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Detecting the Variation in the *lasI* Gene and Their Relation with Biofilm in *Pseudomonas aeruginosa* Isolates

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Abstract

One of the most causative agents for many opportunistic diseases is the *Pseudomonas aeruginosa* which has a high percentage of multidrug resistance disease through construction of biofilm. The current study aimed for evaluating the correlation between quorum sensing genes (which is *lasI* gene) and biofilm formation. The biofilm construction and antibiotics susceptibility test were achieved for all the isolates under the study. The PCR and sequencing techniques were also carried out to detect the type of variation in *lasI* gene for each scheme of biofilm formation (weak, strong, and moderate). High antibiotic resistance was recorded among biofilm producing isolates. The genic pattern for the weak biofilm producer, which is isolate No 19, carries lysine instead of arginine in position 92 and aspartic acid instead of asparagine in position 115. Phylogenetic analysis indicated that the horizontal genetic transfer between isolates was very high. Finally, we conclude that the *lasI* gene does participate in biofilm construction of *P. aeruginosa.*

Keyword: *lasI* gene, *Pseudomonas aeruginosa*, Biofilm formation, Genic variation

تحديد التغايرات في جين *lasI* وعلاقتها مع الأغشية الحيوية في عزلات الزائفة الزنجارية

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الخلاصة

تعد الزائفة الزنجارية أحد أكثر العوامل المسببة للعديد من الأمراض الانتهازية ، حيث تحتوي على نسبة عالية من السلالات المتعددة المقاومة للأدوية المتعددة من خلال تكوين الغشاء الحيوي. هدفت الدراسة إلى تقييم الارتباط بين جينات استشعار النصاب وهو جين *lasI* وتكوين الأغشية الحيوية. تم إجراء اختبار الحساسية للبناء البيوفيلم واختبار الحساسية للمضادات الحيوية لجميع العزلات قيد الدراسة. تم إجراء تقنيات تفاعل البلمرة المتسلسل لاكتشاف نوع التباين في جين *lasI* لكل نوع من تشكيل الأغشية الحيوية (ضعيف وقوي ومعتدل). أظهرنا أن مقاومة المضادات الحيوية كانت عالية جدًا في العزلات التي تكون الأغشية الحيوية. ان النمط الجيني للسلالات المنتجة للغشاء الحيوي بشكل ضعيف يحمل اللايسين بدلاً من الأرجينين في الموضع 92 وحمض الأسبارتيك بدلاً من الأسباراجين في الموضع 115. يشير التحليل الوراثي إلى أن النقل الجيني الأفقي بين العزلات كان مرتفعًا جدًا. أخيرًا ، خلصنا إلى أن جين *lasI* يشارك في تكوين الأغشية الحيوية في بكتيريا الزائفة الزنجارية.

