



## MOLECULAR DETECTION OF *PSEUDOMONAS AERUGINOSA* BY USING *ALGD*, *PLCH* AND *LASB* GENES AND PATHOLOGICAL STUDY OF THE VIRULENT ISOLATE FROM HUMAN BLOOD

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### Abstract

One hundred and thirty human samples obtained from different hospitals from various medically implicated cases, showed that only 29 isolates with the total percentage of (22.30%) found to be *P. aeruginosa* from human blood. *P. aeruginosa* positively identified samples collected from patients suffering from different cancer types, kidney diseases, patients with 2<sup>nd</sup> degree burns, liver diseases, chest disease and type II diabetic patient. Confirm the Biochemical tests obtained by lab work, Vitek were used. The results indicated that there are 29 isolates from human source. Percentage of presence of virulence factors of *P. aeruginosa* in human 51.72%. Histopathological section in animals infected with *P. aeruginosa* infected intra-peritoneal showed in spleen infiltration of inflammatory cells and hemorrhage in interstitial tissue, liver appeared infiltration of inflammatory cells in parenchyma and sinusoids and hemorrhage in interstitial tissue, in intestine intra-peritoneum showed hyperplasia of goblet cells in lung infiltration and aggregation of inflammatory cells and hemorrhage in interstitial tissue in interstitial tissue. This study showed that isolation of *P. aeruginosa* from blood of human with confirm diagnosis by using PCR technique, in addition to virulence isolates causing pathological lesions in internal organs especially intraperitoneal route of infection experimentally in mice.

### Introduction

*Pseudomonas aeruginosa* is a worldwide considered as one of the nosocomial infections an opportunistic pathogen associated with substantial morbidity and mortality rates (Buhl *et al.*, 2015), Specifically this bacterium is the second most frequently recovered pathogen from intensive care unit (ICU), so patients accounts for 10% of all hospital-acquired infections (Gershman *et al.*, 2008; Snyder *et al.*, 2013), Consistent isolation of the organisms infection is confirmed by from normally-sterile sites such as blood. (Stojek *et al.*, 2008). The hospital mortality associated with *P. aeruginosa* bloodstream infections is reported to be greater than 20% in most series, bacterial bloodstream infections associated with significant mortality and health-care and is highest among patients with inappropriate initially antimicrobial treatment (Osmon *et al.*, 2004). In addition *P. aeruginosa* is also largely associated with central line-associated bloodstream infection, urinary catheter-related infection, surgical/transplantation infections and hospital acquired infections including ventilator-associated pneumonia (Trubiano and Padiglione, 2015). Virulence factors makes it highly pathogenic in immune-comprised patients (Driscoll *et al.*, 2007). A number of well-characterized virulence factors of *P. aeruginosa* produces that facilitate the establishment of infections, degrades host-associated phosphatidylcholine (PC) and sphingomyelin occur because the hemolytic phospholipase C (*PlcH*) is a secreted hydrolase that (Bomberger *et al.*, 2009). the *Las* Elastase degrades collagen and no collagen host proteins, can be destroying host physical barriers by facilities spread of infection and to prevent early Clearance of *P. aeruginosa*, inhibits monocyte chemotaxis from wound sites by phagocytosis and then bacterial antigens presentation to the host immune system is stopping (Lyczak *et al.*, 2000). Polymerase Chain Reaction was essential for recognizing etiological genus rapidly by magnification the unique sequence to a specific being (Fadhil *et al.*, 2016) due to the importance of *P. aeruginosa*, therefore the study was designed, and aimed the following: Isolation of *Pseudomonas aeruginosa* from blood stream of

human suffering from chronic diseases, Molecular identification by mentally in mice.

### Materials and Methods

**1. Preparation of culture media:** All media were prepared according to the instructions of the manufacturer company, then autoclaved at 121 °C, 15 pound/Inch<sup>2</sup> for 15 minutes, and distributed into sterile Petri- dishes or test tubes. The media was incubated at 37 °C for 24 hrs to ensure they are contamination free. Some media required special additives (Markey *et al.*, 2014).

