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ORIGINAL ARTICLE

Pseudomonas Aeruginosa Biofilm Formation and Quorum Sensing lasR Gene in Patients with Wound Infection

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ABSTRACT

Background: *Pseudomonas aeruginosa (P.aeruginosa) is one of the most commonly studied bacteria capable of forming biofilms which are important in the establishment of P. aeruginosa infections on different host tissues including burn and surgical wounds.* *P. aeruginosa possesses at least two well-defined, interrelated QS systems, las and rhl that control the production of different virulence factors including biofilm development.* ***Objectives:*** *To determine the biofilm producing ability, antimicrobial susceptibility pattern and the presence of QS lasR gene in isolated P. aeruginosa strains from patients with surgical and burn wound infections.* ***Methodology****: The study was conducted on thirty five clinical isolates of P. aeruginosa from infected burn and surgical wounds in patients attending Ain Shams University Hospitals in the period from December 2013 till February 2015. Isolates from all patients were tested for antimicrobial susceptibility using disk diffusion method, in vitro formation of biofilm in microtiter plates containing Luria Bertani (LB) broth medium, and detection of QS lasR gene using conventional PCR technique.* ***Results****: P. aeruginosa isolates showed high prevalence of resistance against ticarcillin/clavulonate (85.7%), while they showed the lowest resistance to imipenem (20%). Biofilm formation was detected in 32 (91.4%) of P. aeruginosa isolates; 25.7%, 40% ,25.7% and 8.6% of isolates were strong, moderate, weak and non-biofilm producers, respectively. The lasR gene was detected in 33 (94.3%) of P. aeruginosa isolates. There was significant relation between biofilm formation and presence of LasR gene.* ***Conclusion:*** *P. aeruginosa isolates from infected wounds have strong capability of producing biofilms. The QS lasR gene is strongly associated with biofilm formation; such information can help in identifying lasR gene as a useful diagnostic marker for biofilm producing P. aeruginosa strains isolated from infected wounds.*

INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is an increasingly prevalent opportunistic human pathogen causing severe infections in hospitalized patients especially those with underlying disease; cancer, cystic fibrosis, patients with human immunodeficiency virus infections and patients with severe burn wounds. Multidrug-resistant *Pseudomonas* can be deadly for patients in critical care, an estimated 51,000 healthcare-associated *P. aeruginosa* infections occur in the United States each year. More than 6,000 (13%) of these are multidrug-resistant, with roughly 400 deaths per year attributed to these infections [1].

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Bacterial infection following severe thermal injury or surgical wound can be attributed to extensive breaches in the skin barrier[2,3]. *P. aeruginosa* is an extremely likely causative pathogen of burn wound infection as burn hospitals often harbor multidrug-resistant *P. aeruginosa* that can serve as the source of infection[3]. Burn infections caused by *P. aeruginosa* often deteriorate rapidly and lead to systemic spread and death within days or weeks [4]. *P. aeruginosa* contributes substantially to post operative wound-related morbidity and mortality worldwide. The organism enters into the blood, causing sepsis [5].

Biofilms are estimated to be responsible for over 65% of nosocomial infections[6], and 60% of all human bacterial infections[7]. Bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in treating infected skin

wounds[8]. Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, matrix production and detachment[9]. *P. aeruginosa* is one of the most commonly studied bacteria capable of forming biofilms[10]. Biofilm formation is important in the establishment of *P. aeruginosa* infections on different host tissues[11, 12] as well as different medical devices[13]. In these settings, the antibiotic resistance engendered by biofilms presents a serious challenge to the treatment of chronic *P. aeruginosa* infections[11].