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Introduction

Pseudomonas aeruginosa is one of the famous opportunistic pathogen that causes hospital mediating infections [1, 2]. The mortality correlates with nosocomial infections for multidrug-resistant strains of *P. aeruginosa* has increased significantly [3, 4]. *P. aeruginosa* undergoes evolution in cystic fibrosis (CF), therefore their genomic and phenotypic is different from *P. aeruginosa* in other sites of human body [5]. Different morphological colonies have appeared in order to adapt to selective pressure in CF airways. These are small colony variants and mucoid [6]. *P. aeruginosa* possesses many virulence factors that contribute in the development of acute and chronic infections such as pili, alginate/biofilm, lipopolysaccharide, pyocyanin, siderophores and exotoxin [7]. *P. aeruginosa* can persist for a long time inside the host due to their ability to adapt with stress condition such as adaptation to the inflammatory defense mechanism and antibiotic therapy [8]. Overproduction of exopolysaccharide helps in the formation of biofilm in *P. aeruginosa*, hence causing chronic infection in CF [9]. Biofilm is described as accumulation of many organisms under sheath of polysaccharide in presence of exogenous DNA (eDNA), and proteins and it contributes in the resistance to immune system and antibiotic resistance [10]. Biofilm formation consists of different processes starting by reversible binding of the cells then irreversible binding and development of microcolonies, before finally establishing mature biofilm [11]. Transfer the *P. aeruginosa* from planktonic cell to biofilm producer relies on forming extracellular matrix that forms from polysaccharides, proteins, extracellular DNA and lipids [12]. Biofilm of *P. aeruginosa* may lead to multidrug resistance evolution due to spreading of antibiotic resistance genes through horizontal gene transfer among bacterial species [13]. One study showed that the *PA3 782* and *PA37 1* genes upregulated with biofilm formation, therefore they may have a major role in constructing biofilm [14]. Biofilms and quorum sensing manages *P. aeruginosa* behavior in their host through colonizing and evading different tissues [15]. Quorum sensing which is intercellular communication help in formation of biofilm through autoinducers signaling controlling targets genes [16]. Quorum sensing has a major role in bacterial motility in addition to expression of virulence factors, hence forming biofilm [17]. In *P. aeruginosa lasB* plays an important role in biofilm formation by acting as polysaccharide secretion regulator [18]. A study showed that about 1% from total genes in *P. aeruginosa* were enrolled with biofilm formation, among thesewere the *las* and *rhl* which are quorum-sensing genes that mediate biofilm formation [19]. A study showed that MDR ST111 and ST235 formed biofilm in hospital waste-water pipes that leads to serious problems [11]. Due to the lack of sufficient information regarding genic variation of *lasI* gene in isolates under study that formed strong, moderate and weak biofilm, this study aimed for comparing the variation in *lasI* gene among biofilm non-forming and forming isolates and for finding the type of DNA and amino acid variation in non-biofilm producer compared with biofilm producer isolates.

Materials and Methods

Collection of the Isolates

All in all, 20 *P. aeruginosa* isolates were detected in different samples, which were collected from sputum and urine, from different hospital in Baghdad city using morphological and biochemical tests. For morphological detection, all the samples were cultured in different media such as cetrimide agar, MacConkey agar and blood agar. Catalase and fermentative, motility, oxidase and indol biochemical tests were carried out for further identification of isolates.

Antibiotic Susceptibility Test

The susceptibility of different antibiotics against *P. aeruginosa* isolates was tested using disc diffusion method. Four different disc diffusion of antibiotics were used: ceftazidime (30),

amikacin (10), imipenem (10) and colistin (10). Detection of the resistance, susceptible or intermediate isolates, relied on CLSI guideline [18].

Biofilm Forming Assay

Weak, moderate and strong biofilms formation was checked in 20 of *P. aeruginosa* isolates using microtiter plate [20]. 0.25% glucose was mixed with tryptic soy broth for the purpose of cultivating *P. aeruginosa* isolates at 37^0C for 24 h before forming biofilm. Next *P. aeruginosa* suspension with 125 µl was added to 96-well microtiter plates and left at 37^0C for 24 h. The wells of microtiter plates were then washed with distilled water and the plate was inverted on filter paper at room temperature for drying. Staining the wells with 125 µl crystal violet (0.1%) was carried out and left for 5 min. The wells were then de-stained using acetic acid. The density of biofilm that was formed in each well, was measured using ELISA reader at 570nm. For each one of 20 of *P. aeruginosa* isolates, testing the biofilm formation was achieved in triplicate while the media without inoculum represented as a negative control while inoculum without media represented as a positive control and then added to well of microtiter plate. ODi and ODc were measured to detect strong, moderate, weak and non-biofilm producing isolates. ODi refers to average of triplicate ODs of each sample after subtracting from OD of negative control. Whereas ODc refers as three standard deviations plus mean O.D of the control. In case of (ODi < ODc), (ODc < ODi < or = 2 multiply ODc), (2 multiply ODc < ODi < or = 4 multiply ODc), and (4 multiply ODc < ODi) indicate that isolates are non-, weak, moderate or strong biofilm producer respectively.

