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ROLE OF QUORUM SENSING GENES IN *PSEUDOMONAS AERUGINOSA* TO FORM BIOFILM AND THEIR RELATION TO ANTIBIOTIC RESISTANCE

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ABSTRACT

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. In this study, thirty isolates of *P. aeruginosa* were tested for seven types of antibiotics. On the other hand, the ability of *P. aeruginosa* isolates was tested to form biofilm membranes using crystal violet stain. All *P. aeruginosa* (13 isolates), which were positive to form biofilm, submitted to molecular study for detecting of two Quorum Sensing genes (*llsa*I and *rrh*I genes) related to biofilm formation in *P. aeruginosa*. Twenty-nine isolates were found to be resistant to one type of antibiotic, and twenty-six isolates were resistant to more than one antibiotic. The highest resistance was reported against CAZ (90%), followed by AK (80%) and CN (70%). However, the highest sensitive was observed in IPM (73.33%), and CIP (56.67%). The results of biofilm formation reported that 13 (43.3%) produce biofilm, while 17 (56.7%) not produce biofilm. Together *llsa*I and *rrh*I genes were detected in 10 isolates and only one gene of them was detected in three isolates compare with control isolate that was not biofilm producer and lack to these genes. In conclusion, *P. aeruginosa* will be developed antibiotic resistance when a specific QS gene is stimulated to form biofilm.

KEYWORDS: *Pseudomonas aeruginosa*, Quorum sensing, *llsaI* and *rrhII* genes.

INTRODUCTION

Pseudomonas aeruginosa is gram-negative bacterium structure was discovered in 1882 by the French bacteriologist and chemist Carle Gessard and contains a (0.5-0.8µm) diameter and length (1.5-3µm) rod form with one flagellum for mobilization. *P. aeruginosa* distinguishes itself from most gram-negative bacteria by being positive in an oxidase response. Furthermore, *P. aeruginosa* is unable to ferment lactose indefinitely (Diggle and Whiteley, 2020). *P. aeruginosa* may be found in a variety of environments, including water, plants, soil, and animal epidermis (Batrich et al., 2019). *P.aeruginosa* is a gram-negative bacterium that has high adaptability and intrinsic resistance to antibiotics, enables it to colonize a variety of settings(Musa, 2021).A general definition of antimicrobial resistance is the ability of an organism to resist the action of an antimicrobial agent to which it was previously susceptible (Pachori *et al.*,2019).

P. aeruginosais in the "critical" category by WHO “World Health Organization's” priority list of bacterial infections for which new antibiotic research and development are urgently needed (zeina&Khairallah,2023). *P.aeruginosa* has become an important and frequent opportunistic nosocomial pathogen. This organism is characterized by an innate resistance to multiple classes of antimicrobials, causing difficult-to-treat infections, which are therefore associated with significant morbidity and mortality (Mohamad *et al.*,2017). Antibiotic resistance in *P. aeruginosa* includes aminoglycosides (e.g. gentamicin), quinolones (e.g. ciprofloxacin), B-lactams (e.g. Cefepime), and polymyxins (e.g. colistin). Antimicrobial resistance in *P. aeruginosa* is characterized by multifactorial processes such as the synthesis of antibiotic altering enzymes such as B-lactamases, metallolactamases, and other enzymes that effectively alter the aminoglycoside; expression of efflux pumps that may protect the bacterium from antimicrobial agent internalization into the cytoplasm; and the ability to gain antibiotic resistance genes (Taylor *et al.*, 2014). The main way of acquiring drug resistance in multidrug resistance (MDR) *P. aeruginosa* is through acquiring plasmid. Plasmid-mediated resistance has been documented by several authors (Saleh and Balboula, 2017).