Biofilm formation by *P. aeruginosa* involves the cell-to-cell communication quorum-sensing (QS) systems. QS is a cell-density-dependent mechanism through which bacteria coordinate different activities, including bioluminescence, plasmid conjugation and the production of different virulence factors[11,14,15]. *P. aeruginosa* possesses two well-defined, interrelated QS systems, las and rhl that control the production of different virulence factors. Each QS system consists of two components, the autoinducer synthases (lasI and rhlI, respectively) and their cognate transcriptional regulators (lasR and rhlR, respectively). LasI is the synthase for the autoinducer N-(3-oxododecanoyl) homoserine lactone (3OC12-HSL), while rhlI synthesizes the autoinducer N-butyryl homoserine lactone (C4-HSL) [14,15]. The two QS systems of *P. aeruginosa* are hierarchically linked. The las system positively regulates the expression of rhlR and rhlI[16,17,18]. *P. aeruginosa* also possesses an additional signaling molecule, 2-heptyl-3-hyroxy-4-quinolone (PQS). The production and activity of PQS is dependent on lasR and rhlR[19]. It has been suggested that in *P. aeruginosa*, QS is involved in both the initiation of biofilm formation and the maturation of the biofilm[8,20]. The las QS system appears to be especially important during the late stages of biofilm development [20]. This study aimed to determine the biofilm producing ability, antimicrobial susceptibility pattern and the presence of Quorum sensing lasR gene in isolated *Pseudomonas aeruginosa* strains from patients with surgical and burn wound infection at Ain Shams University Hospitals.

METHODOLOGY

1-Bacterial strains, media and growth conditions:

Thirty five non repeat clinical isolates of *P. aeruginosa* were obtained from patients with burn and surgical infected wounds at Ain Shams University Hospital. The isolates were collected within the period from December 2013 till February 2015. *P. aeruginosa* strains were isolated and identified based on standard microbiological techniques [21].

2- Antimicrobial susceptibility testing:

Antibiotic susceptibility of all isolated strains was done by disc diffusion method (discs supplied by Oxoid, UK), using Muller-Hinton agar plates (supplied by Oxoid, UK). After overnight incubation, results were reported and interpretation was done according to CLSI[22]. Amikacin, ceftazidime, levofloxacin, imipenem, ticarcillin /clavulonate were used for the antibiotic susceptibility testing.

3- Biofilm formation and quantification:

In vitro formation of biofilms in 96 well microtiter plates containing Luria Bertani (LB) broth medium was tested. Each strain of *P. aeruginosa* was inoculated in 3 ml Luria Bertani (LB) broth medium and incubated overnight. Cultures were diluted 1: 100 in LB medium and then added into each of three wells of microtiter plate then covered and incubated at 37°C for 48 hours duration. Negative control wells contained sterile broth. The plates were washed to remove planktonic bacteria, and then stained with 0.1% crystal violet solution (125 µl/well) for 10 min at room temperature. After staining, plates were washed with sterile distilled water three times to remove crystal violet solution that was not specifically staining the adherent bacteria. Plates were allowed to air-dry, at this stage, the staining became stable. 200 µl of 95% ethanol were added to each stained well. Dye was allowed to solubilize by covering plates and incubating for 10 to 15 min at room temperature. Lastly, the optical density (OD) of each well was read using a microtiter-plate reader at wavelength 620 nm [23]. The average OD values were calculated for all tested strains and for negative controls, the cut-off value (ODc) was established. It was defined as a three standard deviations (SD) above the mean OD of the negative control; the final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value. Strains were divided into the following categories; non biofilm producers (0) OD ≤ODc, weak biofilm producers (+ or 1) = ODc <OD ≤2×ODc, moderate biofilm producers (++ or 2) = 2×ODc <OD≤4×ODc and strong biofilm producers (+++or 3) = 4×ODc <OD [24] **(Fig 1).**

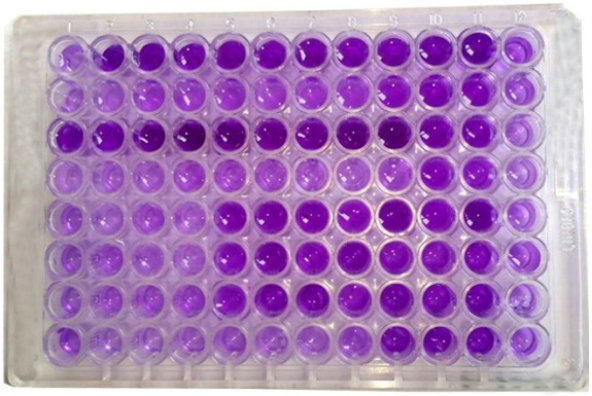

Fig. 1: Microtiter plate showing different grades of biofilm formation

4- PCR for detection of the quorum-sensing lasR gene:

