006). These wounds carry a diverse group of microorganism, which contributes both directly and indirectly to their non-healing phenotype (Stephens *et al.,* 2003). Much attention has recently been focused on the ability of the bacteria within chronic wounds to form and exit within biofilm (James *et al.*, 2008). It had been speculated as early as 2001 that bacteria colonizing human chronic wounds exist as biofilm communities. Kirketerp-Møller *et al*. evaluated specimen wounds of 22 patients suspected of *P. aeruginosa* colonization (Kirketerp-Moller *et al.*, 2008). Biofilm consist of heterogeneous group of bacteria which attached to each other or to surfaces, and enclosed within extracellular polymeric substances. The chronic wound provide moist surface with its proteinaceous substrate, and supply of nutrients, makes ideal environment for biofilm development. Many researchers have demonstrated that bacteria within the wound environment possess the ability to form biofilm in both acute and chronic wounds (Bajarnsholt *et al*., 2008; Davis *et al*., 2008). Biofilm infections, such as pneumonia in cystic fibrosis patients, chronic wounds, chronic otitis media and implant- and catheter-associated infections, affect millions of people in the developed world each year and many deaths occur as a consequence. Further biofilm play important role in resistance to host immune responses and conventional treatment of wounds. The study of pathogenic bacteria associated with wounds biofilm has become an important area of current research, the effective monitoring of bacteria within a biofilm important to develop possible management strategies. Therefore the study has undertaken to isolate and identify *Pseudomonas aeruginosa* from different wounds infection and biofilm development in vitro.

****Corresponding author: Syed Sajeed Ali,***
Department of Microbiology, D.S.M. College Parbhani- 401431 (MS) India.

MATERIALS AND METHODS

Isolation of *Pseudomonas aeruginosa form different wounds infection*

A total 10 *Pseudomonas aeruginosa* were isolated from individual wounds infection by sterile cotton swabs. Swabs were transported into 2 ml trypticase soya bean broth and incubated aerobically at 37°C for 18 hrs. One loopfull from

each sample was streaked on citramide agar; the plates where then incubated at 30°C for 24 hrs, a well isolated colony were selected for identification and characterization. The organism were identified base on combination of colonial morphology, Gram stain characteristics, motility tests, pigmentation, oxidation-fermentation tests, Catalase and oxidizer activity tests and pyocyanin production (Cheesbrough, 1993). Biofilm formation capability of *Pseudomonas aeruginosa* were studied by tube and 96 microtiter plate methods.

Tube method

Pseudomonas aeruginosa isolated from different wounds infection were tested for biofilm formation by a modification of the standard method proposed by Christensen *et al*. (1982). Two milliliters of tryoticase soy broth were inoculated with a loopful culture of isolate from overnight culture plates and incubated for 48 hrs at 37°C, after which the content of tube were decanted and washed three times with phosphate buffer saline (PBS pH 7.3) and left to dry at room temperature. The tubes then stained with 4% solution of crystal violet and each tube was then gently rotated to ensure uniform staining and the contents of tube were decanted. A positive biofilm formation was considered when visible film lined develops on wall and bottom of the tubes. The results were scored visually as 0-absent, 1-weak, 2-moderate, 3-strong (Mathur *et al*., 2006)

Microtiter plate method

Biofilm formation by *Pseudomonas aeruginosa* was studied on 96 microtiter plate a method described by Stepanovic *et al*. (2004). 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 37°C for 24 hrs 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 300 µl of sterile distilled water. Bacteria adhering to the wells were fixed with 250 µl of methanol per well for 15 minutes. Then each well of plates were stained with 250 µl of 0.1% (w/v) crystal violet solution for 5 minutes. Excess stain was removed by washing with sterile distilled water and air dried. To measure the strength of biofilm 250µl of 33% (v/v) glacial acetic acid 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 490nm using an automated Microtiter plate reader. The tests were carried out in triplicate and the results were averaged. The cut-off O.D (O.D.c) was determined as three standard deviations above the mean O.D. of the negative control. Strains were classified as (i) no biofilm producer [O.D.≤ O.D.c]; Weak biofilm producer [O.D.c< O.D.≤ (2xO.D.c); and strong biofilm producer.

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 are extracted by a procedure which consists of saponification in dilute sodium 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

RESULTS AND DISCUSSION

Isolation of *Pseudomonas aeruginosa*

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 1).