It has been demonstrated that *P. aeruginosa*, a significant etiological agent linked to infections connected to healthcare, raises patient mortality and morbidity rates. Due of its capacity to develop several antibiotic resistance mechanisms, it may eventually become multidrug resistant (Costerton et al., 1999; Karatuna and Yagci, 2010; Perez et al., 2013; Kırmusaoğlu et al., 2017; Di Domenico et al., 2017). The pathogen demonstrates the capacity to form biofilms, which are crucial for virulence and bacterial resistance and can have a significant effect on the host's health. Chronic pathology is caused by the thick polysaccharide matrix of the biofilm, which also contributes to the persistence of infection, the ineffectiveness of antimicrobials, and the escape from the phagocytic activities of the host immune system cells (Asati and Chaudhary, 2017; Di Domenico et al., 2017; Lima et al., 2018; Stepanović et al., 2000). *P. aeruginosa i*s a well-known biofilm former, which makes it an excellent model to study biofilm formation (Ghafoor et al., 2011; Crespo et al., 2018). A resilient biofilm is a critical weapon for *P. aeruginosa* to compete, survive and dominate in the cystic fibrosis lung polymicrobial environment (Oluyombo et al., 2019). Infections with *P. aeruginosa* are becoming more difficult to eradicate, this is due to the extraordinary capacity of *P. aeruginosa* to develop resistance against different antimicrobial agents including cephalosporins, aminoglycosides, fluoroquinolones and carbapenems through different mechanisms which are often simultaneously found in clinical bacterial isolates (Potron et al., 2015).

Production of virulence factors in *P. aeruginosa* is a strategy for survival to evade the immune defense of host (Feng et al., 2016). Production of various virulence factors is metabolically costly and requires community involvement. Therefore, they are mainly under the regulatory control of the quorum sensing systems (García-Contreras, 2016). Quorum sensing in this bacterium plays a critical role during pathogenesis for survival and colonization by coordinating progress of acute to chronic phenotype regulating more than 10% of *P. aeruginosa* genes expression mainly involved in production of virulence factor, such as pyocyanin, motility, biofilm formation and antimicrobial agents resistance mechanisms (La-Sarre & Federle, 2013; Barr et al., 2015). *P. aeruginosa* pathogenesis has been extensively studied and proven to be a multifactorial process, mediated by quorum sensing. *P. aeruginosa* possess two quorum sensing systems, las and rhl that facilitate cell - cell communication by signalling molecules production termed autoinducers to target specific receptors for activation (O’Loughlin et al.,2013; Streeter & Katouli, 2016). Therefore, the aim of this study was to estimate the formation of biofilm by *P. aeruginosa* and the presence of Quorum sensing genes, as well as, to determine the antibiotic resistance by *P. aeruginosa*.

MATERIAL AND METHODS

Bacterial isolates and their sources

Samples were collected from patients admitted to Al-Hussein Teaching during the period from September 2022 to January 2023. A total of 120 samples were gathered from different infected sites of patient's body for isolating *P. aeruginosa*. The samples were cultured on blood agar and MacConkey agar and incubated at 37°C for 24hrs. Grown colonies were inoculated into new petridishes contain *Pseudomonas* agar and also incubated for 24hrs at 37°C. *P. aeruginosa* isolates were purified depending on morphology characteristics and final identification was made via biochemical tests and VITIK2 system.

Antibiotics susceptibility test

The antibiotic-resistance profile of *P. aeruginosa* isolates were evaluated by the typical disc diffusion technique according to Bauer et al. (1996). Bacterial isolates were subcultured on nutrient agar and incubated at 37 C for 24 hrs. One colony from each activated isolate was suspended in normal saline 0.085% and adjusted to 0.5 McFarland. One hindered microliters was inoculated on the entire surface of new plate contain of Muller Hinton agar (MHA) and allowed to dry. Then, antibiotic discs (Gentamicin, Amikacin, Ciprofloxacin, Levofloxacin, PiPeracillin, Imipenem, Ceftazidime) were carefully placed on the surface of MHA and incubated in 37 C for 18-24 hrs. inhibition zone was read after incubation period and recorded according to Clinical and Laboratory Standards Institute (CLSI 2022) guidelines 27th (Humphries, 2018).

