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Rapid Detection For *lasI* And *lasR* Genes Of *Pseudomonas Aeruginosa* At Deference Iraqi Hospitals By Polymerase Chain Reaction (PCR) Technique.

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Abstract : *Pseudomonas aeruginosa* is Gram-negative, and it is the most important and opportunistic pathogens that cause a high rate of mortality and morbidity in hospitalized patients with compromised immune systems. This study was to estimate the specificity and accuracy of a rapid detection of the bacterium based on a uniplex polymerase chain reaction (PCR) that amplifies the *lasI* and *lasR* genes. Forty of Clinical samplesas a wound,burn, and earswabs were collected from deference, and the other forty samples were collected from hospital facilities like operation room, bathrooms,and hospital equipment swaps.The primers was evaluated by specific primers (*lasR/I genes)* with percentage 100%.The result showed that the *lasI* and *lasR*geneswere amplified from the genomic DNA of standard *P.aeruginosa*, Clinical Isolates samples by uniplex PCR. The produced amplicons were 600bp,700bp, for the *lasI* and *lasR* genes, respectively. For all of the samples of *P.aeruginosa*, the PCR results were positive.
Keyword : *Pseudomonas aeruginosa,* Polymerase Chain Reaction (PCR),*lasI* and *lasR* Genes, Pathogenic bacteria.

Introduction and Experimental

Pseudomonas aeruginosa is Gram-negative bacteria, facultative anaerobic rods, non-fermentative, non-sporulation, motile by one polar flagellum [1].In recent years, infections caused by this bacterium are one of the major problems in hospitals and are related to high rates of mortality, which range from 18% to 61% [2].*Pseudomonas* infections are treatedwithantibiotics however unfortunately in hospitalized patients; these infections are becoming harder to be treated correctly because of the increasing number of antibiotic-resistant strains. Most of the laboratories, the detection of *P.aeruginosa* is still accomplished by microbiological culture and biochemical tests. Thus early diagnosis and proper medical treatments are the best strategies for fighting against these infections[3].

Although a comparative study has shown that these methods contain reliable detection results, they are time-consuming and require several days to be completed[4]. Studies have shown that inappropriate initial antimicrobial therapies were associated with adverse outcomes for infection treatments. Conversely, false detection can result in the administration of ineffective antimicrobial therapies during the first 48 to 72 hrs. [5].

Moreover,in some cases in which the bacterial count is low, especially in antibiotic-treatedpatients,false negative results can be achieved in routine laboratory tests. Thus access to rapid and specific methods that have

a high sensitivity is of great importance.In recent decades, the detection and identification of *P.aeruginosa*in clinical samples by polymerase chain reaction (PCR) has been increased substantially[4]. Since 1992, when [6]Reported PCR detection of *P.aeruginosa* for the first time, multiple genes have been reported as PCR targets for the identification of this bacterium[7]. Afterward, various studies have shown that these genes do not have complete sensitivity or specificity for bacterial detection and thus have false negative and false positive results. It should note that *P.aeruginosa*'s genome has a highly polymorphic nature, which can influence the reliability and specificity of the PCR.Therefore, the use of a single gene target could lead to unexpected errors, including cross reactions with other bacterial species and false negative or false positive results, and a highly stringent and distinctive PCR assay is needed [8].According to studies,quorum sensing QS is necessary for the development of infection by *P.aeruginosa*, and the QS genes are exclusive and conserved for each bacterial species [9].The *lasI*and *lasR*genes are quorum sensing essential (QS) genes of the bacterium.Studies have shown few clinical isolates with quorum sensing deficient system [10].

Sample collection & bacterial isolation

The samples were obtained isolatedfrom deferent hospitals.Clinical samples were collected by Sterile swap from the surface of burn's patients, flooring of Burns Unit and Tools of Burns Unit, wound, ear. Swabthen injected in 15 ml nutrient broth and incubated for overnight at 37 ^{o}C.Samples were collected in sterile tubes containing nutrient broth from patients of various hospitals, 50 clinical samples including (15 wound swap, 25 burn swap, tenear swap) and 50 samples were collected from hospital facilities including (15 operation room swap, 25 bathroom room, ten hospital equipment).This samples included *P. aeruginosa*,*Pseudomonas spp*. And others strains.All these samples were cultured by spreading on different media (Blood agar base, MacConkeysagar, Nutrient agar, Nutrient broth, Brain heart infusion broth and Brain heart infusion agar). Plates were incubated 24hrs at 37°C.After the incubation streaked on selective media (Cetrimide agar) and incubated at 37°C for overnight, the pigmentation related to *P. aeruginosa*.

