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Research article

Association of Imipenem Resistance with *LasR/RhIR* Quorum Sensing, Biofilm Formation, and Adhesion to Human Epithelial Cells in *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is responsible for a high rate of burn wound infection. Biofilm formation enhances this pathogen's resistance to antibiotics. Quorum-sensing (QS) genes regulate biofilm production. Carbapenem resistance, particularly to imipenem (IMP), is a major challenge for physicians. The study aims to investigate the relationships among IMP resistance, *lasR*/rhlR QS genes, biofilm formation, and adhesion of *P. aeruginosa* to human oral mucosal epithelial cells (OMECs). Eleven *P. aeruginosa* isolates were obtained from burn wound infections; 45.5% were IMP-resistant. All IMP-resistant isolates were strong biofilm producers. A significant negative correlation was observed between IMP susceptibility, measured as the diameter of the inhibition zone (r = - 0.84, P < 0.005), and biofilm formation, measured as optical density at 590 nm. This finding indicates that biofilm formation in *P. aeruginosa* decreases IMP susceptibility. The current study also revealed that strong biofilm producers showed high adhesion to human OMECs. Polymerase chain reaction (PCR) screening detected *lasR* in 72.7% of *P. aeruginosa* isolates and *rhIR* in 81.8%, with both genes co-occurring in 72.7% and absent in 18.2%. The study revealed that one isolate had a *lasR*-negative/*rhIR*-positive profile. The study concluded that there is a strong relationship among QS gene carriage, biofilm production, adhesion to epithelial cells, and IMP resistance in *P. aeruginosa*.

Keywords: Biofilm formation; Imipenem resistance; Burn wound infection; Epithelial cell adhesion; *Pseudomonas aeruginosa*; Quorum sensing (*lasR/rhlR*).

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1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterial isolate and is responsible for several nosocomial infections, especially in immunosuppressed patients, burn patients, and cystic fibrosis patients [1]. This pathogen acquired antibiotic-resistance mechanisms, such as reduced outer-membrane permeability, overexpression of efflux pumps, antibiotic-resistance enzymes such as ß-lactamases, and biofilm formation. *P. aeruginosa* has been remarkable as one of World Health Organization's priority bacteria, which requires serious research and therapeutic attention [2,3].

Imipenem (IMP) is a wide-spectrum carbapenem antibiotic. It has a bactericidal effect against various Gram-negative bacteria.

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This antibiotic destroys the bacterial cell wall by binding to penicillin-binding proteins (PBPs). It is stable against most β-lactamases, making it an important medicine for severe and multidrug-resistant bacterial infections.

Quorum sensing (QS), a communication system in bacteria, which assists bacteria in communicating with each other based on how densely they are packed together, directs both the expression of genes that produce toxins and the structure of the biofilm production of *P. aeruginosa* [5,6]. Within the connected QS systems of this pathogen, the *LasI*/*LasR* and *RhlI*/*RhlR* QS systems adjust the majority of gene expression for toxins and other virulence factors responsible for biofilm development in each bacterium [6,7]. The regulators *LasR* and RhlR are both part of the family of LuxR of transcriptional regulators and when the autoinducers (acyl-homoserine lactones) bind to these two regulators, *LasR* and RhlR activate the expression of these genes those encode different virulence factors e.g. elastase, pyocyanin, rhamnolipids, and exopolysaccharides to contribute to the maturate biofilms and provide the structural integrity of biofilms [6,7]. The lack of either LazR or RhlR has been shown to significantly reduce formation of biofilms and reduce the amount of tissue destruction of human epithelial cells caused by biofilm-associated bacteria where these QS communication systems are utilized, indicating that QS signaling is strongly associated with host–pathogen interactions [6].

The biofilms matrix is a primary mechanism of antibiotic resistance because the polysaccharide matrix of the biofilm limits uptake of antibiotics and creates a group of metabolically inactive cells called persisters [8,9]. Furthermore, *P. aeruginosa* has several mechanisms to attach to human epithelial cells, employing pili, flagella, and lectins, which constitute the first step in colonizing a human host and leading to chronic infection [1,10,11].