DNA extraction was done using Qiagen DNeasy (Qiagen, USA), for DNA extraction from bacterial cultures according to manufacture instructions[25]. Genomic DNA was prepared from overnight cultures grown on Tryptose Soy broth, 20 µl protease were added to the bacterial suspension and incubated for 1 h at 55°C, 200 µl AL buffer for 10 min at 55°C were added, then 200 µl ethanol were added; the mixture was transferred to spin column, centrifuged at 8000 rpm/1 min. The rest of the sample was transferred to spin column and centrifuged. Then 500 µl of wash 1 buffer was added and centrifuged. Transfer to new wash tube and 500 µl wash 2 was added. Spin column was transferred to new wash tube, centrifuged at 14000/3 min, then transferred to elution tubes and 100 µl preheated elution Buffer (70°C) was added. Incubation for 3 minutes at room temperature; and then centrifugation 8000 rpm/1 min were done. PCR amplification was carried out using thermal cycler (BioRad, USA) with specific primers for lasR (table 1). PCR was performed in 25 µl of reaction mixture containing 150-200 ng (10.5uL) of extracted DNA, 12.5uL Taq PCR Master Mix, 100 pmol (1µl) of each primer. PCR conditions for the amplification step were: denaturation at 94°C for 1 min, annealing for 1 minute at 60°C, and extension at 72°C for 1 minute. Cycling was followed by a final extension at 72°C for 10 minutes. Agarose gel electrophoresis was used for examining the amplified products; PCR products were run on 2% agarose gel, stained with ethidium bromide visualized under UV light and photographed[26].

Table 1: Primer sequence used in the study:

Gene	*Primer direction*	*Primer sequence*	*Length of Primer (bp)*	*Size of amplified product (bp)*
lasR	Forward	5′ aagtggaaaattggagtggag 3′	21	130
	Reverse	5′gtagttgccgacgacgatgaag 3′	19	

5- Statistical analysis:

Statistical Package for the Social Sciences (SPSS) of Windows computer program was used for analysis of data using Chi-square test. Chi-square test was used for analysis of qualitative variables and P-values were calculated. A P-value <0.05 was considered as statistically significant.

RESULTS

This study was conducted on thirty five clinical isolates of *P.aeruginosa* from burn and surgical wound infected patients attending Ain Shams University Hospitals in the period from December 2013 till February 2015. Out of the 35 isolates; 21(60%) were isolated from burn wounds, while 14 (40%) were isolated from surgical wounds.

Antibiotic susceptibility of *P. aeruginosa* isolates:

P. aeruginosa isolates showed high prevalence of resistance against ticarcillin/clavulonate [30 strains (85.7%)], while 12 (34.3%) strains were resistant to ceftazidime, 10 (28.6%) strains were resistant to amikacin, 9 (25.7%) strains were resistant to levofloxacin and 7 (20%) strains were resistant to imipenem (table 2).

Table 2: Antibiotic susceptibility results of *P. aeruginosa* isolates.

Antimicrobial agent	*Sensitive No. (%)*	*Intermediate No. (%)*	*Resistant No. (%)*
Ticarcillin /Clavulonate	0	5 (14.3%)	30 (85.7%)
Ceftazidime.	20 (57.1%)	3 (8.6%)	12 (34.3%)
Levofloxacin	24 (68.6%)	2 (5.7%)	9 (25.7%)
Amikacin	25 (71.4%)	0	10 (28.6%)
Imipenem	27 (77.1%)	1 (2.9%)	7 (20%)

Biofilm formation:

Out of 35 *P. aeruginosa* isolates; 32 (91.4%) isolates were biofilm producers; 9 isolates (25.7%) were strong biofilm producers, 14 (40%) were moderate biofilm producers and 9 (25.7%) were weak biofilm producers, whereas 3 (8.6%) isolates were non biofilm producers. Among the 9 (25.7%) strong biofilm producers; 6 isolates (17.1%) were from burn wounds and 3 isolates (8.6%) were from surgical wounds. Among the 14 (40%) moderate biofilm producers; 10 (28.6%) isolates were from burn wounds and 4 isolates (11.4%) were from surgical wounds. Among the 9 (25.7%) weak biofilm producers; 5 isolates (14.3%) were from surgical wounds and 4 isolates (11.4%) were from burn wounds. Among the 3 (8.6%) non-biofilm producing isolates; 2 (5.7%) isolates were from surgical wounds while one isolate (2.9%) was from burn wound. There was no statistically significant correlation between type of infected wound (surgical vs burn wound) and the biofilm strength (table 3).

