Association between Genotypic and Phenotypic Formation of Biofilm in *Pseudomonas aeruginosa* Isolated from Diabetic Foot Patients

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Abstract

The purpose of this research is to determine the relationship between genotypic and phenotypic characteristics associated with biofilm formation in Pseudomonas aeruginosa isolated from diabetic foot patients. Methods: The identification of Pseudomonas aeruginosa isolates was performed using morphology, microscopic examination, biochemical assays, and the vitek-2 system. In addition, the phenotypic detection of biofilm by CRA and TCP. Finally, biofilm genes (quorum-sensing) identification by PCR (Las I and Las R genes). Results: In accordance with the results obtained from 100 samples based on morphological and cultural characteristics as well as biochemical characteristics and the vitek-2 system only 47 (47%) isolates were identify as Pseudomonas aeruginosa. Biofilm formation found 41 (87.23%) isolates from Ps. aeruginosa able to produce biofilm by CRA and TCP are the number of isolates showing high biofilm formation was 33 (70.2%), moderate biofilm formation was 7 (14.9%) and non-biofilm formation was 7 (14.9%). Finally, the PCR data showed that LasI gene high prevalence 43/47 (91.4%), also LasR gene was positive in 33/47(70.2%) which encode for Biofilm formation in this study.

Keywords: Pseudomonas aeruginosa, biofilm, Congo red agar and tissue culture plate.

1. Introduction

Insufficient insulin production by the pancreas or inadequate insulin production induces diabetes mellitus (DM). Increased blood glucose levels cause damage to many biological systems, including blood vessels and nerves, and are the most common cause of diabetes-related hospitalization [1]. Resistance mechanisms developed by bacteria in mature biofilms make them difficult to eliminate from the host. Pseudomonas aeruginosa is a frequent infection in chronic wounds that can build resistant biofilms. Ps. aeruginosa is an opportunistic pathogen commonly associated with respiratory and urinary infections, burn wounds, and chronic wounds [2].

Scientists have been studying Pseudomonas aeruginosa because it can chronically invade and infect cystic fibrosis sufferers [3]. Adaptability of this bacteria to its environment is a result of extensive and intricate transcriptional control of virulence genes in the host environment. In the Ps. aeruginosa community, the quorum-sensing (QS) cell-to-cell communication mechanism serves to coordinate activity. In fact, two AHL-dependent QS systems, LasI/R and RhlI/R, govern the transcription of numerous virulence genes [4].

As part of the LasI/R system, lasI synthesizes 3-OXODODECANOL, which interacts with and activates the cognate response regulator LasR, therefore controlling the expression of target genes. RhlI initiates the production of N-(butanoyl)-homoserine lactone (C4-HSL) in the RhlI/R system, which then interacts with RhlR, influencing target gene transcription. Pseudomonas aeruginosa's pathogenicity is dependent on these two QS systems, which are among the most extensively studied in bacteria [3].

2. Materials and Methods

2.1. Patients and clinical specimens

There was a total of 100 samples isolated from AL-Sader hospital in Najaf City, Iraq, from 1/11/2020 to 1/4/2020, patients with diabetic foot ulcer. Physical and clinical examinations were conducted for each patient, and data were entered in a data sheet. This study coincided with the ethics of the Health Ministry and the AL-Sader hospital.

All respondents were notified verbally. Specimens were carried by sterile transport swabs and inoculated with a direct inoculation method on the cultivation of selective media such as MacConkey, blood and nutrient agar, and inoculated for 18-24 hours at 37°C [5].

2.2. Identification of bacteria

From each primary positive culture on the MacConckey, blood and nutrient agar, a single colony was taken to reinforce pure culture and was identified according to morphological and cultural characteristics (blood hemolysis, lactose fermentation, colonial form, size, color, frontiers and texture) and examined under microscopic therapies and then further identified by biochemical tests [6]. Finally, identified by vitek-2 system.

2.3. Biofilm formation

1- **Congo Red Agar method (CRA):-** According to Freeman et al. [7].