Tested and then collected another non-pseudomonal species this includes: *Escherichia coli*, *Klebsiella pneumonia*,*Neisseria gonorrhoeae* and *Lactobacillus acidophilus* (these species diagnosis by API system) for comparison with *P. aeruginosa*by *lasI*, and *las R*sequence genes for molecular diagnosis.

Primer Design Dilution

The sequences ofthe *lasI; lasR* genes primerswere adoptedfrom GenBankaccessionnumber NC 002516.2designed by [11]. Primers were supplied by Bioneer Company as a lyophilizedproduct of different picomol concentrations and resuspending using deionized water to reach a final concentration for 100 picomols / µl of suspension, then diluted to10 picomols / µl according to the following equations [12].

$\mathbf{x} = \frac{MW/10}{(OD)\ 33}$= ml **x:** Dilution factor

MW: Molecular Weight

$\frac{1000}{X}$ = µl

the amount was in µl of sterile deionized water added to the tube of dried primer to obtain 100 picomols/ µl then diluted to 10 picomoles/ µl by following the equation below:

($Concentration_1$ * $Volume_1$=$Concentration_2$* $Volume_2$).

Stoke solution the lyophilized primer was dissolved in 1 ml Deionized water and stored at-20 ºC. for the working solution, small aliquots were prepared at a suitable concentration and stored at -20 ºC until use to avoid repeated freezing and thawing.

Component and Concentration In Mixture Of PCR

The component used in mixture PCR were Deionized water. GoTaq® Green Master Mix with Concentration 5 mM Tris- HCl, pH 8.3, 200 µM (each) dCTP, dGTP, dATP , and dTTP; 1.5mM MgCl2, 1.25 U o f Taq DNA polymerase. Forward Primer and a reverse primer with Concentration 10 pmole, DNA Sample with Concentration 50 ng.

PCR Amplification of Gene Sequences

Initially, PCR amplification conditions were optimized by separately varying, annealing temperature, primer concentration, and DNA template concentration. Optimization of PCR reaction was accomplished after several trials. Pre-Denaturation the Temperature was 95°C for 5 min and PCR cycles 1, for Denaturation 95°C in 30 sec, Annealing 57°C; 60°C; 62°C in 30 sec. Extension 72°C 30sec and the PCR cycles for all of the last three steps were 30. For the final extension the temperature 72°C in 5 min and PCR cycles 1.

Detection of amplicons

Following amplification, from each reaction (10 µl) aliquots were removed and phage ladder 100 bp are examined by electrophoresis (70V and 60 min) in gels composed of 1.5% (w/v) agarose (Promega, USA) in 1X TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.3), stained with ethidium bromide (2µg/100ml). Gels were visualized under UV illumination using a gel image analysis system [13].

Agarose Gel Electrophoresis

The size of the amplicon was confirmed by electrophoresed PCR-amplified products, and a DNA molecular–weight marker (100bp DNA ladder) on an agarose gel.PCR products were detected by agarose gel electrophoreses using Tris-borate-EDTA (TBE) as the running buffer. Agarose was weighted to prepare 1.5 % gel in 1X TBE in a glass conical flask. It was then placed in a microwave oven to melt the agarose completely, upon cooling approximately 4µl of 10 mg/ml ethidium bromide solution was added, and the molten agarose was decanted into a gel casting tray with a gel comb. The comb was carefully removed. The running tray with gel was placed in the electrophoresis tank, and the gel was immersed in 1X TBE. The DNA molecular weight marker was also loaded in one of the wells[14]. The power was set in the power supply usually at 60 voltages to resolveDNA under an electric field. Following electrophoresis, the products were visualized using UV light on a transilluminator and photographed.

Results and Discussion.

Extraction Chromosomal DNA

Chromosomal DNA Extraction from 80 identified was confirmed as *Pseudomonas spp*as showed in Figure 1. For PCR technique to detection the genes (*lasI* and*lasR*genes) and extraction chromosomal DNA from anther bacteria such as Escherichia *coli, Klebsiella pneumonia, Pseudomonas putida*and(these species diagnosis by API system) for comparison with P. *aeruginosa* by*lasI,lasR* sequence genes for molecular diagnosis.

