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Detection of (*LasI*) Gene in *Pseudomonas aeruginosa* and its Role in Biofilm Formation.

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ABSTRACT

The present study included the collection of 120 samples from various clinical sources. The samples collected from some hospitals in Baghdad city which included burns ,wound , cystic fibrosis, ear infections and urinary tract infections (52 ,28, 4, 23 and 13). Samples respectively. Samples were identified based on the morphological and microscopical characteristics of the colonies when they were culturing in a number of culture media as well as biochemical tests. The molecular detection was used to confirm diagnostic test for the isolates based on the *16SrRNA* gene, as a detection gene, which has specific sequences for DNA of *P.aeruginosa* bacteria.The results of molecular detection showed that 51 isolates belong to target bacteria were distributed as follow 21 of burns, 14 isolates of wounds, 7 isolates of ear infection, 4 isolates of cystic fibrosis, 5 isolates of urinary tract infection. The results of phenotypic detection of biofilm formation showed that 44/51 (86.2%) of isolates differences in their ability to biofilm formation. The results of screed QS genes by Polymerase chain reaction (PCR) amplify based on specific sequences for *las*I gene showed that 48 (94.1%) of isolates have *las*I gene.

INTRODUCTION

Pseudomonas aeruginosa is Gram negative bacteria, aerobic, rod, and motile, belongs to Pseudomonadaceae family, since the revisionist taxonomy based on the conserved macromolecules (e.g. 16s ribosomal RNA) (1). *P. aeruginosa* is one of the opportunistic human pathogen that preferentially infects patients with cancer or AIDS, immunocompromised patients by surgery, cytotoxic drugs or burn wounds, people with cystic fibrosis, eye, ear and urinary tract infections (2). *P. aeruginosa can* produce any of the opportunistic extra-intestinal infections caused by members of the Enterobacteriaceae and may progress to bacteremia (3). Septicemia and endocarditis may occur in patients who are debilitated due to concomitant infection, malignancy or immunosuppressive therapy (4).

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In some cases of *P. aeruginosa* bacteremia, cutaneous papules develop that progressto black, necrotic ulcers (5). *P. aeruginosais* also one of the most common causes of infection in environmentally contaminated wounds , eg: osteomyelitis after compound fractures or nail puncture and body wounds (3). The success of *P. aeruginosa* in diverse environments is attributed to its impressive arsenal of virulence factors , which include multiple cell-associated factors such as alginat, lipopolysaccharide , flagella and pili, and secreted virulence factors, including toxins, elastases, alkalin protease, hemolysin, pyocyanin, as well as small molecules that include phenazines , rhamnolipid , and biofilm formation (6). More advanced approaches to more identification have been developed, including polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and DNA sequencing (12). Molecular tools such as *16SrRNA* gene sequencing provide trusty results although it might

have problems to assign at specie, Selective amplification of *Pseudomonas 16SrRNA* gene by PCR has been used to detect the differentiate of *Pseudomonas* species (13).

Biofilm is a community of microorganisms attached to substrate surface and submerged into extracellular slimy matrix (7). Genetic diversity of organisms that form the biofilm and variety of environmental conditions where it emerges prove that biofilm is an ancient ubiquitous life form of microorganisms (8).

The formation of biofilms contributes to the high resistance of *P. aeruginosa* to antibiotics making the treatment of biofilm infections more difficult (9). In addition, bacteria in biofilm were demonstrated to show elevated resistance to the host immune system clearance (10). Factors which explain the high antimicrobial resistance of biofilms include decreased oxygen and nutrient decreased diffusion of antibiotics through the biofilm matrix,decreased growth rates and metabolism (11).

Bacteria have chemical molecule which mediated cell to cell communication system to regulate gene expression and group activates within communities (14).Quorum sensing (QS) was first discovered in the marine bioluminescent bacterium *Vibrio fisheri* in the period early 1970 (15).QS is widely spread in the bacteria, and considered as a "speaking" systems, QS plays major role in virulence factor production, and biofilm formation (16).