### 2. Bacterial identification

Growing isolates were identified according to their colonial morphology, pattern of Gram

**2.1 Bacterial isolation :** Bacteria isolated from human and animal sources were taken from an overnight culture of transoprt media and streaked on Macconkey agar, blood agar, after that, isolates suspected to be of interest were subculture onto cetrimide media and *pseudomonas* agar to ensure pure growth.

**2.2 Identification with analytical profile index (API) 20E system :** The bacterial isolates were further identified by using analytical profile index (API 20E) system according to the procedure suggested by the manufactured company (Bio-Merieux). This system is designed for the performance of 20 standard biochemical tests that are listed in Appendix 1. The bacterial suspension was prepared for all the isolates from purified isolated colonies by using API suspension medium, and the turbidity was adjusted to 0.5 McFarland tube (1x10<sup>8</sup> CFU/ml). By using a sterile Pasteur pipette, then bacterial suspension was transferred to the 20 microtubes and inoculated according to the manufactures' instructions. A period of incubation at 37 °C for 24 hrs. The isolates identification was done using the API system (Numerical coding) for confirmatory identification at species levels.

**2.3 Identification of isolates using VITEK®2 system :** Automated system VITEK-2 Compact system (Biomereux,

France) was used to confirm the identification obtained earlier manually and laboratory identified samples were sent to Central Health Laboratory for confirmation.

### 3. Specific primers

Primers used in this study for molecular diagnosis were lyophilized and investigated by IDT (Integrated DNA Technologies company, Canada); three primers were used for *las*, *plcH* and *algD* primer sequences are described in tables (1, 2 and 3) respectively (Faraji *et al.*, 2016).

**Table 1:** The specific primer for *lasB* gene.

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'- GGAATGAACGAAGCGTTCTCCGAC - 3'	60	54.2
Reverse	5'- TTGGCGTCGACGAACACCTCG - 3'	62.1	61.9

**Table 2 :** The specific primer *plcH* gene.

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'- GCACGTGGTCATCCTGATGC - 3'	58.9	60
Reverse	5'-TCCGTAGGCGTCGACGTAC - 3'	58.9	63.2

**Table 3 :** The specific primer *algD* gene

Primer	Sequence	Tm (°C)	GC (%)
Forward	5'-CGTCTGCCGCGAGATCGGCT - 3'	64.7	70
Reverse	5'-GACCTCGACGGTCTTGCGGA - 3'	61.7	65

### 3. Extraction of genomic DNA

The bacterial suspension was prepared by inoculating the isolate into Brain Heart Infusion broth (BHIB), which was incubated at 37 °C for 24 hr, and then the turbidity was adjusted to obtain approximately  $1 \times 10^9$  CFU/ml. After that, 1 ml of the suspension was transferred to an eppendorf tube and centrifuged at 14000-16000 rpm for 1 min, and the supernatant was discarded. This process was repeated twice, and then 200 µl of GT buffers was added to the deposit, which was re-suspended by shaking vigorously and incubated at room temperature for 5 minutes. G- Spin DNA extraction kit, intron biotechnology was used for total DNA extraction, the contents of kit is listed below.

#### 3.1 Sample preparation

3 µl of the processor loading buffer (Intron / Korea) has been mixed with 5 µl of the extracted DNA to be electrophoresis (loading dye), after the mixing process, the loading process is now to the holes of the gel. An Electric current of 7 v/c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by a source of the UV with 336 nm after put the gel in pool contain on 3µl Red safe Nucleic acid staining solution and 500 ml from distilled water.

#### 3.2 Amplification of DNA.

##### PCR PreMix Kit

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution. *Maxime* PCR Pre Mix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq DNA polymerase, dNTP mixture, reaction buffer, and so on in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do

PCR just add a template DNA, primer set, and D.W.; The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. It is suitable for various samples' experience by fast and simple using method; the components of the Components of the *Maxime* PCR PreMix kit (*i*-Taq) are illustrated below.

### 4. Histopathological Examination

Samples with dimensions  $1 \text{cm}^3$  taken from the internal organs including: kidney, spleen, skin, intestine and the tissues repair in a 10% solution of formaldehyde immediately after removal. after 72 hours of installation ,and wash samples with tap water and then treatment was routinely done by (Luna, 1968).