2- Tissue culture plate method (TCP):- According to Nibras et al. [8].

2.4. Total DNA Extract

By using the Genomic DNA Extraction kit (Intronbio/USA), DNA may be extracted from bacteria.

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2.5. PCR Amplification and Gel Electrophoresis

All isolates' DNA was subjected to polymerase chain reaction (PCR) to detect quorum-sensing (QS) genes such as (*LasI* and *LasR* genes). Tables 1 and 2 detail the primers and reaction conditions used in this study.

Table (1): The Primer was utilized in this research.

Target Gene	Sequence	bp	Reference
Las I	5'-CGTGCTCAAGTGTTCAAGG-3' 5'-TACAGTCGGAAAAGCCCAG-3'	295	MacFaddin [6]
Las R	5'-AAGTGGAAAATTGGAGTGGAG-3' 5'-GTAGTTGCCGACGACGATGAAG-3'	130	

Table (2): Thermocycling conditions for PCR

Gene Name	Temperature (°C) / Time					Cycles Number
	Initial Denaturation	Cycling Conditions			Final Extension	
		Denaturation	Annealing	Extension		
Las I* and *Las R	94 Cº for 2 min.	95 Cº for 40 Sec	50Cº/60 for 1 min	72 Cº for 2min.	72Cº for 10min.	30

3. Results

3.1 Description of study samples

This research was conducted with a total of (100) samples, sourced from individuals who had diabetes foot ulcers (DFU). Patients who attended to AL-Sader Hospital in AL-Najaf City, Iraq, between 1/ 11/ 2020, and 1/ 4/ 2021, during the time. The 47 (47%) Pseudomonas aeruginosa-positive samples, another 53 (53%) samples were also considered positive (causes by other causative agent).

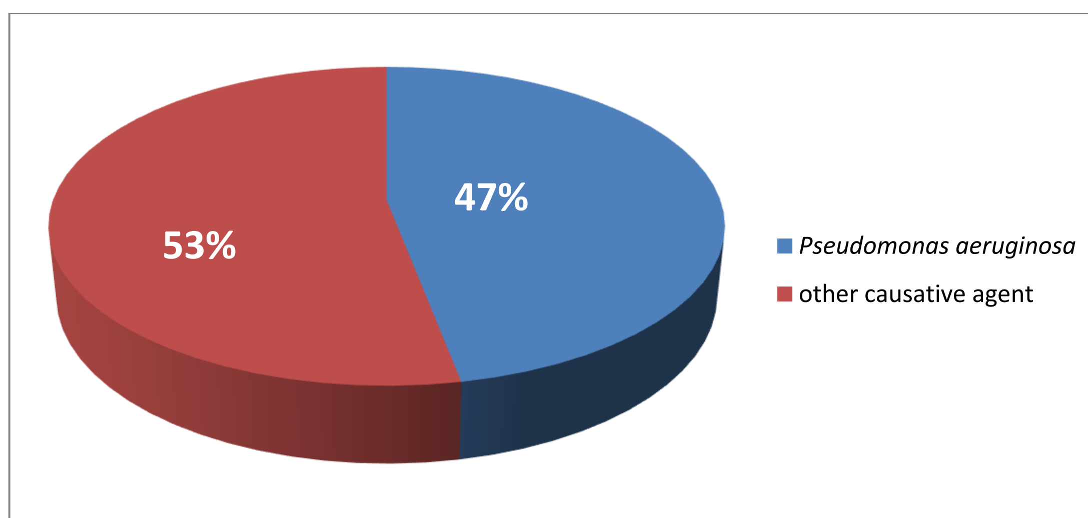

Fig. (1): The occurrence of Pseudomonas aeruginosa in 100 clinical samples.

Negative growth may be due to the presence of other infectious agents, such as anaerobic bacteria, fungi, and viruses [7], or it may be because the wounds were not infected at the time of the study or the medicines administered were successful [8].