Table 1. Morphology and biochemical tests of *P. aeruginosa*

S. No.	Biochemical Test	Results
1	Gram staining	Gram Negative, Single rods
2	Motility	Motile
3	Colony Morphology on Nutrient Agar	Bluish green colours colonies
	MacConkey agar	Non lactose fermenting colonies
	Cetrimide agar	Bluish green colour colonies
4	Oxidase	Positive
5	Catalase	Positive
6	Growth at different temperature	
	5°C	Negative
	15°C	Positive
	37°C	Positive
	42°C	Positive
7	Growth at different pH	
	5.7	Positive
	6.8	Positive
	8.0	Positive
8	Growth on Nacl(25%)	Positive
9	Simmon's citrate medium	Positive
10	Urease	Negative
11	Indole	Negative
12	Methyl red	Negative
13	Vogues Prosker	Negative
14	Nitrate Reductase	Positive
15	Gelatin Hydrolysis	Positive
16	Glucose	Positive
17	Lactose	Negative
18	Manitol	Positive
19	Arginine dihydrolase	Negative
20	Starch hydrolysis	Negative

Tube method

The study of biofilm formation by *Pseudomonas aeruginosa* in tube method reveal that all ten isolates shown significant growth in tubes 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 color 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 2). The ability of *P. aeruginosa* to form biofilm in wound has confirmed on the result of Harrison

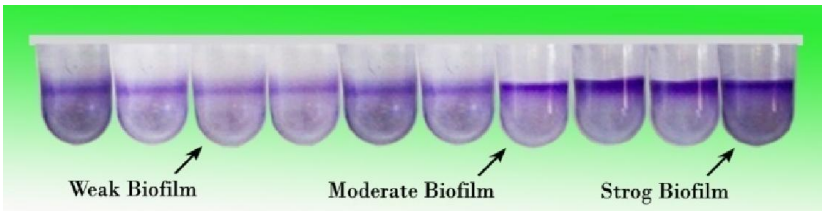

Fig. 1. Biofilm production by *Pseudomonas aeruginosa* in tube

Table 2. Strength of biofilm produced by *Pseudomonas aeruginosa* in tube method

Strains of *P. aeruginosa*	Category of biofilm formation
PA01	3
PA02	3
PA03	3
PA04	2
PA05	1
PA06	2
PA07	1
PA08	1
PA09	1
PA10	2

+++ Strong, ++ Moderate, + Weak

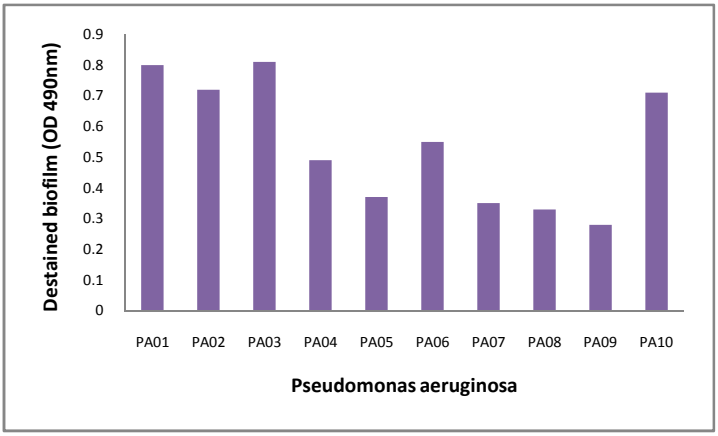

Fig. 2. Crystal violet OD of biofilm destaine produced by *Pseudomonas aeruginosa*

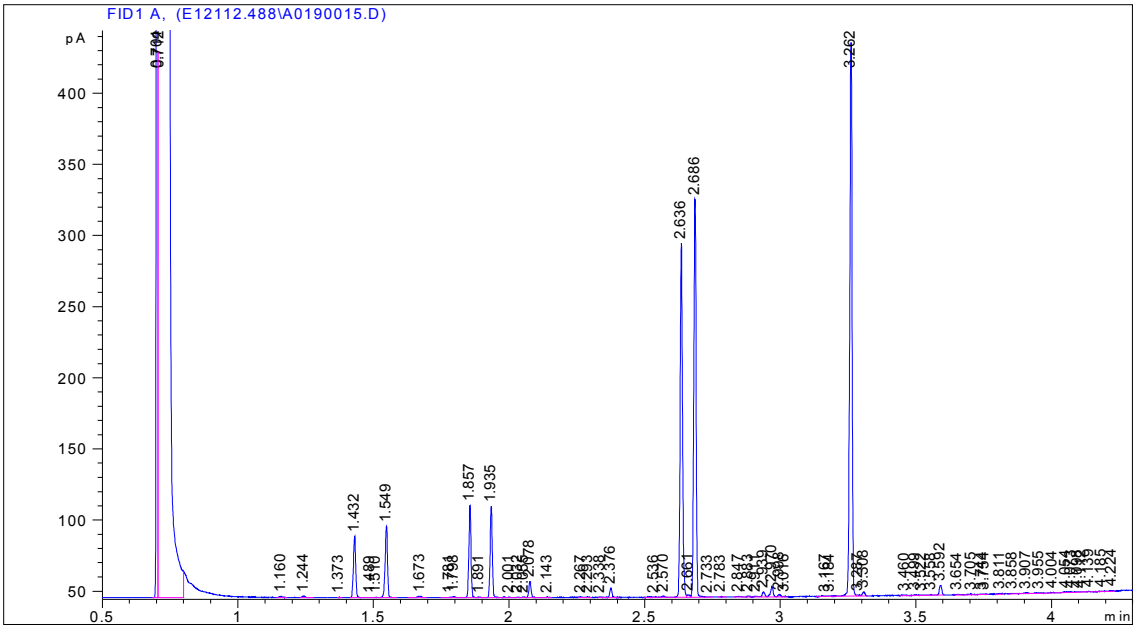

Fig. 3. Chromatogram of *P. aeruginosa* fatty acid obtained from Gas Chromatography