The communication occurs through small molecules called acyl homoserine lactones (AHL) also known as "Autoinducers" that diffuse freely across the membranes of bacteria when bacterial density increase, these molecules when reach to threshold concentration act as a cofactors of transcriptional regulators (17).

P.aeruginosa has two of QS system *las* and *rhl* system (18).The las system consist of *lasI*, which is responsible for the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL), and the transcriptional activator *LasR* (19), the *las*R binds to (3-oxo-C12-HSL) molecule and regulates expression of specific genes (20). While a second QS system in *P. aeruginosa* consists of the *rhlI* and *rhlR* genes (21, 22). The *rhlI* synthase responsible for produce of the AHL (N-butyryl-L-homoserine lactone (C4-HSL), and *rhlR* is the transcriptional regulator only when *rhlR* is complexed with C4-HSL does it regulate the expression of several genes (rhamnolipid, elastase cyanide, alkaline protease, and pyocyanin production (23).

The role of QS in biofilm formation was first reported in 1999 by Davies *et al*. who showed that *las*I mutants act to formed flat and thin biofilms (24). The rhl system maintains biofilm archictecture by preventing microbial colonization of open channels surrounding colonies (25).

The *lasI* mutants are defective in activating Pel transcription and biofilm formation in *P. aeruginosa* (26), also the *lasI* and *rhlI* double mutants produce less eDNA which indicating effect in compromised biofilm integrity (27). Recently, Gilbert *et al*. found that *LasR* binds to the promoter region of the psl (28).

Methodology:

The samples (120) were collected from clinical state including both gender with different ages, who suffered from; cystic fibrosis, urinary tract infections, wounds, burns and ear infection from different teaching hospitals in Baghdad city. The study was carried out through September 2016 till May 2017.

Laboratory and Molecular Diagnosis

The samples cultured in Pseudomonas agar and Cetramide agar. Lab. diagnosis was done according to Holt *et al*. (29). While the molecular diagnosis was done based on *16SrRNA* gene as a detection gene.

Detection of biofilm formation:

Biofilm formation determinate according to Bose *et al* (30). The results were calculated according to following equation: Capacity of biofilm formation = Absorption of the sample test - absorption for control (Broth only.)

DNA extraction method:

The bacterial DNA extracted according to Genomic DNA mini Kit which provides by Geneaid Company.

PCR technique used for Detection of *lasI* Gene and *16SrRNA*gene

Primers (table1) were designed using NCBI and supplied by the Bioneer Company as a lyophilized product of different picomol concentrations.

Table 1: Sequence of primers and their size

Gene		Sequence of forward and reverse Primer(5´- 3´)	Size (bp)	Annealing Temp.˚C
16SrRNA	F	GGGGGATCTTCGGACCTCA	956	58
	R	TCCTTAGAGTGCCCACCCG		
Las I	F	GCGCGAAGAGTTCGATAAAA	537	60
	R	ATCTGGGTCTTGGCATTGAG		

F: Forward sequences, R: Reverse sequences

PCR program for *16SrRNA* gene and *lasI* gene:

The PCR tubes containing the mixture were transferred to preheated thermocycler and started the program as in the following table (2).

Table (2): PCR amplification program for *lasI* gene and *16SrRNA* gene

Gene	Initial denaturation	No.of cycles	Denaturation	Annealing	Extension	Final extension
LasI	94 °C for 3min	35	94 °C for 45 sec	60 °C for 45 sec	72 °C for 45 sec	72°C for 7 min
16SrRNA	94 °C for 3min	35	94 °C for 45 sec	58°C for 45 sec	72 °C for 45sec	72°C for 7 min

Five microleters of the product were subjected to electrophoresis

Results and Discussion:

The collected samples were cultured in some media agar to initially diagnosis based on morphological characteristics of the colonies table (3). Molecular detection used to confirm final diagnosis of all isolates. The result of gel electrophoresis (figure 1) for amplification PCR product showed that presence of bands in same level for all isolates.

Table (3): Results of the Biochemical Tests and Culture of *P.aeruginosa* Isolates.