In spite of the increase in the evidence of the correlation between QS, biofilm formation, and adhesion to biotic surfaces, the specific correlation between IMP resistance and *LasR*/*RhlR*-dependent biofilm and adhesion to human epithelial cells is scantily covered in the literature. Therefore, the present project aims to study the association between resistance to IMP, the *LasR*/*RhlR* QS genes, biofilm production, and attachment to human epithelial cells in *P. aeruginosa* isolates from infected burn wounds.

2. MATERIALS AND METHODS

2.1. Bacterial Isolates

The present study is cross-sectional and descriptive-analytical. A hundred swabs were collected from burn wound infection of indoor patients, residents in the Baghdad Teaching Hospital and Burn Center, Baghdad, Iraq. The samples were inoculated onto MacConkey agar, and the non-lactose-fermenting isolates were re-cultured on cetrimide agar. The biochemical tests (oxidase, catalase, and pigment production test). The VITEK II system (bioMérieux's VITEK® systems, France) was used to prove the identification of bacterial species. The purified bacterial growth was stored short-term by inoculating onto nutrient agar plates and keeping them at 4°C for a week. For long-term storage, they were suspended in 20% glycerol in broth of nutrient and kept at -20°C for a year.

2.2. Biofilm Formation

The microdilution and spectrophotometric methods, post-staining with 0.1% crystal violet, were applied to check the ability of different *P. aeruginosa* isolates to form biofilms on polystyrene surfaces. Briefly, 100 µL of Tryptic Soy Broth (TSB, HiMedia, India) containing 0.5 % glucose was added to each sterile flat-bottom well. Five microliters of standard inoculum of bacterial suspension (optical density 0.1 at 600 nm) were put to each well. The plates were incubated at 37 ºC for 24 h. The wells of plates were washed gently with distilled water and incubated for 1 h at 65 ºC to dry and fix the biomass. One hundred microliters of 1% crystal violet (HiMedia, India) were added to the wells and incubated at 21 ºC for 16 min, and then washed in triplicate with distilled water. The plates were dried, and 100 µL of absolute alcohol (ethanol 99%, Fluke, UK) was put in the wells. The OD at 590 nm (Bio-rad, USA) was measured [14].

2.3. Adhesion to Human Epithelial Cells

To evaluate *P. aeruginosa* isolates for their ability to adhere to human epithelial cells, human oral mucosal epithelial cells (OMECs) were implemented. The isolation and preparation of these cells were described in detail previously [14]. Human OMECs were seeded in a sterile 24-well tissue culture plate using Dulbecco's Modified Eagle Medium (DMEM) supported with 10% fetal golf serum albumin (final volume: 1 mL per well). One hundred microliters of bacterial suspension (107 CFU/mL) were put in the wells. Plates were incubated for 2 h at 37 °C. After incubation, the human OMECs were harvested and washed four times with sterile phosphate-buffered saline (PBS; pH 7.2; 0.1 M). The final volume was modified to 1 mL with PBS, and the solution was divided into two parts. The first was mixed with Triton X-100, and the viable bacterial count was measured by plate count. The second was used to prepare slides, which were stained with Leishman stain to visualize and count the bacteria attached to each human epithelial cell. The light microscope (Carl Zeiss, Germany). The smartphone, Honor 400, was used to snap the pictures.

2.4. *LasR* and *RhlR* Gene Detection

Bacterial DNA was extracted using an extraction kit (iNtRON Biotechnology, Korea) based on the manufacturer's instructions were followed. The purity of extracted DNA was checked spectrophotometrically (A260/280 nm). Table 1. Showed the primers used to target the *lasR* and *rhlR* genes. The primers were synthesized by Macrogen Inc. (Korea). PCR was performed in a twenty-microliter reaction containing 8 µl Master Mix (SYNTOL, Russia), 2 µl primer mix, 7.5 µl $MgCl_2$, 0.5 µl nuclease-free water, and 2 µl template DNA. The running cycling condition is, initial denaturation at 94 ºC for 5 min; 35 cycles of 94 ºC for 30 second (s), 55 ºC for also 30 s, and 72 ºC for another 30 s; and a final extension at 72 ºC for 5 min. Amplicons were resolved on a 1.5% agarose gel stained with ethidium bromide, electrophoresed at 160 V for 40-50 min alongside a hundred base pair ladder, and the gel was visualized under UV transillumination.