Biofilm formation by Crystal violet assay

A 96-well microtiter plates with Brain heart infusion broth (HiMedia) were employed to detect the development of biofilms. Semi-quantitative evaluations of biofilm development were made according to Hemati et al. (2016). Each P. aeruginosa isolate was grown in triplicate wells of 96-well plates containing Brain heart infusion broth medium with 1g of glucose at 37 C for 24 hrs. The plates were vigorously washed three times with regular saline to get rid of any free-floating bacteria. After that, 15 minutes of staining at room temperature with 100 ml of 0.1 percent (w/v) crystal violet solution. The crystal violet was then removed from the wells with 150 µl of 95 % ethanol and acetone [8:2 (v/v)]. Using a microplate reader, the plates were measured at 630 nm for bacteria and at 570 for yeast. The results were classified as following: non-biofilm, weak biofilm, moderate Biofilm, and strong biofilm according to optical density of microorganisms' cells. The results were interpreted as follows: if OD ≤ODc had no biofilm development, ODc < OD ≤2 ×ODc had weak biofilm, 2 ×ODc < OD ≤4 ×ODc had moderate biofilm and OD > 4 × ODc had high biofilm (Hemati et al., 2016).

Genomic DNA extraction from Bacterial Culture

All *P. aeruginosa* isolates (13 isolates), which were positive to form biofilm, were submitted to molecular study to find out of two Quorum Sensing genes (*llsaI* and *rrhII* genes) related to biofilm formation in *P. aeruginosa* (Table 1) (Rodrigues *et al.*, 2020). Genomic DNA was extracted from Bacterial Culture isolates by using Geneaid Genomic DNA extraction Kit (Taiwan). The quality of extracted DNA was tested using an electrophoresis 1.5 % gel agarose.

Table 1: The primers used, annealing temperature and the products size for Genes.

Gene	Primer Sequence ^{3} to ^{5}	Product size Annealing
llasI	F: CGTGCTCAAGTGTTCAAGG	295 bp
	R: TACAGTCGGAAAAGCCCAG	60 C
rrhII	F: TTCATCCTCCTTTAGTCTTCCC	155 bp
	R: TTCCAGCGATTCAGAGAGC	60 C

Statistics analysis

The result was statistically analysis by SPSS version 26, based in using both descriptive and non-parametric chi-square at *p*. value < 0.05. The multiple antibiotic resistance indices (MARI) were also estimated to find out the multidrug antibiotics resistance according to the formula below (Woh, Yeung and Goggins, 2023).

$$MARI = a/b \quad [Eqn\ 1]$$

Where; a = Total number of antibiotics to which an isolate shows resistance

b = Total number of antibiotics to which the isolate was exposed.

RESULT

High prevalence of antibiotic-resistant *P. aeruginosa* that produce biofilm membranes was demonstrated in the current study. Thirty *P. aeruginosa* isolates were identified and tested to antibiotic resistance and biofilm formation.

Antibiotic resistance profiles for 30 *P. aeruginosa* isolates were evaluated against seven types of antibiotics. The results indicated that 29 isolates were resistant to at least to one type of antibiotics. Among these resistant isolates, 26 isolates were classified as multidrug resistant isolates depending on multiple antibiotic resistance indices (MARI ≤ 0.43 classified as multidrug resistant isolate). The highest resistance was reported against CAZ (90%), followed by AK (80%) and CN (70%). However, the highest sensitive was observed in IPM (73.33%), and CIP (56.67%). Statistically, high significant difference was recorded at p. value < 0.05 (Table 2, 3).

Table 2: Antibiotics susceptibility of *P. aeruginosa* for 7 antibiotics locally used in Nasiriyah city, Iraq.

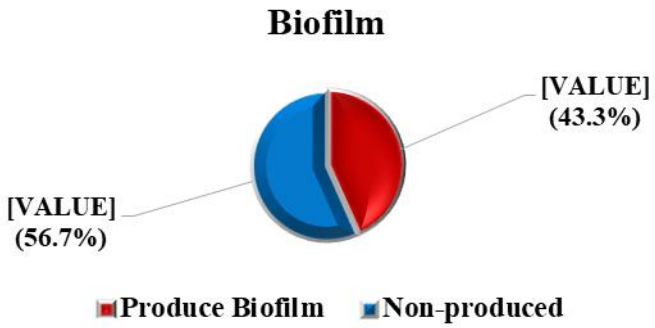
Antibiotics	Resistance		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
GN	21	70.0	1	3.33	8	26.67
PRL	9	30.0	16	53.33	5	16.67
IPM	8	26.67	0	0.00	22	73.33
CIP	13	43.33	0	0.00	17	56.67
CAZ	27	90.0	1	3.33	2	6.67
AK	24	80.0	4	13.33	2	6.67
LEV	11	36.67	9	30.0	10	33.33
Susceptibility %	53.81		14.76		31.43	
$CalX^2$= 108.146 $TabX^2$= 21.03 DF= 12 p. value < 0.001**						