Test	Result
MacConkey agar medium	Pale colony, non-lactose fermented
Pseudomonas agar	Growth +
Cetrimide agar medium	Growth +
Hemolysin	β-hemolysis
Oxidase	Purple color (+)
Catalase	Babbles (+)
Indol	-
Methyle red	-
Vogas-proskauer	-
Citrate utilization	+
Growth at 42°C	Growth (+)
Growth at 4°C	Growth (-)

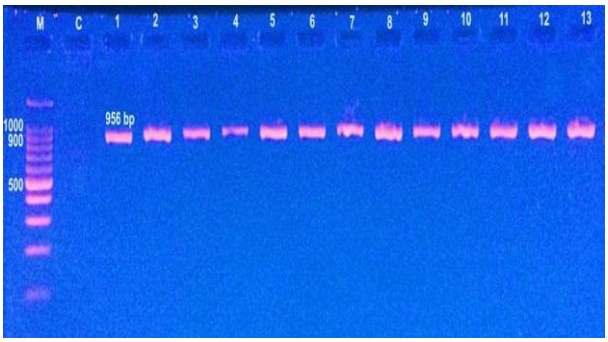

Figure1: Agarose gel electrophoresis (1.5%agarose, 7 V/cm^{2}for 90min) of *16SrRNA* gene (956bp). Lane M: represent 1500bp DNA ladder, Lane C: represent negative control, Lanes 1-13: represent bands of *P.aeruginosa* gene.

The results (table 4) showed that 44/51 (86.2%) of isolates produce biofilm, but which varying degrees compared to negative control.

Table (4) ability of *P.aeruginosa* isolates to biofilm formation.

No. of isolates	Value of biofim formation	No. of isolates	Value of biofim formation	No. of isolates	Value of biofim formation
P1	0.44	P18	0.24	P35	0
P2	0.21	P19	0.18	P36	0.05
P3	0.33	P20	0.11	P37	0.17
P4	0.24	P21	0.06	P38	0

P5	0.36	P22	0.42	P39	0.25
P6	0.25	P23	0.55	P40	0.09
P7	0.26	P24	0.22	P41	0.12
P8	0	P25	0.16	P42	0
P9	0.13	P26	0	P43	0.32
P10	0.08	P27	0.18	P44	0.33
P11	0	P28	0.27	P45	0.30
P12	0.14	P29	0.18	P46	0.34
P13	0.09	P30	0.30	P47	0.28
P14	0.22	P31	0.26	P48	0.05
P15	0	P32	0	P49	0.15
P16	0.07	P33	0.13	P50	0.19
P17	0.11	P34	0.62	P51	0.11

Molecular detection of QS *lasI* gene by used specific primers for *lasI* gene showed that 48 (94.1%) of isolates were positive for this gene, where are the bands appeared within the expected size of the gene (537bp) for all positive isolates (Figure 2).

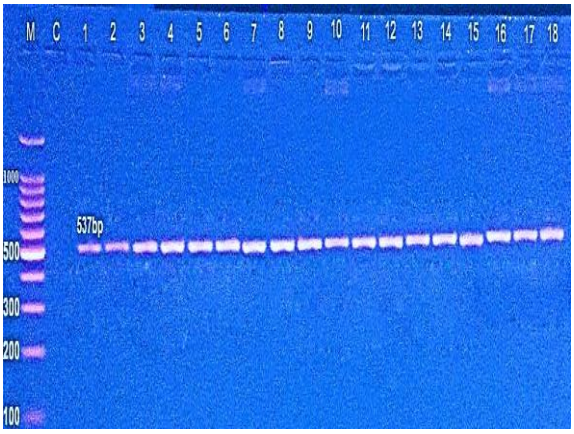

Figure2: Agarose gel electrophoresis (1.5%agarose, 7 V/cm^{2}for 90min) of *lasI* gene (537bp).
Lane M: represent 1500bp DNA ladder, Lane C: represent negative control, Lanes 1-18: represent bands of *P.aeruginosa* gene.