Balestra *et al*. (2003), who reported that biofilm formation by certain pathogens such as *P. aeruginos*a 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.

Microtiter plate method

Biofilm formation by *Pseudomonas aeruginosa* was also studied on polystyrene, 96 well-flat bottom tissue culture plates at 37^{o}C for 24 hrs. It was found that all ten isolates were positive for biofilm formation. The OD of destained solution of biofilm varying according to isolates shown in Figure 2. The strains PA01, 02, 03, 10 were shown strong biofilm, while the strain PA04, 06, shown moderate and strain PA05, 07, 08, 09 showed weak biofilm formation. However the difference in biofilm formation shown by above strain of *P. aeruginos*a 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 (Rice *et al,* 2005; Heydorn *et al*., 2002). Burmolle *et al*. (2006) and Fux *et al*. (2005), demonstrate that the biofilm's strengths are found in its heterogenicity (different protein expression), interspecies cooperation and intercellular matrix structure.

FAME analysis

Further the strong biofilm producing strains 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.).

Conclusion

Current study suggests that *Pseudomonas aeruginosa* where consistent microorganism of wounds infection and their prevalence in wound contribute delaying in wound healing. The strong biofilm forming capability of *Pseudomonas aeruginosa* considered as marker of clinically relevant infection. The above finding confirmed the presence of *Pseudomonas aeruginosa* and their growth in the form of strong biofilm, which emphasizes that their characterization is an important aspect of infection control. Furthermore, the information on the capacity of a clinical isolate to produce biofilm would help a clinician to evaluate the measure of its virulence and devise an appropriate treatment plan for the patients.

REFERENCES

Bjarnsholt, T., Kirketerp-Moller, K., Jensen, P. O., Madsen, K. G., Phipps, R., Krogfelt, K., Hoiby, N. & Givskov, M. 2008. Why chronic wounds will not heal: a novel hypothesis, *Wound Repair Regen,* 16, 2-10.

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

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

Cristensen, G.D., Simpson, W.A., Bisno, A.L., Beachy, E.H. 1982. Adherence of biofilm producing strains of *Staphylococci epidermidis* to smooth surfaces. *Infect Immun*, 37, 318-326.

Davies, C. E., Hill, K. E., Newcombe, R. G., Stephens, P., Wilson, M. J., Harding, K. G. & Thomas, D. W. 2007. A prospective study of the microbiology of chronic venous leg ulcers to reevaluate the clinical predictive value of tissue biopsies and swabs, *Wound Repair Regen,* 15, 17-22

Davis, S. C., Ricotti, C., Cazzaniga, A., Welsh, E., Eaglstein, W. H. & Mertz, P. M. 2008. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. *Wound Repair Regen*, 16, 23-29.

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

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

Heydorn, A., Ersbøll, B., Kato, J *et al.* 2002. 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,* 68**,** 2008-2017.

Howell-Jones, R. S., Price, P. E., Howard, A. J. & Thomas, D. W. 2006. Antibiotic prescribing for chronic skin wounds in primary care, *Wound Repair Regen*, 14, 387-393.

James, G.A., Swogger, E., Wolcott, R., Pulcini, E.D., Secor, P., Sestrich, J., Costerton, J. W. & Stewart, P.S. 2008. Biofilm in chronic wounds, *Wounds Repair Regen*, 16, 37-44.

Kirketerp-Møller, K., Jensen, PØ., Fazli, M., Madsen, KG., Pedersen, J *et al.* 2008. Distribution, organization and ecology of bacteria in chronic wounds, *Journal of Clinical Microbiology*, 46(8): 2717-2722.

Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A. 2006. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods, *Indian J Med Microbiol,* 24, 25-29.

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

Stepanovic, S., Cirkovic, I., Ranin, L., Svabic-Vlahovic, M. 2004. Biofilm formation by *Salmonella spp*. and *Listeria monocytogenes* on plastic surface, *Letters in Applied Microbiology*, 38, 428-432.

Stephens, P., Wall, I. B., Wilson, M., Hill, K. E., Davies, C. E., Hill, C. M., Harding, K. G. & Thomas, D. W. 2003. Anaerobic cocci populating the deep tissues of chronic wound impair cellular wound healing responses in vitro, *Br J Dermatol,* 148, 456–466.

****