***LasI* gene Detection and Sequencing**

Genomic DNA mini extraction kit (Geneaid Company kit) was used to extract the DNA from 6 different *P. aeruginosa* isolates. Some of these isolates were multidrug resistance isolates (MDR). From these 6 different *P. aeruginosa* isolates, two isolates were moderate biofilm producers and the rest four were 2 weak-biofilm producers and 2 non-biofilm producers. In the PCR, the primers were taken from Uzunbayir-Akel *et al.* 2020s which are ATGATCGTACAAATTGGTCGGC for forward primer and GTCATGAAACCGCCAGTCG for reverse primer for amplification amplicon with size of 605 bp. PCR contents were 5 µl bacterial DNA as template, 1 µl from forward primer, 1 µl from reverse primer, 25 µl master mix and 18 µl distilled water with final volume of 50 µl. PCR was carried out with different cycles (Table 1). Visualization and staining were carried out on gel electrophoresis with 1% agarose and ethidium bromide respectively. Purified amplicons were then submitted to sequencing. The alignment of *lasI* gene was carried out using BioEdit program version 7.2 and BLAST tool was used to align our target gene with reference genome.

Table 1: PCR cycles to amplify *lasI* gene

Cycles	Temperature	Time
initial denaturation	95 ^{0}C	2 min
denaturation	95 ^{0}C	1 min
annealing	60 ^{0}C	1 min
Extension	72 ^{0}C	90 s
Final extension	72 ^{0}C	10

Statistical test

Comparing the proportions of the data under the study was achieved using Chi-squared test (Campbell, 2007).

Results

Biofilm results for 20 *P. aeruginosa* isolates showed that isolates non-forming biofilm had OD less than 0.15 and their percentage was 20%, while isolates forming weak biofilm had OD between 0.15 and 0.3 with 45% of the total isolates and isolates forming moderate biofilm had OD between larger than 0.3 with 35% of the total isolates (Table 2). Comparing the percentage of isolates forming and non-forming biofilm showed significant value with P = 0.0002.

Table 2: Percentage and OD of isolates forming and non-forming biofilm.

Isolates	percentage	OD
non-biofilm producers	20	< 0.15
weak biofilm producers	45	> 0.15 - < 0.3
moderate biofilm producer	35	> 0.3

Antibiotic susceptibility test (Figure 1) showed that not only all isolates were resistant to colistin antibiotic (100%) but also high resistance rate was detected against ceftazidime, amikacin and imipenem with (80%), (75%) and (55%) respectively (Table 3). The percentage of MDR among 20 isolates of *P. aeruginosa* was 5 (25%) (multidrug resistance is the resistance to 3 or more antibiotics belong to different classes for each isolate). The resistance for ceftazidime, amikacin and imipenem antibiotics was significantly higher through comparison between resistance and sensitive isolates for these antibiotics with P = 0.0002, P = 0.006 and P = 0.02 respectively.

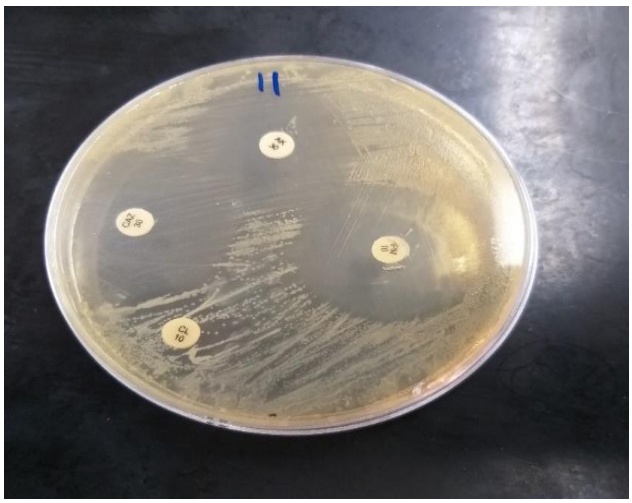

Figure 1: Susceptibility pattern for different antibiotics. Inhibition zones were seen for ceftazidime, amikacin and imipenem antibiotics.

Table 3: Antibiotic susceptibility patterns against *P. aeruginosa* isolates.