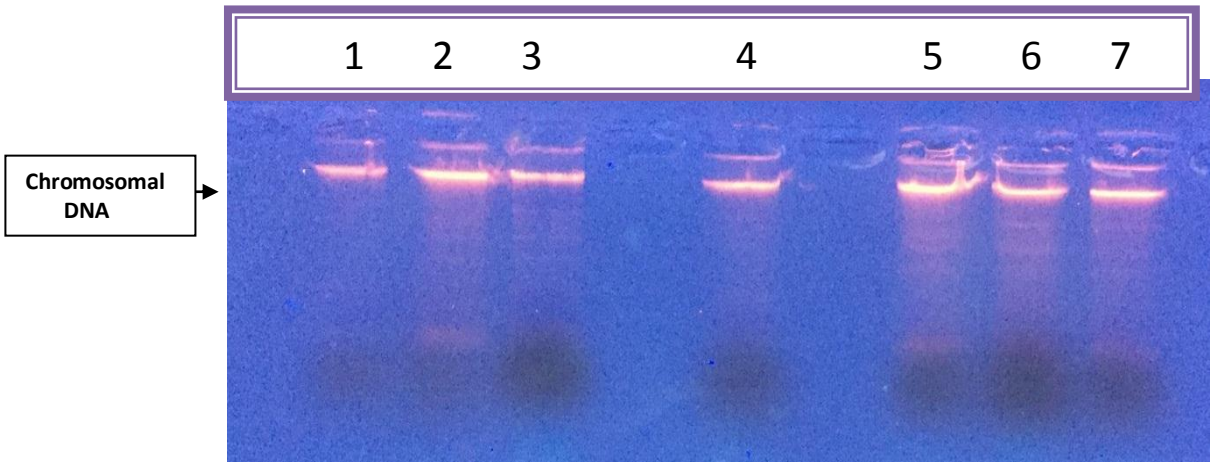

Figure 1 Extracted DNA electrophoresis on 1% agarose (70 vol/ 30 min) to check purity and integrity.

Molecular Identification *P. aeruginosa*

The result showed that the *lasI*and *lasR*geneswere amplified from the genomic DNA of standard *P.aeruginosa*, Clinical Isolates samples by uniplexPCR. The produced amplicons were 600bp, 700bp, for the *lasI* and *las R*genes, respectively. For all of the samples of *P.aeruginosa*, the PCR results were positive 100% as showed in Figure2 and Figure 3.

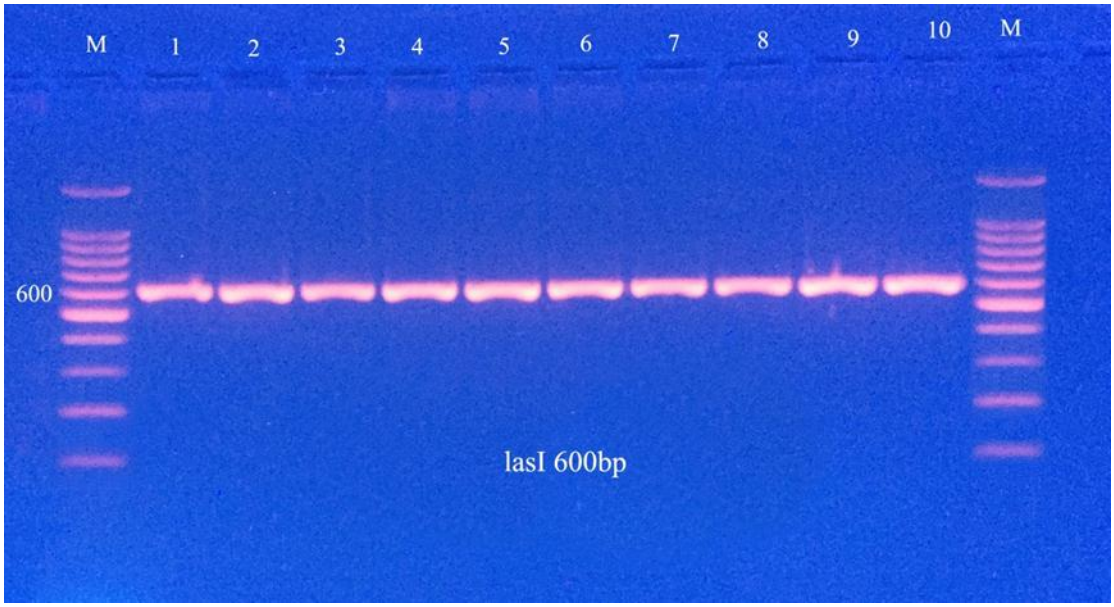

Figure 2.PCR amplification products of *P. Aeruginosalas I* 600 on Agarose gel electrophoresis of Lanes1-10: show positive results, lanes M: ladder 100 bp.