3.2. Identification of bacteria specimens.

Initially, bacterial specimens were recognized using a variety of criteria, including Gram stain, cultural, morphological, and biochemical tests, before being identified using the vitek-2 method. Only 47 (47%) of the 100 clinical specimens contained isolates of Pseudomonas aeruginosa.

This study in same line with some other studies have shown Ps. aeruginosa predominant in DFIs such as Iyanar et al. [9], who demonstrated that Ps. aeruginosa was most common in DFI Also in line with Al- Shabaki [10]. Those who suffer from foot ulcers are more susceptible to bacterial infections that spread quickly, causing permanent tissue damage [11].

Because the colonies of Ps. aeruginosa look diffuse-haemolytic when grown on blood agar, but not when grown on MacConkey agar, this indicates that Ps. aeruginosa is unable to ferment lactose sugar, as light colored tiny colonies are formed [12].

3.3. Bioflim production

3.3.1- Congo Red agar (CRA) detection of Bioflim

It was found 41 (87.23 %) of the isolates from *Ps. aeruginosa* were capable of producing bioflim when tested on CRA, as shown in figure (2).

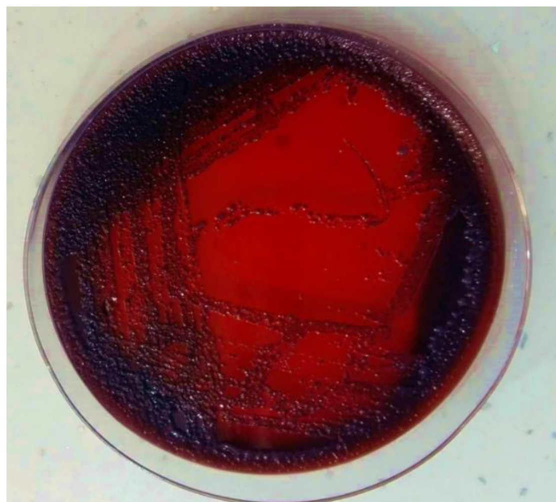

Fig. (2): The bioflim production by Congo Red assay

Microcolony development, biofilm maturation, and detachment (also known as dispersion) are all phases of biofilm formation. Sessile bacteria are in a stationary or latent development phase and have different characteristics from planktonic bacteria [13]. Lima et al. [14] obtained similar results, demonstrating that 75% of *Ps. aeruginosa* clinical isolates were biofilm makers.

3.3.2- Tissue Culture Plate (TCP) Detection of Biofilm Formation:

A semi-quantitative microtiter plate test to measure biofilm growth on polymetric surfaces was tested (biofilm assay). To boost the accuracy of the assay, three distinct runs were completed for each of the strains to be assayed. The results as determined by the mean of OD value (630nm) had a mean OD value of >0.240, 0.120, and <0.120.

According to table (4-6), only 33/47 (70.2 percent) Ps. aurogenosa formed strong biofilms, whereas 7/47 (14.9 %) and 7/47 (14.9 %) of *Ps. aurogenosa* formed moderate biofilms and non-formation, respectively (figure 3).This study dissimilar with Perez et al. [15] biofilm production was being distributed in the following categories: 60% (51/85) poorly adherent, 25.9% (22/85) moderately adherent, and 14.1% (12/85) strongly adherent.