Table 1. PCR primers and their sequences, with concentrations of the forward and reverse primers (Grosso-Becerra *et al*., 2014).

Amplicon	Primer	Sequence 5'-3	Position	Length
RhlR	rt_*rhlR*-F2	CTGGGCTTCGATTACTACGC	112	124
	rt_*rhlR*-R2	CCCGTAGTTCTGCATCTGGT	215	
LasR	rt_*lasR* –F	CGGTTTTCTTGAGCTGGAAC	15	100
	rt_*lasR* –R	GCCGAACAGGATCTTCGAG	114	

7. Statistical Analysis

The Origin 8 software was used for statistical analysis. The data of the current study was showed in means ± standard deviation. The differences were evaluated by using a student t-test and one-way ANOVA. Correlation coefficient values were also calculated. A value of P<0.05 was reported as a statistically significant.

3. RESULTS

3.1. Bacterial Isolates, Susceptibility and Biofilm Formation.

Table 2 shows the *P. aeruginosa* response to antibiotic (IMP). The results showed that five (45.5%) were resistant, one isolate (9.1%) was intermediate, and five isolates (45.5% were susceptible to IMP according to the diameter of the inhibition zone. The results showed that five isolates (45.5%) were categorized as strong biofilm producers, a similar number exhibited moderate biofilm formation, and only one isolate (9.1%) was categorized as a weak biofilm producer. It was observed that all imipenem-resistant isolates (PA1, PA2, PA6, PA7, and PA10) showed strong biofilm formation, with OD590 values ranging from 0.414 to 0.525. On the other hand, susceptible isolates exhibited moderate or weak biofilm formation, suggesting a positive association between imipenem (IMP) resistance and bacterial biofilm formation.

Table 2. The imipenem (IMP) susceptibility of eleven isolates of *P. aeruginosa* isolated from infected burn wounds. The table presents the diameter of the inhibition zone in millimeters, susceptibility interpretations, optical density (OD) at 590 nm, which corresponds to biofilm formation of the isolates, and categorization of biofilm-former isolates.

No	No of Isolates	Diameter of inhibitory zone (mm)	Interpreted	Biofilm formation OD at 590 nm	Categorizes
1	PA1	12.15 ± 1.9	Resistant (R)	0.414 ± 0.12	Strong
2	**PA2**	**8.7 ± 2.4**	**Resistant (R)**	**0.525 ± 0.09**	**Strong**
3	PA3	32.5 ± 3.9	Susceptible (S)	0.216 ± 0.012	Moderate
4	PA4	31.9 ± 4.2	Susceptible (S)	0.193 ± 0.1	Weak
5	PA5	28.6 ± 3.8	Susceptible (S)	0.262 ± 0.089	Moderate
6	**PA6**	**11.5 ± 2.1**	**Resistant (R)**	**0.485 ± 0.14**	**Strong**
7	PA7	9.1 ± 0.9	Resistant (R)	0.45 ± 0.11	Strong
8	PA8	17.2 ± 0.8	Intermediate (I)	0.319 ± 0.09	Moderate
9	PA9	19.1 ± 1.1	Susceptible (S)	0.327 ± 0.13	Moderate
10	**PA10**	**12.2 ± 1.9**	**Resistant (R)**	**0.455 ± 0.103**	**Strong**
11	PA11	22.3 ± 2.1	Susceptible (S)	0.31 ± 0.12	Moderate

To support the results of Table 2, which proposed a negative correlation between the diameter of the inhibition zone (a measure of susceptibility to imipenem) and biofilm production, the correlation coefficient was calculated between biofilm formation in eleven *P. aeruginosa* isolates and susceptibility to imipenem, as measured by the diameter of the inhibitory zone. A high negative correlation was observed between biofilm formation and the diameter of the inhibition zone (r = -0.84, P<0.005) (Fig. 1). The isolates with higher biofilm formation exhibited smaller inhibition zones, indicating greater IMP resistance. Enhanced biofilm production contributes to decreased antibiotic susceptibility.