#### 4.1 Experimental infection in mice

Twenty mice of both sexes, were randomly divided into two groups each group contain 10 mice treated with *P aeruginosa*  $1 \times 10^7$  CFU/ml according to (Matsumoto *et al.*, 2008) were prepared by using the first tube of Mcferland tube for dilution of bacterial suspension as follows: First group were injection intra peritoneal with (0.3 ml) bacterial suspension pathogen, and second as control negative group, animal were sacrificed after 72 hours after infection, then organ takes and study gross change, then pathological study all organ fixed in 10% formalin, samples with dimensions  $1 \text{cm}^3$  taken from the internal organs and the tissues repair in a 10% solution of formaldehyde immediately after removal, after 72 hours of installation, and were washed samples with tap water and then treated was routinely done by (Luna, 1968).

### 5. Statistical Analysis

The Statistical Analysis System (SAS, 2012) program was used to detect difference factors in the parameters employed in this study, Chi-square test was used to compare significances between percentages in this study.

## Results

### 1. Isolation of *P. aeruginosa*

The first part of this study included the isolation of *P. aeruginosa* from various medically implicated cases, the results of cultural examination of 130 samples from human blood showed that only 29 isolates with the total percentage of (22.30%) found to be *P. aeruginosa* from human blood; these results are detailed in table (4).

**Table 4 :** Showed the isolates of *P. aeruginosa* from blood samples of human.

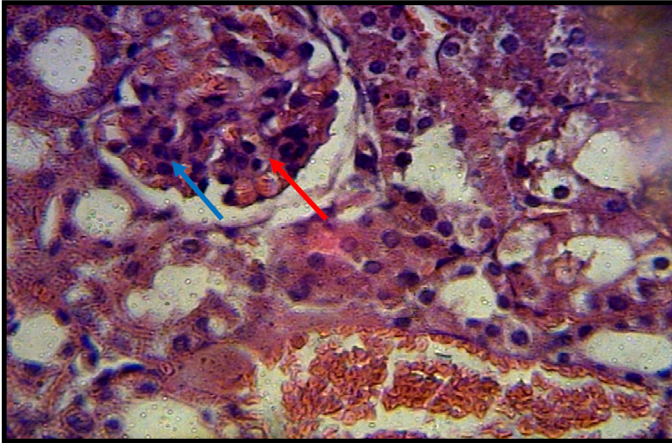
Source	Number of samples	No. of Positive isolates	Percentage%
Human	130	29	22.30
Chi square value			7.01
Probability			0.008

### 3. Pathological study

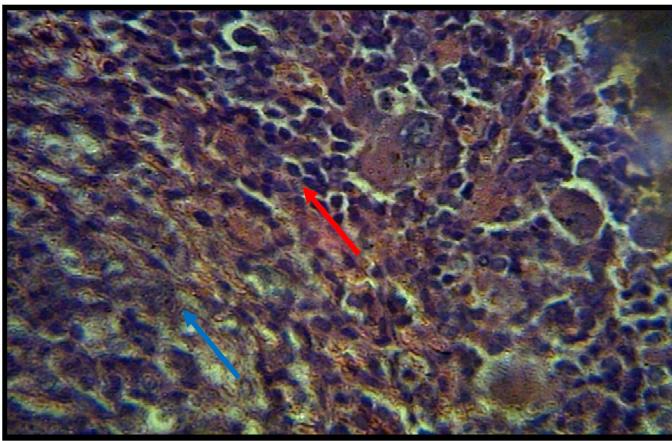
The Histopathological section of animal infected with *P. aeruginosa* intra-peritoneal showed infiltration of inflammatory cells and hemorrhage in kidney (Fig. 1), in spleen intra-peritoneal showed infiltration of inflammatory cells and hemorrhage in interstitial tissue (Fig2), liver showed infiltration of inflammatory cells in liver parenchyma and sinusoids and hemorrhage in interstitial tissue (Fig 3),in