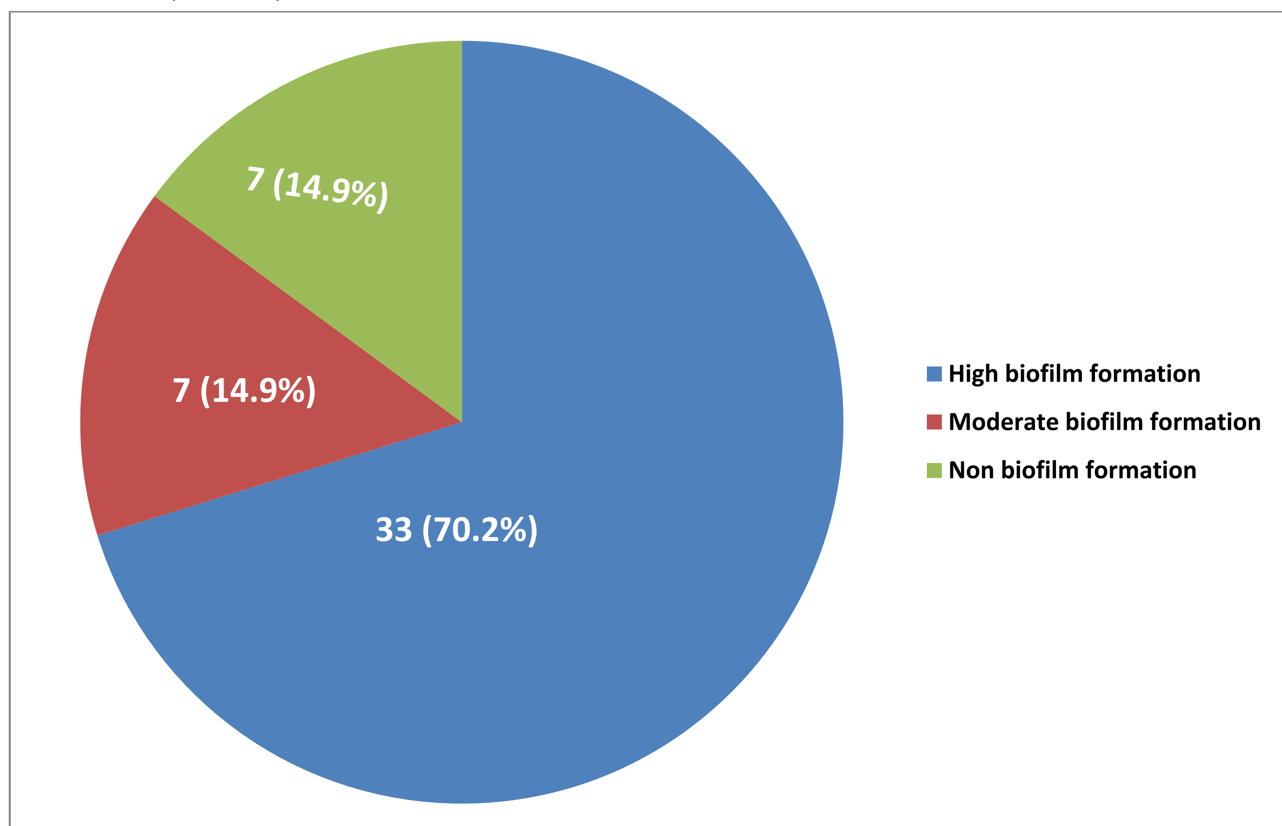

Fig. (3): Frequency of the biofilm formation in Ps. aurogenosa.

A biofilm can protect bacteria from immunological reactions and antibiotics. Microbes can live alone or form micro-communities inside a biofilm matrix of extracellular polymeric molecules. The ability to form biofilm is an important virulence trait because it protects against medicines. Multidrug-resistant bacteria thrive in these biofilms, causing persistent infections like diabetic foot ulcers and treatment failure [9]. Due to the difficulty of eliminating biofilms with traditional antibiotics, finding biofilm producers among clinical isolates may help control wound infections in diabetics who have failed antibiotic therapies [10]. Biofilms form more easily in diabetic wounds, says Ashok [16].

3.4. Molecular detection of bacterial isolates

3.4.1. Detection of the *LasI* and *LasR* genes

Using particular forward and reverse primers, *LasI* and *LasR* genes express acyl-hemoserine lactone (acyl-HSL) enzymes. In the current investigation, 43/47 (91.4%) of the bacterial isolates had the *LasI* gene, while 33/47 (70.2%) had the *LasR* gene.