Table (3): Biofilm formation in burn and surgical wound samples.

Sample / Biofilm	Burn wounds	Surgical wounds	Total	X^2 value	P value
Strong	6 (17.1%)	3 (8.6%)	9 (25.7%)	2.72	0.8
Moderate	10(28.6%)	4 (11.4%)	14 (40%)		
Weak	4 (11.4%)	5 (14.3%)	9 (25.7%)		
None	1 (2.9%)	2 (5.7%)	3 (8.6%)		
Total	21 (60%)	14 (40%)	35 (100%)		

PCR results:

The *lasR* gene was detected in 33 (94.3%) out of 35 *P. aeruginosa* isolates collected, while only 2 (5.7%) isolates did not harbor the gene (Figure 2&3).

LasR gene was detected in all biofilm producing isolates (100%) and in only one (33.3%) non biofilm producing isolate. There was significant relation between biofilm formation and detection of LasR gene (figure 4).

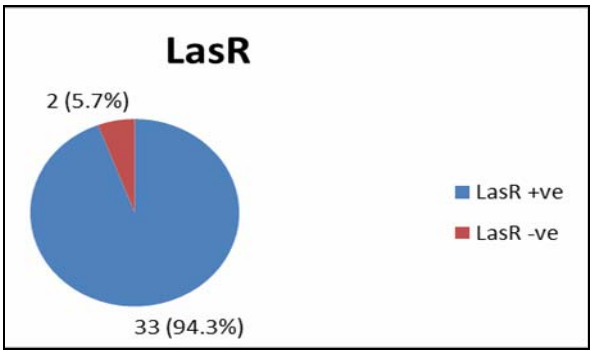

Fig. 2: LasR gene detection among the 35 studied *P. aeruginosa* isolates

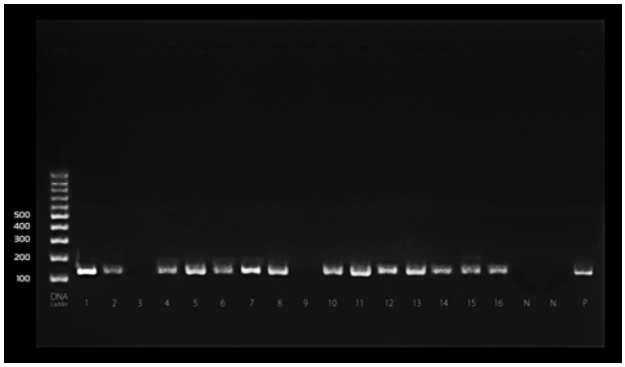

Fig. 3: Agrose gel electrophoresis for lasR gene in *P.aeruginosa* isolates

P: positive control, N: negative control

100bp DNA ladder; different strains of P.aeruginosa with las R gene products detected at 130 bp, strains 3& 9 are negative for the lasR gene

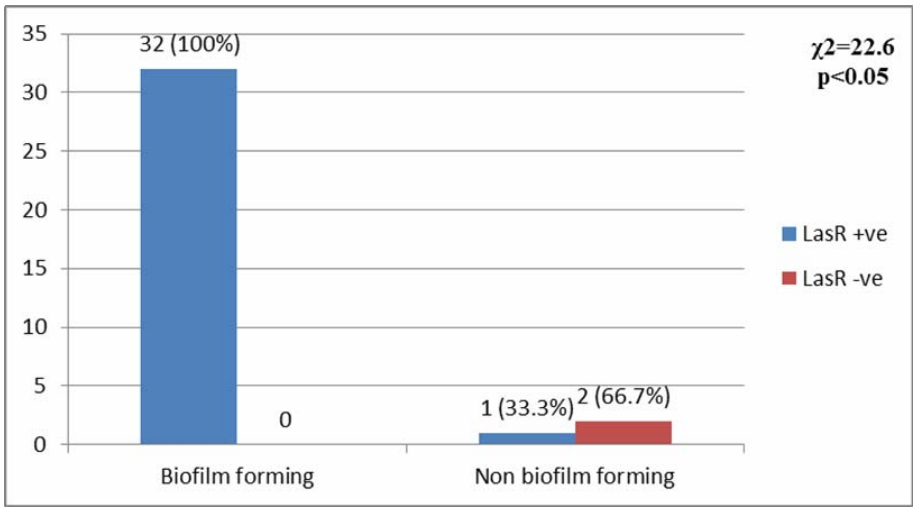

Fig. 4: LasR gene detection among biofilm forming and non biofilm forming isolates

The only non biofilm producing LasR gene positive isolate was isolated from burn wound; meanwhile 62.5% of the biofilm producing LasR gene positive isolates were isolated from burn wound. There was insignificant relation between biofilm formation and the site of the isolate (surgical or burn wound) ($p>0.05$) (figure 5).