Ceftazidime (CAZ), Imipenem (IPM), Piperacillin (PRL), Levofloxacin (LEV), Gentamicin (GN), Amikacin (AK), Ciprofloxacin (CIP).

Table 3: The calculation of multiple antibiotics resistance index (MARI) for detecting Multidrug resistance in *P. aeruginosa*.

Category	Number of Isolates	MARI index	Multidrug resistant
Resistant isolates against ≤ 3 antibiotics	24	0.43 - 1.00	Yes
Resistant isolates against ≥ 3 antibiotics	3	0.14 - 0.29	No

On the other hand, the ability of *P. aeruginosa* isolates was tested to form biofilm membranes using crystal violet assay. The results reported that 13 (43.3%) produce biofilm, while 17 (56.7%) not produce biofilm. Statistically, there was no significant difference at *p.* value < 0.05 between producer and non-producer isolates of biofilm (Fig. 1). Three classes of biofilm formation were detected among *P. aeruginosa* isolates (strong and moderate biofilm (2 isolates for each class), weak biofilm 10 isolates).



CalX^2= 1.96 TabX^2= 3.84 DF= 1 p. value 0.162$^{Non\text{-}Si}$

Fig 1: Percentage of *P. aeruginosa* isolates that produce or non-produce biofilm.

All *P. aeruginosa* (13 isolates), which were positive to form biofilm, submitted to molecular study for detecting of two Quorum Sensing genes (*llsaI* and *rrhII* genes) related to biofilm formation in *P. aeruginosa*. Together *llsa*I and *rrh*I genes were detected in 10 isolates and only one gene of them was detected in three isolates. In contrast, the control isolate was negative to both *llsa*I and *rrh*I genes (Table 4, Fig 2-A, B).

Table 4: Results of antibiotic resistance (No of resisted antibiotic), QS genes detection and biofilm formation classified as weak, moderate and strong to biofilm formation.

Isolates	Antibiotic			QS genes		Biofilm Formation		
	R	S	I	*llsaI*	*rrhII*	Weak	Moderate	Strong
6	42.86	42.86	14.29	-	+			
8	14.29	71.43	14.29	+	+			
10	42.86	42.86	14.29	+	-			
11	85.71	0.00	14.29	-	+			
12	71.43	14.29	14.29	+	+			
13	85.71	14.29	0.00	+	+			
15	14.29	71.43	14.29	+	+			
17	28.57	71.43	0.00	+	+			
22	14.29	85.71	0.00	+	+			
25	71.43	14.29	14.29	+	+			
26	85.71	14.29	0.00	+	+			
28	57.14	28.57	14.29	Control isolate				
29	85.71	0.00	14.29	+	+			
30	57.14	14.29	28.57	+	1			

High Resistance, + QS genes, Strong Biofilm
High Resistance, + QS genes, Moderate Biofilm
High Resistance, + QS genes, Weak Biofilm