Antibiotics	Isolates Number %		
	Sensitive	**Intermediate**	**Resistance**
Imipenem	5 (25%)	4(20%)	11(55%)
Amikacin	4(20%)	1(5%)	15(75%)
Colistin	-	-	20(100%)
Ceftazidime	4(20%)	-	16(80%)

Six *P. aeruginosa* isolates by which 4 forming biofilm and 2 non-forming biofilms, were subjected to different concentration of antibiotics. The DNA from 6 *P. aeruginosa* isolates was

then extracted and the PCR technique was used for detecting *lasI* gene presence. Results showed the *lasI* gene was present in all isolates except isolates 12 and 15 which were weak and moderate biofilm producer isolates (Figure 2).

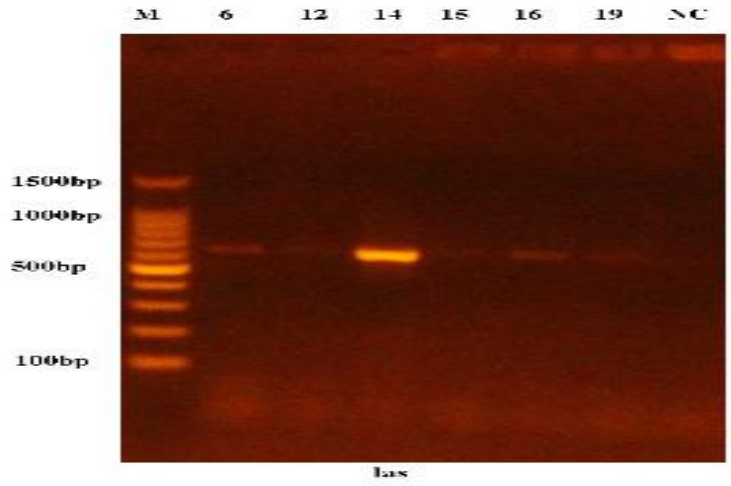

Figure 2: Gel documentation for *lasI* gene. lane 1: the ladder, lanes with numbers 6, 12, 14, 15, 16 and 19 are the isolates while the last lane: negative control. The concentration of agarose in gel was 1% and the gel was run on 80 V for 1 hour.

To confirm the deletion in *lasI* gene in isolates 12 and 15, the extracted DNA from 6 *P. aeruginosa* isolates was sent for sequencing. The sequencing results confirmed that the deletion only occurred in *lasI* gene in isolate belonging to 15.
The genic variation among isolates under the study showed that three substitutions occurred at positions 92, 115 and 130 in isolate 19 which is a weak biofilm producer isolate (Figure 3 and Table 4).

Table 4: Analysis of the *lasI* gene and its protein sequence through comparison with reference genome.

Position of DNA Change	DNA Change	Position of Amino Acid Change	Amino Acid Change
9	G to A	3	No change
23	A to G	8	L change to S
47	A to G	16	L change to S