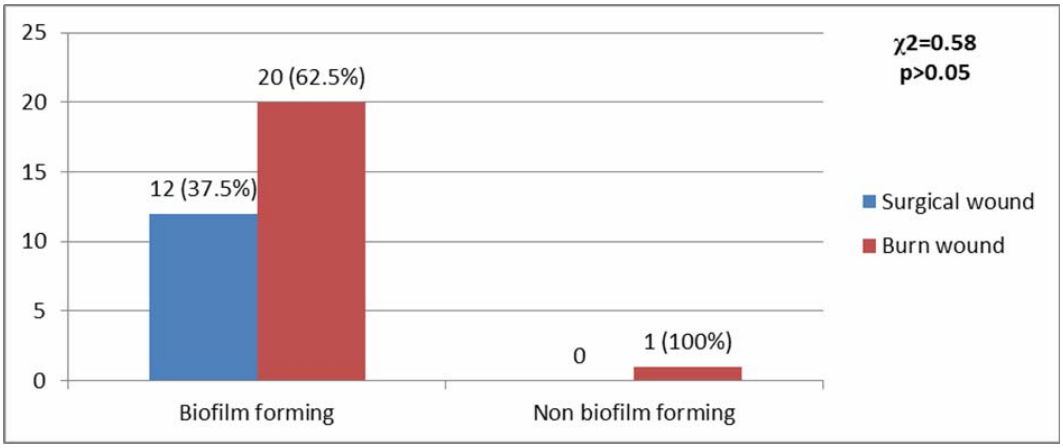

Fig. 5: Relation between biofilm formation and the site of isolates among LasR positive *P. aeruginosa* isolates

DISCUSSION

The las QS system is important for development of fully differentiated biofilms[8]. The expression of QS genes, especially of the las signaling system, in clinical isolates of *P. aeruginosa* is strongly associated with biofilm formation and resistance to antibiotic treatment [27].

This study included 35 isolates of *P. aeruginosa* from burn and surgical wound infected patients attending Ain Shams University Hospitals in the period from December 2013 till February 2015.

As regards antibiotic susceptibility results in this study, *P. aeruginosa* isolates showed high prevalence of resistance against ticarcillin/clavulonate (85.7%). These results agree with those done by Behera et al [28] in India and Salah et al [29] in Egypt who found that 63% and 60% of *P. aeruginosa* isolates were resistant to ticarcillin /clavulonate respectively. In contrast, Xi-chun et al [30] in China showed that only 22.8% of isolates were resistant to ticarcillin-clavulanate combination. High level of ticarcillin-clavulanate resistance in this study may be due to the increasing and the widespread use of this antibiotic combination in the hospitals. Regarding ceftazidime and amikacin in this study; 34.3% and 28.6% of *P. aeruginosa* isolates were resistant respectively. Salah et al. reported similar results where 34.4% and 30.6% of isolates were resistant to ceftazidime and amikacin respectively [29]. Anjum and Mir [31] in Pakistan also showed similar results, as they reported that 38% of *P. aeruginosa* isolates were resistant to ceftazidime. A higher incidence was detected by Makram et al. [32] in Egypt who reported that 49% and 37.7% of their *P. aeruginosa* isolates were resistant to ceftazidime and amikacin respectively The high activity of amikacin may be attributed to the presence of the aminohydroxybutyryl group, which generally prevents the enzymatic modification of amikacin at multiple positions without interfering with binding to the A site of rRNA [33]. Resistance to levofloxacin among isolates in the current study was 25.7%. Another study in Egypt showed a resistance rate of 31% to levofloxacin [34]. In contrast, a study done by Murugan et al.[35] showed that the percentage of resistance to levofloxacin was 57.1%. The lowest rate of resistance in the current study was to imipenem; where only 20% of isolates showed resistance making imipenem the last resort of therapy. Similarly, Salah et al. [29] found 20% resistance rate to imipenem [29]. A higher incidence was detected by Makram et al. who reported resistance rate of 35.6 % [32]. In contrast, *Murugan* et al. reported imipenem resistance rate of 71.4% [35]. Carbapenems are the last line of effective therapy available for the treatment of infections caused by multi drug resistant *P.aeruginosa* [36]. The low level of resistance to levofloxacin and imipenem in this study may be linked to the decreasing use and rotation of these antibiotics in our hospital as a part of an effective antimicrobial stewardship.