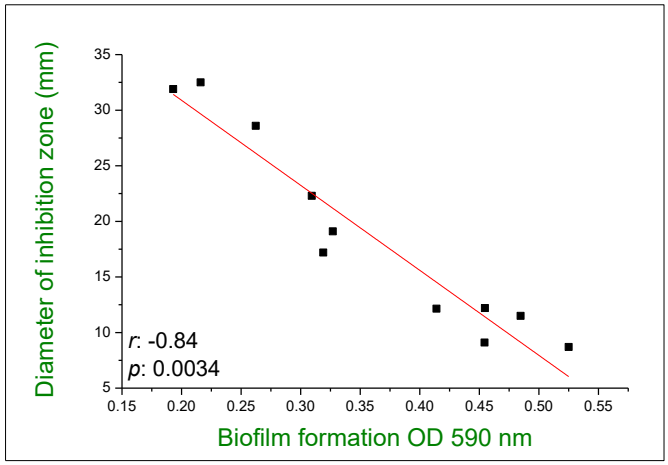

Fig. 1. Correlation between the susceptibility of eleven *P. aeruginosa* isolates to imipenem (IMP), expressed as the diameter of the inhibition zone in millimeters (mm), and biofilm formation in the same isolates, expressed as optical density at 590 nm. The analysis showed a significant correlation between biofilm production and susceptibility to IMP. *r*, correlation coefficient; *P* < 0.005 considered significant.

3.2. Bacterial Adhesion to Human OMECs

In this experiment, the ability of three isolates that were resistant to imipenem and strongly produced biofilm to adhere to human OMECs in vitro. Fig 2a shows the viable adhered bacteria of *P. aeruginosa* (CFU/mL) isolates to human OMECs. Fig 2b showed the mean of visualized adhered bacteria (total adhered bacteria) per epithelial cell determined by microscopic examination. The result displayed that isolates exhibiting higher viable counts also showed a higher number of visualized adhered bacteria per human OMEC, which indicates that the enhanced adhesive capacity of the bacterial isolates to human epithelial cells is variable dependent on the isolate. These results indicate a correlation between bacterial adherence to a biotic surface, the ability to produce a biofilm, and resistance to imipenem, as the isolates PA2, PA6, and PA10 are highly resistant to IMP and strongly produce a biofilm.

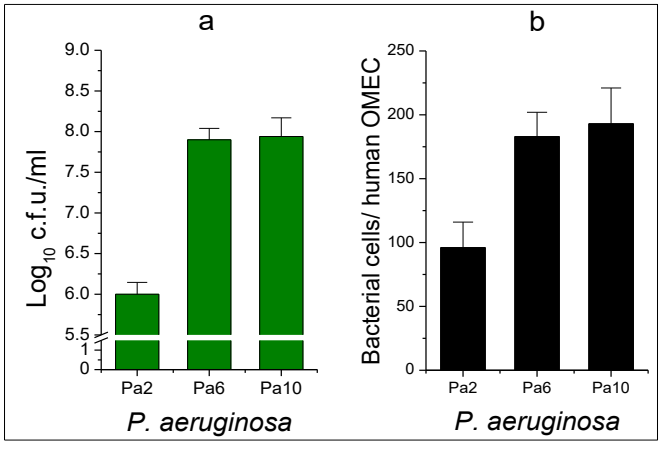

Fig 2. *P. aeruginosa* count (CFU/ml) that attached to human OMECs was measured using the plate count method (a). The mean of the visualized total number of adhered bacteria per human OMEC (b).

Fig. 3 supports the finding that *P. aeruginosa* has a high ability to adhere to human OMEC, indicating that the isolate may be highly virulent, as it possesses the ability to adhere to epithelial cells, which is the first step of infection.