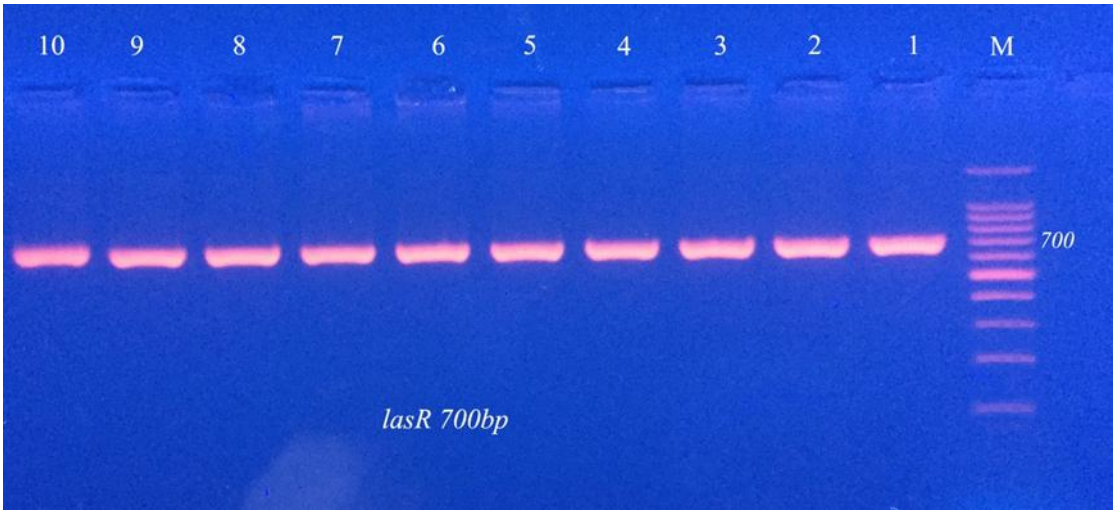

Figure 3. PCR amplification products of *P.aeruginosalasR* 700 on Agarose gel electrophoresis of Lanes1-10: show positive results, lanes M: ladder 100 bp

Results showed that designed primers for the *lasI* and *lasR* genes have a complementary region in all of the *P.aeruginosa* strains also have no similarity in non-*P.aeruginosa* species and other bacteria. In this study, in all of the samples, the target sequences were amplified, which was parallel with the results of biochemical analyses and microbial cultures. Based on these studies, the sensitivity of uniplex PCR was high. Additionally in PCR reactions, using genomic DNA of non-*P.aeruginosa* species and other bacteria as templates, no false-positive results were observed, which demonstrates100% Specificity.For the first time, we reported the detection of this bacterium via the *las* genes by a uniplexPCR.In comparison to previous studies; these results showed that the selected genes are appropriate candidate targets for epidemiologic purposes and regional.

Discussion

Study for [15] evaluated the detection of *P.aeruginosa* by triplex PCR using primers that were designed based on two outer membrane lipoprotein genes *oprL* and *oprI*,And reported that two amplicons which were produced by the *oprI* and *oprL* genes, were observed only in *P.aeruginosa* isolates, while only the *oprI* gene was amplified from the other *Pseudomonas* species. Likewise, the PCR results for all of the other bacteria were negative. The lowest detection level for *P.aeruginosa* was estimated to be102 cells/ml. On the other hand, similar to [6] also evaluated the identification of *P.aeruginosa* in clinical samples that were obtained from patients with cystic fibrosis by PCR using primers for the *algD* gene. He reported that the specificities of the primers were as high as100%, and the method could detect 100 pg of bacterial DNA(good sensitivity). However, in comparison to new primers in our study, they show less sensitivity (10 pg of bacterial DNA for our primers). It should be noted that based on BLAST analysis, the reverse primer in their study is 100% identical to the part of the genome of *Pseudomonas putida*, and thus, it is not specific for *P.aeruginosa.*

Many studies have been developed to provide appropriate genes for the identification of *P.aeruginosa* using specific primers with high sensitivity and specificity. The *lasI* and *lasR* genes specifically detect only the *P.aeruginosa* strain. It should be noted that *Pseudomonas* species have similar quorum sensing system but the QS genes in each species are unique and conserved[16].

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