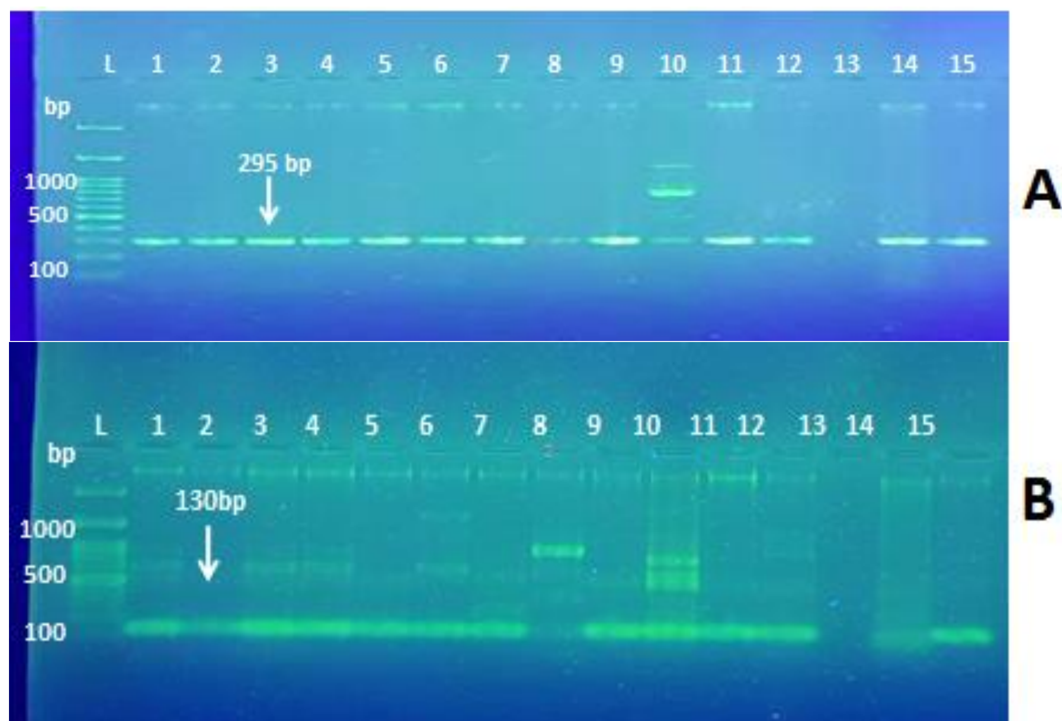

Fig. (4) Product from bacterial isolates that amplified with A) LasI gene and B) LasR gene primer were dyed with red safe dye, a blue gel stained with various dyes, and agarose gel electrophoresis with a PCR amplification product as an analytical tool.

When quorum sensing is activated, the expression of different virulence factors in *Ps. aeruginosa* is regulated. Quorum sensing is thought to be mediated mostly by N-acyl-l-homoserine lactones (AHLs) as the signaling molecules in cell-to-cell communication through quorum sensing [17].

In terms of the presence of QS genes, our findings are consistent with those of MacFaddin [6] who observed that 90.1% (82/91) of the examined isolates possessed *lasI* genes. In comparison, the results provided by Hasan [18] indicated that the identification of QS genes was not as high as those obtained in our work (81.25% for the *lasI* and *lasR* genes, 68.75% for the *rhlI* gene).

Reference

1. Spichler A, Hurwitz BL, Armstrong DG, Lipsky BA. Microbiology of diabetic foot infections: from Louis Pasteur to 'crime scene investigation'. BMC Medicine. 2015;13(1):2. https://doi.org/10.1186/s12916-014-0232-0

2. Thomsen TR, Aasholm MS, Rudkjøbing VB, Saunders AM, Bjarnsholt T, Givskov M, Kirketerp-Møller K, Nielsen PH. The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. Wound repair and regeneration. 2010;18(1):38-49. https://doi.org/10.1111/j.1524-475X.2009.00561.x

3. Smith RS, Iglewski BH. P. aeruginosa quorum-sensing systems and virulence. Current opinion in microbiology. 2003;6(1):56-60. https://doi.org/10.1016/S1369-5274(03)00008-0

4. Fuqua C, Greenberg EP. Listening in on bacteria: acyl-homoserine lactone signalling. Nature reviews Molecular cell biology. 2002;3(9):685-95. https://doi.org/10.1038/nrm907

5. Cheesbrough M. District Labor Practice in Tropical Countries Part two. Cambridge: Cambridge University Press U. S, 2010.