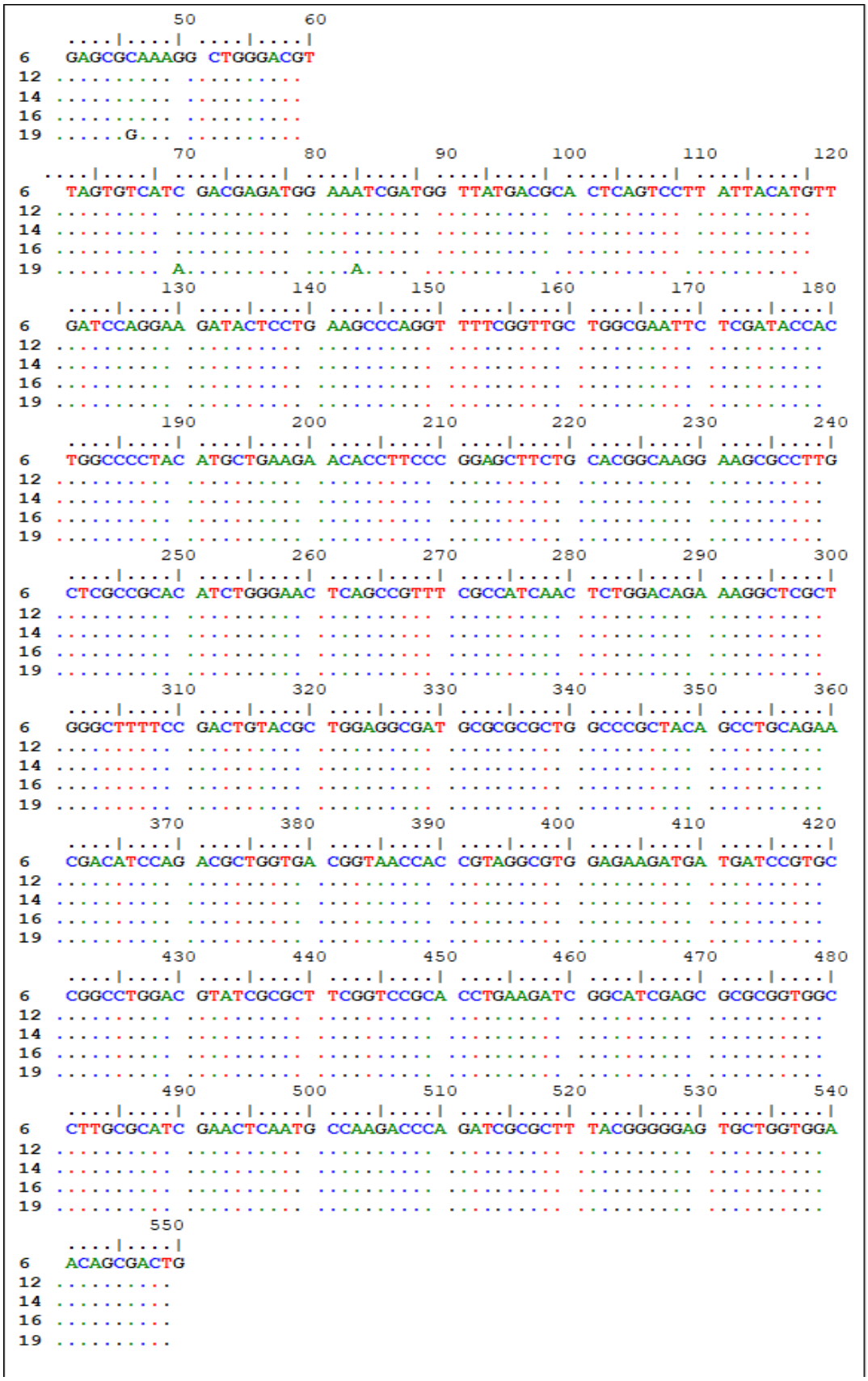

Figure 3: Alignment of the DNA sequence for *lasI* gene to the isolates under the study. The patterns of genic variation showed in isolate 19 compared with other isolates.

The variation at position 92 involved A converted to G by which lysine converted to arginine. At position 115, G converted to A by which aspartic acid converted to asparagine, while C converted to A at position 130 and it was missense mutation.
Further analysis was carried out through comparing the DNA sequence of our isolate with reference genome in the databases and the results showed three substitutions changes that wereobserved at positions 9, 32 and 47 converting G to A, A to G and A to G respectively (Figure 4 and Table 3).

