IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 8, Issue 2 (Mar. – Apr. 2022), PP 10-14
www.iosrjournals.org

Assessment of Biofilm Formation and Related Biofilm Encoding Genes in *Pseudomonas aeruginosa* Clinical Field Isolates

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Abstract:

Background*: Pseudomonas aeruginosa (P. aeruginosa) is a pertinent opportunistic and hardly to treat pathogen because of its high resistance to widespread antibiotics. Pseudomonas aeruginosa infection is getting graver due to presence of virulence factors as the ability to form biofilm. The current study addressed biofilm formation of Pseudomonas aeruginosa isolates with relation to its encoding genes.*
Materials and Methods*: Multiplex polymerase chain reaction was applied on six clinical Pseudomonas aeruginosa isolates for detection of three biofilm encoding genes; algD, pelF, and pslD. The positive genes harboring isolates were induced to form biofilm and examined by scanning electron microscopy. The biofilm formation ability was assessed by a microtiter plate assay.*
Results*: The three out of tested six Pseudomonas aeruginosa isolates were found to carry two types of biofilm encoding genes; two isolates harbored algD while the third carried pelF. The three isolates were associated with wounds and abscesses. The three isolates displayed two patterns of biofilm formation demonstrated quantitatively by microtiter plate assay and morphologically by scanning electron microscopy.*
Conclusion: *Pseudomonas aeruginosa clinical isolates usually harbored biofilm encoding genes and have the ability to perform biofilms which help the pathogen to resist the adverse environmental conditions and antibiotic administration.*
Key Word*: Biofilm, Biofilm encoding genes, Pseudomonas aeruginosa.*

Date of Submission: 15-03-2022 Date of Acceptance: 31-03-2022

I. Introduction

Pseudomonas aeruginosa (P. aeruginosa) is a ubiquitous Gram-negative bacterium that implemented in various serious nosocomial and zoonotic infections. It is widely found in different environmental circumstances but in certain conditions as immune-compromised individuals may become an opportunistic pathogen [1]. *P. aeruginosa* strains of animal origin is considered of great public health importance and reported as priority pathogen in the last decades as threaten One Health Concept because of the organism possesses a special pathogenic criteria [2].

Interestingly, *P. aeruginosa* can resist the habitual antimicrobial agents via its unique intrinsic antibiotic resistance, rather adaptive and acquired resistance mechanisms and also its potency to form biofilms [3].

The formation of biofilm is considered as virulence feature of *P. aeruginosa* which favors protection from surrounding external stressors, prohibits phagocytosis, promotes colonization and prolongs persistence. The biofilm is commonly associated with chronic conditions [4].

The biofilm is a complex assemblage of microorganisms wrapped in a spontaneous formed extracellular polymeric matrix. It has been demonstrated that the biofilm matrix of *P. aeruginosa* is predominately comprises polysaccharides, proteins, lipids and extracellular nucleic acids [5]. The biofilm matrix assists cell-to-cell communication and facilitates a bridge for adhesion to biotic and abiotic surfaces such as

industrial equipment or medical implants. Moreover, it gives a sanctuary for protecting bacteria in adverse environmental circumstances as well as antibiotics administration and host immune activities [6].

The biofilm matrix is frequently composed from three exopolysaccharides; Pel, Psl and alginate. Alginate is negatively charged linear polymer made up mannuronic acid and guluronic acid and gives the structural stability and protective functions of biofilm. Alginate synthesis by *P. aeruginosa* strains is of complicated genetic control systems. One of those systems is the *algACD* operon. *alg*D gene promote primarily the control of alginate biosynthesis and transcription of the alginate associated proteins while *psl*D and *pelf* are responsible for formation of biofilm matrix substantial exopolysaccharides; Psl and Pel [7, 8].

Our study was directed to assess the existence of biofilm encoding genes of *P. aeruginosa* clinical isolates besides evaluation of the formed biofilms.

II. Material And Methods

The current study was applied on previously recovered six *P. aeruginosa* isolates [9] obtained from wound and abscesses, tracheal and lung tissues. DNA extraction was performed by using kit of the QIAamp DNA Mini (Qiagen, Germany, GmbH) with amendments from the manufacturer's notes.

DNA extraction and multiplex PCR

The six *P. aeruginosa* isolates were assessed for three biofilm- encoding genes, *alg*D, *pel*F, and *psl*D by multiplex polymerase chain reaction (PCR) technique, using specific primers (Metabion international AG, Germany) [10] and the reactions were conducted on 30 cycles of thermal conditions as described in (Table 1) in a T3 Biometra thermal cycler. The positive and negative controls represented by *P. aeruginosa* (ATCC 27853) and distilled water (Merck, Germany) respectively. The amplified PCR products were analyzed using gel documentation system (Alpha Innotech, Biometra) through computer software.

Table 1: Multiplex PCR: Primers sequences, target biofilm- encoding genes, amplicon sizes, and cycling conditions.