R Resistant
S Sensitive
I Intermediate

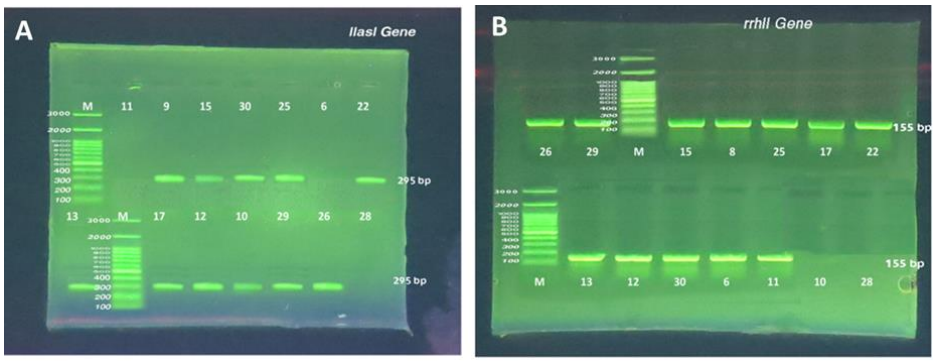


Fig 2: Detection Quorum sensing genes in *P. aeruginosa* isolates. A *llsaI* gene and B *rrhII* gene. No. 28 indicates to Control sample.

DISCUSSION

High antibiotic resistance was revealed among *P. aeruginosa* isolated from clinical samples. *P. aeruginosa* was resistant for Ceftazidime, which is third generation cephalosporins, and this result was in agreement with Abdul-Wahid et al., (2015) in Nasiriyah, Al-Saffar and Jarallah (2019) in Babylon, Al-Janahi et al., (2020) and Alhamdani et al., (2020) in Basrah, that *P. aeruginosa* showed high resistant to ceftazidime with percentage ranged from 70% to 100%. The lowest resistant rate was found with carbpenem: Impenem (26.67%).This study coincided with Al-kazrage, (2022) in Baghdad city, the resistant rate to Impenem was 30%. Overall, *P. aeruginosa* isolates show high resistance to almost of antibiotics tested here. These results indicate the ability of P. aeruginosa increases by the time because several Excessive use of antibiotics in Iraq (Al-Kaisse et al., 2015: Abdel et al., 2016; Hasan et al., 2020; Al-Azzawi, 2018; Attiah et al., 2021; Hussein et al., 2018; Al-Wasity, 2018; Hosu, et al., 2021; Al-Dahmoshi et al., 2018; Khudair and Mahmood, 2021).

A sophisticated virulence mechanism known as biofilm development needs quorum sensing, a chemical social networking system employed by P. aeruginosa to control gene expression (QS) (Asfahl & Schuster 2018; Siehnel et al. 2017; Ding et al. 2018). Changes in the expression of these genes related to the QS network or the presence of mutations can reduce the production of biofilms (Lima et al., 2018, Jácome et al., 2012, Castillo et al., 2019).

In this study, the genotypic analysis of 30 isolates showed (84.61%) of isolates were positive for *llasI* and (92.30%) of them were positive for *rrhII*. The *llasI* gene plays an important role in maintenance of *P. aeruginosa* biofilm, where that the signaling 3-oxo-C12-HSL (synthesized by *llasI*) is necessary for the establishment of *P. aeruginosa* biofilm, whereas a *llasI* mutant forms a flat and thin biofilm, and *llasI* is expressed in a large number of cells during the initial stage of biofilm formation (Davey et al., 2003). In *P. aeruginosa*, the regulation of many virulence associated factors, as well as biofilm formation, are under regulation of two hierarchically arranged N-acyl homoserine lactone (AHL) quorum-sensing (QS) systems, namely the LasI/R and RhlI/R systems (Smith, 2003). Each system contains one gene encoding AHL sensor/transcriptional regulator, i.e. lasR and rhlR, and a gene encoding an autoinducer synthase, i.e. lasI and rhlI, required for the synthesis of the autoinducer molecules N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-(butanoyl)-L-homoserine lactone (C4-HSL), respectively (reviewed by (Smith, 2003;Juhas,2005;Venturi,2006).At high cell density in a situation of quorum AHL concentrations, 3-oxo-C12-HSL and C4-HSL interact directly with the cognate LasR and RhlR sensor/regulator affecting transcription of target genes. The

QS system regulate hundreds of genes many of which coding to biofilm formation in *P. aeruginosa* (Miranda *et al.*, 2022).

CONCLUSION

P. aeruginosa isolates have the ability to develop antibiotic resistance when their genome contain QS genes associated with biofilm formation, leading to the development of the ability of bacteria to resist several types of antibiotics.

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