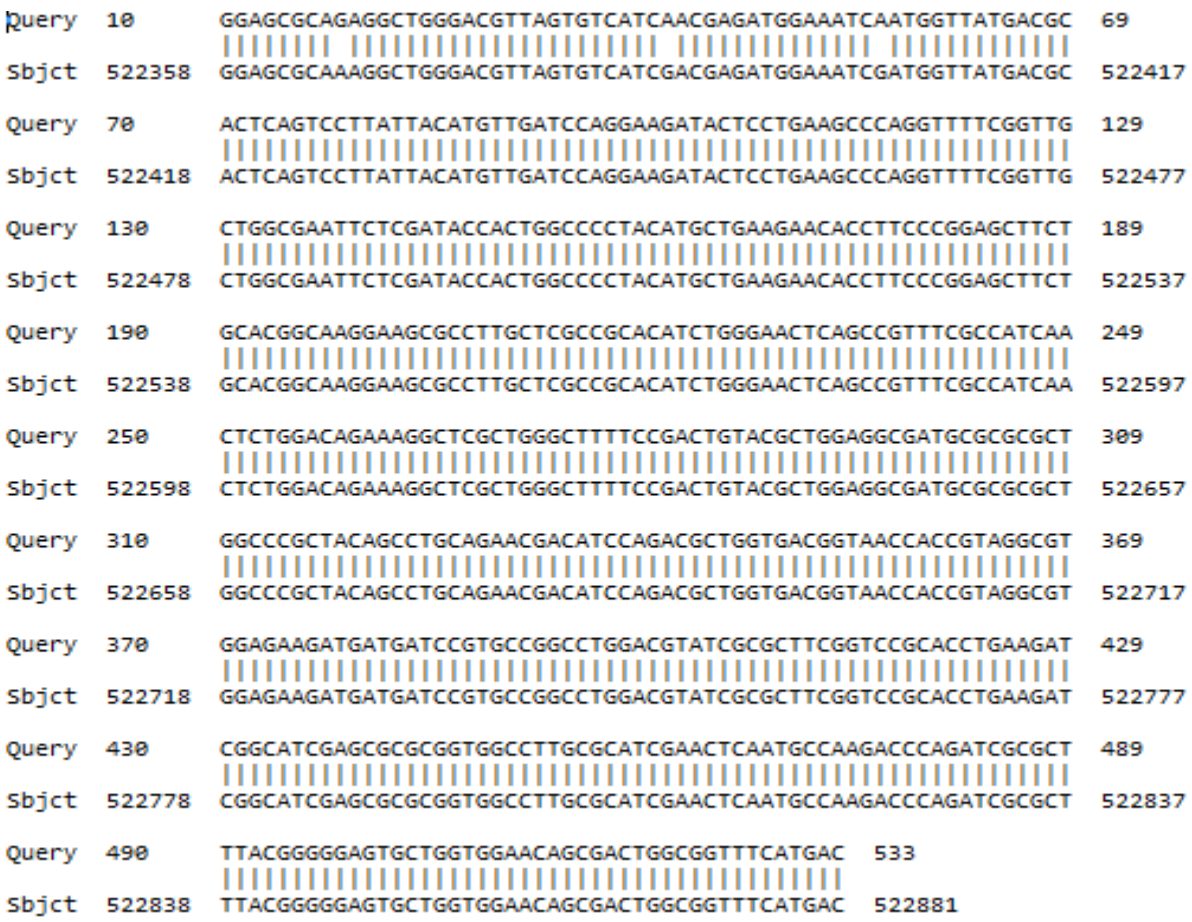

Figure 4: Alignment pattern between reference genome and one of our isolate. The genic variation showed in three different positions.

Table 3: Analysis of the *lasI* gene and its protein sequence through comparison with reference genome.

Position of DNA Change	DNA Change	Position of Amino Acid Change	Amino Acid Change
9	G to A	**3**	**No change**
23	A to G	**8**	**L change to S**
47	A to G	**16**	**L change to S**

Finally, phylogenetic tree was constructed between isolates under study and isolates in databases which showed high similarity index with our isolates. In general, the tree showed two clusters, the first cluster for the isolate under the study with one isolate collected from Iraq as well highlighted with red color while the second cluster showed the isolates collected from USA (Figure 5).