Target gene	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension
			Secondary denaturation	Annealing	Extension	
*alg*D	593 bp	95 ℃ for 5 minutes	94 ℃ for 30 seconds	60 ℃ for 40 seconds	72 ℃ for 40 seconds	72 ℃ for 5 minutes
*pel*F	789 bp					
*psl*D	369 bp					

Quantitative assessment of biofilm formation

The three positive biofilm genes *P. aeruginosa* isolates culture were grown overnight in Tryptic soy broth (Himedia, India). After that, the overnight cultures were adjusted to the turbidity of a 1 McFarland standard, and then diluted to 1:100 in Luria Bertani (LB) broth medium (Himedia, India) for biofilm assays. The quantitative evaluation of biofilm formation was carried out via colorimetric assay [11]. On hundred microliter from each was added to each well in the sterile flat bottomed polystyrene 96-well microtiter plate (Thermofisher) and incubated for 24 and 48hrs at 28°C. The control wells were filled with LB broth alone.

After the two incubation periods, the supernatants (containing non-adherent cells) were taken out and wells were rinsed with sterile phosphate buffered saline (PBS, pH 7.3) three times. The adherent biofilms were fixed by methanol 99%, let air-dried, and then stained with crystal violet 0.1% (Sigma-Aldrich) for 15 minutes, at room temperature. After that, the free stain was removed by water washing and let to dry. All tests were conducted in triplicate and repeated three times for each isolate and the optical densities (OD) values were measured using microtiter plate reader ELx 800 UV (Bio-Tek, USA) at 570 nm and evaluated as mentioned [12].

Scanning electron microscopy

The planktonic cells free formed bacterial biofilms were examined by scanning electron microscopy (SEM). 3% phosphate-buffered (v/v) glutaraldehyde (Himedia, India) was used to fix the samples for 24 hours at 4 °C. After that, fixed samples were dehydrated in graded cold ethanol /water series [13]. Desiccated dried biofilm samples were coated and inspected under SEM (QUANTA FEG 250, Japan), at a voltage of 20 kV and magnifications ×12000.

Statistical analysis The statistical analysis of data was performed by applying one-way ANOVA, and the results were presented as means ± SD.

III. Result

Biofilm-encoding genes were determined in 3 out of 6 tested *P. aeruginosa* (Fig. 1); 50 %. Two isolates displayed amplified band at 593 bp represented *alg*D and one isolate showed amplified band at 789 bp presented *pel*F gene. Concerning the biofilm formation the three isolates showed two criteria; two isolates PA1 and PA2 that obtained from ill sheep showed strong biofilm while the third isolate which obtained from diseased goat displayed a much weaker biofilm. All three isolates demonstrated increasing in biofilm formation at second day in comparison to the first day incubation (Fig. 2), pillar-like matured intact cells biofilms in SEM images (Fig. 3).

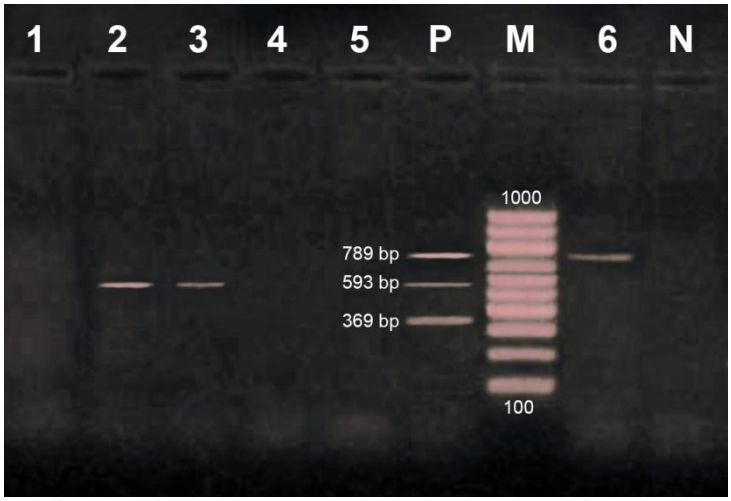

Fig. 1: PCR amplification of three biofilm-encoding genes in six clinical isolates of *P. aeruginosa* obtained from wound, abscesses, tracheal and lung tissues as representative. Lane 1–5: sheep origin's isolates Lane 6: goat origin's isolate. Lane P: PCR products (369, 593 and 789 bp) for the *psl*D, *alg*D and *pel*F genes respectively, in *P. aeruginosa* reference strain as control positive. M: 100 bp DNA ladder. Lane N: distilled water as control negative.