In the present study examined the role of QS gene (*lasI*) in biofilm formation of *P. aeruginosa* in various clinical infections. Initially collected of 120 samples and, final diagnosis based on molecular detection of *16SrRNA* gene which is considered one of the basic criteria in the classification because of highly constant and unable to change over time of its regions (31) showed that 51 isolates belong to target bacteria and, highest percentage of clinical isolates belong to burns followed by wounds, this may explain that *P.aeruginosa* is one of the more bacterial species that cause burns and wounds infection in hospitals.

The ability of *P. aeruginosa* isolates to biofilm formation tested by Micro-titer plate (MTP) method. This method is a quantitative analysis to detect biofilm formation where it gives numerical value for absorption at 630 nm by ELISA reader to determine the amount of membranes formed through adhesion of bacteria to surfaces of microtiter plate, where absorbance represents the thickness of the membranes which formed on surface by *P.aeruginosa* isolates. The isolates capable to form biofilm ranged in intensity among high, medium and weak adherent. The high productivity of biofilm formation may be back to sensitivity of way to measure the few quantities formed, and considered an important method in studying the early stages of biofilm formation (32), this results agrees with Heydari and Eftekhar (33).

Many studies and reports which indicate that there is a strong correlation among QS system and biofilm formation, where the results of biofilm formation showed that 43 of isolates which are able to biofilm form were positive to *lasI* gene, while the isolates that are defect in biofilm form were negative for *lasI* gene, these indicate to strong correlation among biofilm formation and the presence of *lasI* gene .

The *las* gene plays an important role in maintenance of *P.aeruginosa* biofilm, where that the signaling 3-oxo-C12-HSL (synthesized by *LasI*) is necessary for the establishment of *P. aeruginosa* biofilm, whereas a *lasI* mutant forms a flat and thin biofilm, and *lasI* is expressed in a large number of cells during the initial stage of biofilm formation (34). The results of present study confirm that the important role of QS systems in the pathogensis of *P. aeruginosa* bacteria and biofilm formation.

Conclusions

Detection of bacteria by *16SrRNA* gene is very simple and rapid technique compare with other conventional method. Most of local isolates of *P.aeruginosa* were positive for QS gene (*lasI*). The *lasI* gene has an important role in the production of biofilm.

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الكشف عن جين (*LsaI*) في بكتيريا الزوائف الزنجارية ودوره في تكوين الغشاء الحيوي

مثنى حامد حسن حسن فلاح لاحج لمى عامر ياسر

الخلاصـــة

تضمنت الدراسة الحالية جمع 120 عينة من مصادر سريرية متنوعه لغرض التحري عن وجود بكتيريا *P.aeruginosa* في تلك المصادر ، حيث جمعت العينات السريرية من بعض مستشفيات بغداد وتضمنت اخماج الحروق والجروح والتهاب الاذن والتهاب المجاري البولية والتليف الكيسي بواقع (52,28 ,23 ,13 ,4) عينة على التوالي. شُخصت العينات بالاعتماد على صفات المستعمرات المظهرية والمجهرية عند تنميتها في عدد من الاوساط الزرعية فضلا عن الفحوصات البايوكيميائية. تم استخدام التشخيص الجزيئي كتشخيص تأكيدي للعزلات على المستوى الجيني بالاعتماد على الجين التشخيصي*16SrRNA* والذي يحمل تتابعات خاصة ببكتيريا *P.aeruginosa* اظهرت نتائج التشخيص النهائي ان 51 عزلة تعود للبكتيريا المستهدفة توزعت بواقع 21 من حالات الحروق 14 عزلات للجروح و7 عزلات لألتهاب الاذن و4 عزلات للتليف الكيسي و5 عزلات تعود لألتهاب المجاري البولية . وبينت نتائج الكشف المظهري لتكوين الغشاء الحيوي تبايناً في قابليتة العزلات على تكوينه حيث كانت العزلات المكونة للغشاء 44 عزلة (86.2%). واظهرت نتائج التحري عن جين تحسس النصاب باستخدام تقنيه تفاعل البلمرة التسلسلي وبأستخدام بادئات خاصة بتسلسلات معينه لجين *lasI* ان 48 عزلة تملك جين *lasI* والتي تمثل نسبة (94%) من العزلات.