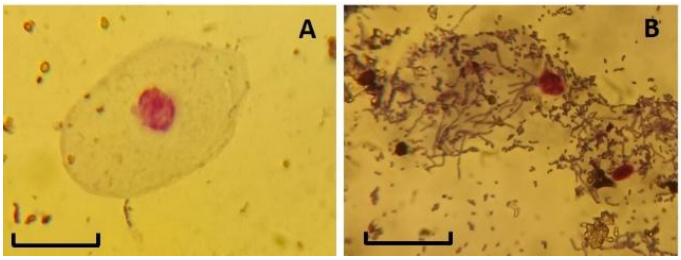

Fig. 3. Light micrographs of *P. aeruginosa* (PA6) interacting with human OMECs. A. The negative control of human OMECs treated with PBS, showing the typical structure of epithelial cells with a central nucleus and a clear cytoplasmic membrane surrounding the cytoplasm. B. The positive control of human OMECs exposed to a bacterial suspension, showing the high ability of bacteria to adhere to epithelial cells. Bars, 100 µL.

3.3. QS Genes Distribution

Fig. 4 shows the agarose gel electrophoresis image confirming the QS genes presence in the three selected isolates. Both genes were found in the three P. aeruginosa isolates shown in Fig. 4. The image showed that the *lasR* gene was 150 bps (Fig. 4a), while the simplified *rhlR* was 220 bps.

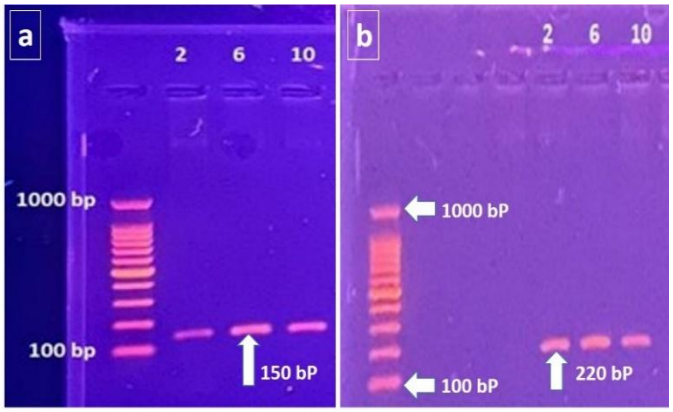

Fig 4. Agarose gel electrophoresis image of the agarose gel of the amplification of *lasR* at 57 °C (a) and *rhlR* at 55 °C (b) of *P. aeruginosa* isolates (Pa2, Pa6, and Pa 10). The amplified genes were run on a 1.5% agarose gel stained with Ethidium bromide. M: 100 bp ladder marker.

Table 3 shows the PCR screening of the studied isolates of *P. aeruginosa* (11 isolates). The table shows that the *lasR* gene was detected in 8 out of 11 isolates (72.7%). The study revealed that the *rhlR* gene was higher prevalent than the *lasR* gene; it was detected in 9 out of 11 isolates (81.8%) and absent in only two isolates (18.2%). The current study showed that the both genes (QS genes) were simultaneously present in 8 isolates (PA1, PA2, PA6, PA7, PA8, PA9, PA10, and PA11) (72.7%). The both genes were absent in two isolates (PA3, and PA4) in 18.2%.

4. DISCUSSION

Antibiotic resistance is a global threat to public health. The resistance to imipenem increased over time, which is associated with negative consequences for the treatment of bacterial infections. Several reports have linked biofilm production to antibiotic resistance and the QS phenomenon. The current study showed a strong correlation between imipenem resistance and QS gene distribution, ability of bacteria to form biofilm, and adhesion to human epithelial cells in *P. aeruginosa* isolated from infections of burn wound. It was observed in the study that around half of studies isolates (45.5%) were resistant to imipenem (IMP), which can be explained by the ability of these bacterial isolates to acquire multiple resistance mechanisms [15,16]. *P. aeruginosa* is a WHO priority bacterium that needs serious research and antimicrobial stewardship programs [3]. The study showed a strong negative correlation between biofilm production and the diameter of the inhibition zone (r = -0.84; P < 0.005), which is consistent with previous studies showing that the biofilm matrix serves as a barrier to antibiotic penetration, reducing the antibiotic's ability to reach bacterial cells. This phenomenon helps bacterial cells persist in the site of infection [17,18]. This finding agrees with a previous study that showed the role of biofilm formation of clinical and environmental isolates of *P. aeruginosa* in increasing the rate of multidrug resistance [18].