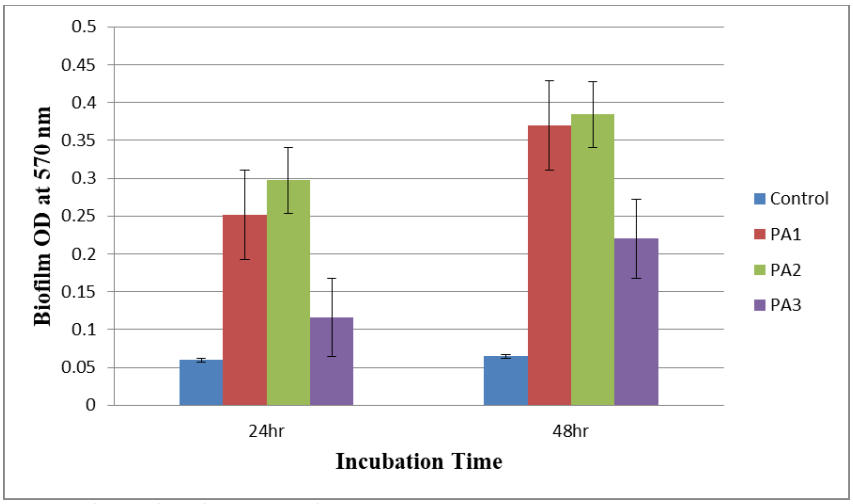

Fig. 2: (OD_{570}) values for biofilm formation of the three *P. aeruginosa* clinical isolates which carried one of biofilm encoding genes by colorimetric assay. Control: LB medium, PA1 and PA2 isolates showed high values (indicated strong biofilm formation) while PA3 showed lower values (indicated weak biofilm formation). Values are mean ± SEM.

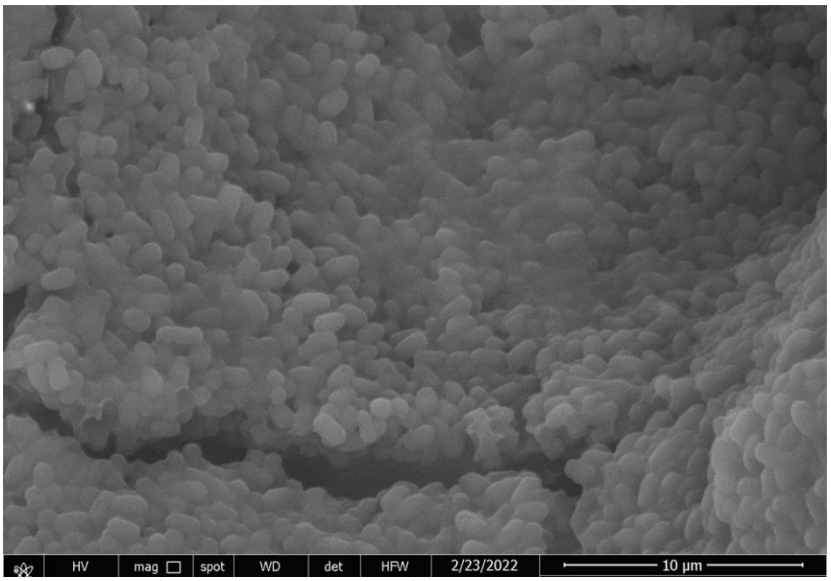

Fig. 3: SEM for *P. aeruginosa* biofilm cells grown in sterile flat bottomed polystyrene 96-well microtiter plate 24-48 hours (magnification ×12000).

IV. Discussion

A considerable number of examined isolates (50%) carried biofilm encoding genes; the present study displayed two isolates harbors *alg*D while one carried *pel*F gene (Fig. 1). The usual existence of at least one of biofilm encoding genes among *P. aeruginosa* clinical isolates was demonstrated in different reports [14-18]

Interestingly, the positive isolates were associated with clinical wound or abscess lesions; two obtained from sheep and one from goat. It was illustrated that *P. aeruginosa* one of the most common bacteria correlated with biofilm formation in chronic wounds [19].

Data obtained in (Fig. 2) displayed two biofilm strengths; one strong represented by the two isolates recovered from sheep clinical cases and the other weak displayed by the third isolate obtained from goat case.

In coincidence with other researches [20-22], our data displayed a significant correlation between the biofilm forming ability and the existence of relevant genes.

In our study; SEM examination exposed small rod-shaped cells with diverse arrangements within the biofilms (Fig. 3), in harmony with our data, studies observed intact cells surrounded by matrix and interwoven with matrix fibers in the biofilm [20, 23-25].

V. Conclusion

The significance of the One Health concept depends on the investigation of highly antibiotic resistant zoonotic pathogens which can transmit from animals to human. One of the most public health hazards is *P. aeruginosa* which implemented in various acute and chronic animal and human serious infections. The clinical *P. aeruginosa* isolates usually carry biofilm encoding genes as virulence factors. Also they have the ability to form biofilms under unsuitable conditions. Future insight should be directed to develop different ways to overcome the biofilm formation to improve therapy of *P. aeruginosa* infections.

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Sohier M. Syame, et. al. "Assessment of Biofilm Formation and Related Biofilm Encoding Genes in Pseudomonas aeruginosa Clinical Field Isolates." *IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB), 8* (2), (2022): pp. 10-14.