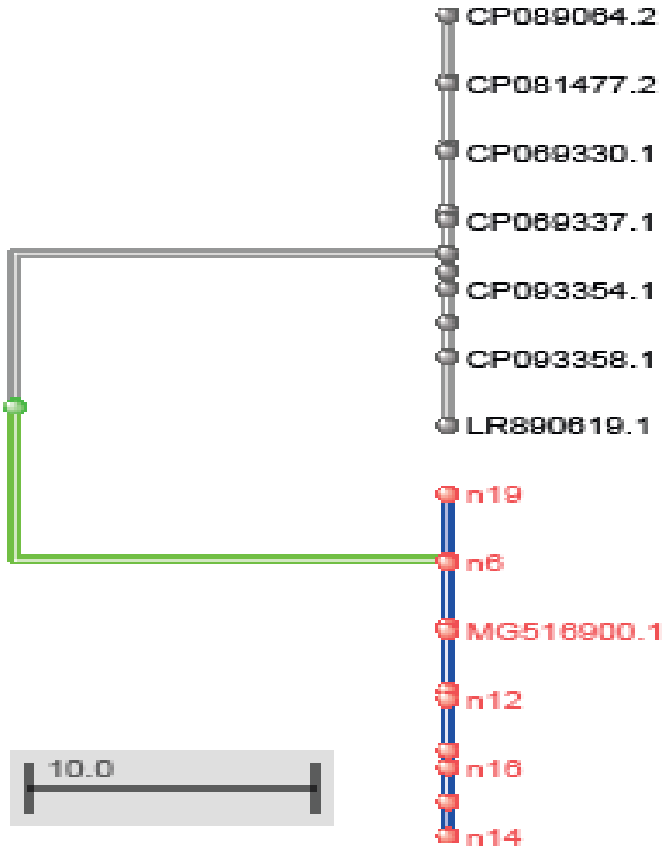

Figure 5: Construction phylogenetic tree among isolates under the study and the isolates collected from database. Isolates that highlighted with red color represented the isolates under the study.

Discussion

In this study, 80% of *P. aeruginosa* isolates formed biofilm and this percentage was approximately close to the study conducted by O'Toole [20], and showed that 93.4% of the isolates form biofilm. Percentage of MDR isolates in our study was 25% which is higher than the percentage reported in another study with 6.52 % [21]. However, it was significantly lower than that reported by Banar *et al*. [22] with 87%. In addition, there was no correlation with MDR and biofilm formation in our isolates and this results were compatible with a previous

study outcome [23]. However, most of the isolates were resistant to different antibiotics with high percentages and this agreed with Saffari *et al*.[21] but disagreed with O'Toole's study [20]. For the isolates under study, the high percentage of the resistance for the antimicrobial agents and high rate of forming biofilm indicated that these isolates have high pathogenicity. In addition, 66.6% from isolates forming and non-forming biofilm carried *lasI* gene. This result indicated that there was no correlation between construction of biofilm and the *lasI* gene prevalence. In addition, presence of gene in non-biofilm producer could be due to mutations affecting the formation of biofilm. However, Lahij *et al*.[24] showed that there is a strong correlation between construction of biofilm and the presence of *lasI* gene. Moreover, Perez *et al*. [25] showed that most of isolates carried *lasI* gene with 90%. Our result agreed with Sabharwal *et al.* who showed that only 75% of isolates carried *lasI* gene. However, Aboushleib *et al.* [26] found that only 48% of formed isolates carried *lasI* gene. The *lasI* gene was not detected in isolate 15 which is a moderate biofilm producer. We, however, think that the isolate 15 was able to form moderate biofilm in the presence other quorum sensing genes such as *rhII*. This result was compatible with *Senturk et al*. [27] who showed some missing quorum sensing genes were replaced by the presence of other in *P. aeruginosa* therefore this bacterium still has the ability to produce biofilm. The mutation pattern in *lasI* gene was detected in isolate 19 which is weak biofilm producer isolate, therefore we think that *P. aeruginosa* to produce weak biofilm, in position 92 lysine should be replaced by arginine and at position 115 aspartic acid should be replaced by asparagine. Another study showed that the mutation in quorum sensing genes converted *P. aeruginosa* from biofilm producer isolate to non-biofilm producer isolate [28]. Many other studies showed that the mutation in quorum sensing genes can have a direct effect on construction of biofilm in *P. aeruginosa* [22]. We detected genomic variation in DNA sequence through comparing our isolates with the database and this variation may reflect that *P. aeruginosa* may change in their pathogenicity to adapt with their host defense strategies. Finally, phylogenetic analysis showed that the isolates clustered relying on geographic distribution by which all Iraq isolates aggregate in one cluster, this gives an indication that *lasI* gene highly transferred among isolates in specific regions through horizontal gene transfer.

Conclusion

Studying the relation between *lasI* gene and biofilm formation showed that there is a powerful correlation between antibiotic resistance and construction of biofilm and between *lasI* gene and biofilm formation for the isolates under study. In addition, lysine replaced arginine in position 92 and aspartic acid replaced asparagine in position 115 for weak biofilm producer isolate.

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Disclosure and Conflict of Interest

The authors declare that they have no conflicts of interest

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