Regarding biofilm results in this study, 32 (91.4%) of *P. aeruginosa* isolates were biofilm producers, 25.7%, 40% and 25.7% of isolates were strong, moderate and weak biofilm producers, respectively, whereas, 3 (8.7%) of isolates were non-biofilm producers. These findings come in accordance with Jabalameli et al [37] who found that among 96 burn isolates of *P. aeruginosa*, 96% showed biofilm formation, among which 47%, 26% and 22.9% were strong, moderate and weak biofilm producers respectively. In contrast, Heydari and Eftekhar [38] showed biofilm formation in only 43.5% out of 62 burn isolates of *P. aeruginosa*, of which 66.7% produced strong and 33.3% formed weak biofilm. Perez et al [39] showed biofilm formation in 68% isolates of *P. aeruginosa* from sputum samples; 98% of them were weak biofilm producers. Zaranza et al [40] showed biofilm formation in 86% out of 100 *P. aeruginosa* isolates of different sources; 22.1% were strong biofilm producers, 47.7% were moderate and 30.2% were weak biofilm producers. In a study done by Kaur and Wankhede[41]

biofilm formation was found in 65% out of 60 isolates of *P. aeruginosa* of different sources; among them 45% were strong biofilm producers and 20% were weak biofilm producers. The discrepancy in results between different studies may be attributed to many factors such as the different countries from which the samples were collected, the number and the type of clinical specimens from which the isolates were obtained and also the differences in isolates capability to form biofilm. The primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that were produced from each isolate may also play an essential and an important role [42].

The *lasR* gene was detected in 33 (94.3%) out of 35 *P. aeruginosa* isolates collected, while only 2 (5.7%) isolates did not harbor the gene. LasR gene was detected in all biofilm producing isolates (100%) and in only one (33.3%) of non biofilm producing isolates. There was significant relation between biofilm formation and detection of LasR gene. Sabharwal et al**.** [43] detected lasR gene in 75% of *P. aeruginosa* isolates from urinary tract infection and found that lasR gene was related to the presence of some virulence genes as lasB (responsible for the production of elastase) and toxA gene (responsible for production of exotoxinA)**.** Zhu et al. [26] studied QS genes in 12 *P. aeruginosa* strains isolated from patients with corneal infection and found that 2 strains (16.7%) did not harbor lasR gene and that these strains were less virulent. Our results are supported by a study conducted by Senturk et al. [23], as they found that out of 6 *P.aeruginosa* isolates that were not biofilm producers, 2 were negative for lasR gene and 4 had lasR gene mutation. De´nervaud et al. [44] found that all QS (lasR and rhlR) deficient genotypes which represented 19% of the total isolates obtained from intubated patients were defective in the biofilm forming ability. These data suggest that the loss of the QS system severely impacts the biofilm forming ability of *P. aeruginosa*. Li et al. [27] observed a significant positive correlation between *P. aeruginosa* biofilm formation and the expression of the lasR and lasI signaling genes. The reason for the presence of lasR gene in one of the 3 non-biofilm producing isolates in our study may be due to mutation in the lasR gene or due to low expression of the gene due to enviromnetal factors.

In conclusion, our results indicate that *P. aeruginosa* isolates from infected wounds has a strong capability of producing biofilms and that the QS lasR gene is strongly associated with biofilm formation and could therefore be used as a as a useful diagnostic marker for biofilm producing P. aeruginosa strains isolated from infected wounds. Further studies regarding the molecular mechanisms involved in biofilm formation including studying expression and sequencing of QS genes will give a better understanding of the pathogenesis of biofilm formation which will ultimately lead to novel strategies for controlling recalcitrant biofilms.

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