Table 3. Variations in the presence of QS genes (*lasR* and *rhlR*) among 11 isolates of *P. aeruginosa*. The genes were detected by PCR. +, presence of gene; -, absence of gene.

No	No of Isolates	*lasR* Gene	*rhlR* Gene
1	PA1	+	+
2	**PA2**	**+**	**+**
3	PA3	-	-
4	PA4	-	-
5	PA5	-	+
6	**PA6**	**+**	**+**
7	PA7	+	+
8	PA8	+	+
9	PA9	+	+
10	**PA10**	**+**	**+**
11	PA11	+	+

Here, the *lasR* and *RhlR* was detected in 72.7% and 81.8% of *P. aeruginosa* isolates, respectively. It was also found that both genes were present in most isolates. This agrees with previous publications documenting that the *LasR/RhlR* regulate different virulence determinants, e.g. elastase, pyocyanin, rhamnolipids, and polysaccharides, which are involved in biofilm formation [5, 7].

As the regulation via *RhlR* can happen with a partial independence from *LasR* being activated, it is expected that isolate PA5 has a profile which does not match *LasR*, yet matches *RhlR*, similar to previous "*LasR*-null" phenotypes which are still capable of *rhlR*-mediated virulence and have been characterized as being biofilm-capable [10]. Likewise, the disruption of QS signaling has been shown to significantly decrease biofilm biomass quantity and lessen the degree of bacterial injury to the host epithelial cells [11]. This also supports the biological importance of carrying QS genes of the isolates in terms of potential to cause disease in the isolates studied here.

It was seen in the current study that three IMP-resistant, strong biofilm-producing isolates (PA2, PA6, PA10) had high adhesion to human oral epithelial cells supports the idea that adherence to host epithelial cells via pili, flagella, and lectins represents a critical first step of colonizing the host and occurs earlier in QS-proficient, biofilm-capable strains [1, 14]. These findings suggest that the synergy of biofilm creation and epithelial adhesion that is under QS-control and IMP-resistance mechanisms together enhance the pathogenic ability of *P. aeruginosa* caused by burn wound infections.

There are many limitations to the current study. First, the limited number of bacterial isolates included weakens the conclusions drawn. Second, the presence of genes does not necessarily mean they are active; therefore, future studies will include gene expression analysis.

5. Conclusion

The study indicates a strong relationship among imipenem (IMP) resistance, the distribution of the QS genes (*lasR*/rhlR), the ability to form biofilms, and the adhesion of *P. aeruginosa* isolates to human OMECs. About 50% of the isolates were IMP-resistant and had a strong capacity to form biofilms. This demonstrates a robust inverse correlation between ability of bacterial isolates to form biofilm and antibiotic response. The *rhlR* gene was more common than the *lasR* gene across all tested samples and both genes were co-detected in the most isolates; however, one of the isolates had a non-co-inherited *lasR*-negative/*rhlR*-positive genotype, suggesting that the two QS systems are not obligately co-inherited. All three IMP-resistant/biofilm-strong isolates exhibited the highest epithelial cell adhesion; thus, it can be inferred that QS-regulated virulence, biofilm formation, and IMP resistance are interconnected. Taken together, these data support the notion that QS genes may be new therapeutic targets for the curing biofilm-associated, MDR *P. aeruginosa* infections in burn wound infections.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

The current study was conducted following approval from the Ministry of Health, Baghdad, Iraq (Reference number 1123, Date: 14, 10, 2023).

Author contributions

Talib MM. Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Project administration, Visualization, Writing – original draft, Writing – review & editing.

Attia MS. Investigation, Validation, Supervision, Resources, Writing – review & editing.

Abdulrraziq AA. Investigation, Validation, Supervision, Resources, Data curation, Writing – review & editing.

All authors reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work.

AI declaration

The authors used Claude AI to improve the manuscript's academic tone and readability. The authors also used Gemini Pro to prepare the manuscript's graphical abstract. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Data availability

Data will be made available on request.

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