6. MacFaddin JF. Biochemical tests for identification of medical bacteria. 3rd ed. Lippincott Williams & Wilkins, Philadelphia, 2000. Available from: https://worldcat.org/en/title/41327723

7. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989;42(8):872-4. https://doi.org/10.1136/jcp.42.8.872

8. Nibras YA, Basaad AA-f, Aqeel AA. Effect of Phenolic Compounds of Conocarpus on DNA Concentration and Biofilm Formation of Pathogenic Bacteria. International Journal of Pharmaceutical Research. 2020;12(3). https://doi.org/10.31838/ijpr/2020.12.03.137A

9. Iyanar K, Premavathy R, Cecilia S, Jayalakshmi M, Priyadarsini SS, Shantha S. Isolation and antibiotic susceptibility of bacteria from foot infections in the patients with diabetes mellitus type I and type II in the district of Kancheepuram, Tamil Nadu, India. International Journal of Research in Medical Sciences. 2014;2(2):457-61. http://dx.doi.org/10.5455/2320-6012.ijrms20140515

10. Al- Shabaki J, F. A. Bacteriological evaluation of diabetic foot infections of patients in Al-Salam hospital in Mosul city/Iraq and antibiotic sensitivity pattern. International Journal of Advance Research. 2014;2(5):614-23. Available from: https://www.journalijar.com/uploads/307_IJAR-3275.pdf

11. Rodrigues RL, Lima JLdC, Sena KXdFRd, Maciel MAV. Phenotypic and genotypic analysis of biofilm production by Pseudomonas aeruginosa isolates from infection and colonization samples. Revista da Sociedade Brasileira de Medicina Tropical. 2020;53:e20200399. https://doi.org/10.1590/0037-8682-0399-2020

12. Jneid J, Lavigne J, La Scola B, Cassir N. The diabetic foot microbiota: a review. Human Microbiome Journal. 2017;5:1-6. https://doi.org/10.1016/j.humic.2017.09.002
13. Turhan V, Mutluoglu M, Acar A, Hatipoglu M, Onem Y, Uzun G, Ay H, Oncul O, Gorenek L. Increasing incidence of Gram-negative organisms in bacterial agents isolated from diabetic foot ulcers. The Journal of Infection in Developing Countries. 2013;7(10):707-12. https://doi.org/10.3855/jidc.2967
14. Lima J, Alves LR, Jacomé P, Bezerra Neto JP, Maciel MAV, Morais MMC. Biofilm production by clinical isolates of Pseudomonas aeruginosa and structural changes in LasR protein of isolates non biofilm-producing. Braz J Infect Dis. 2018;22(2):129-36. https://doi.org/10.1016/j.bjid.2018.03.003
15. Perez LRR, Machado ABMP, Barth AL. The presence of quorum-sensing genes in Pseudomonas isolates infecting cystic fibrosis and non-cystic fibrosis patients. Current microbiology. 2013;66(4):418-20. https://doi.org/10.1007/s00284-012-0290-5
16. Ashok D. Why diabetic foot ulcers do not heal. JIMSA. 2011;24(4):205.
17. Edmonds M, Foster A. The use of antibiotics in the diabetic foot. The American journal of surgery. 2004;187(5):S25-S8. https://doi.org/10.1016/S0002-9610(03)00300-3
18. Hasan AS. Antibiogram of Pseudomonas aeruginosa Isolated from Burn & Wound Infections Among Inpatients and Outpatients Attending to Ramadi Teaching Hospital in Ramadi, Iraq. Egyptian Academic Journal of Biological Sciences, G Microbiology. 2019;11(1):13-22. https://dx.doi.org/10.21608/eajbsg.2019.28794