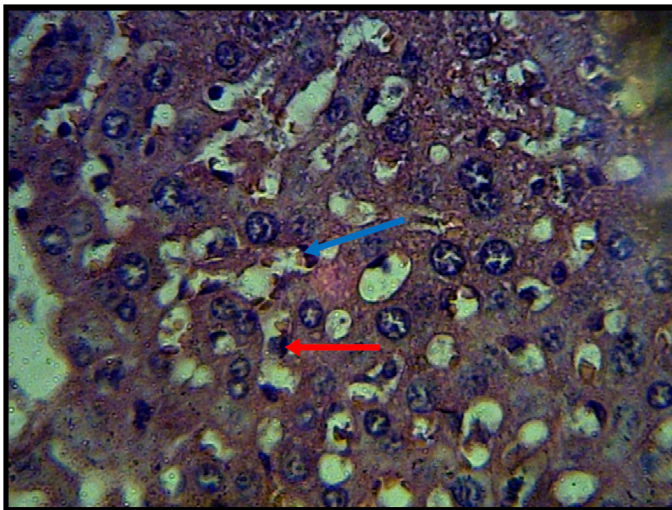
intestine intra-peritoneum showed hyperplasia of goblet cells (Fig 4) in (Fig 5) lung infiltration and aggregation of inflammatory cells and hemorrhage in interstitial tissue in interstitial tissue.



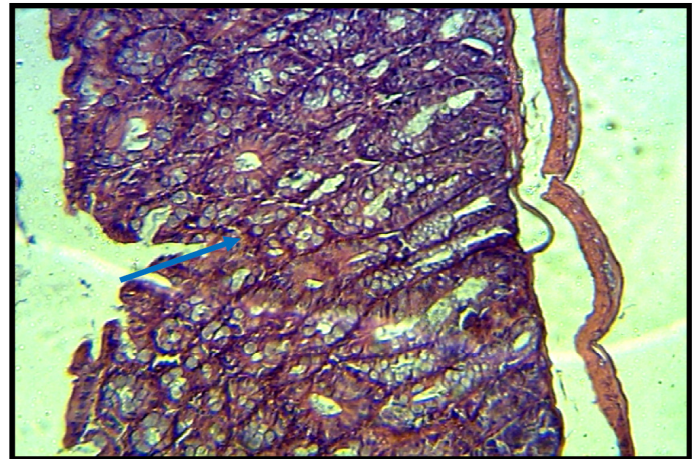
**Fig. 1 :** Histopathological section in kidney of group animal infected with *P. aeruginosa* intraperitoneal showed infiltration of inflammatory cells (red arrow) and hemorrhage (blue arrow) (H&EX400 ).



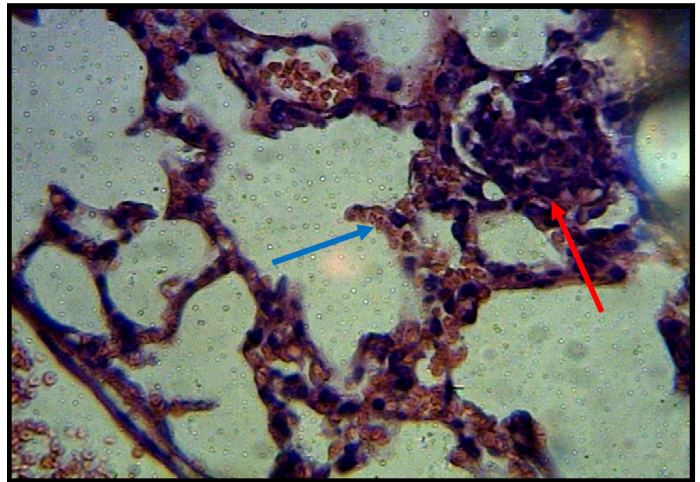
**Fig. 2 :** Histopathological section in spleen of group animal infected with *P. aeruginosa* intraperitoneal showed infiltration of inflammatory cells (red arrow) and hemorrhage in interstitial tissue (blue arrow) (H&EX400 ).



**Fig. 3 :** Histopathological section in liver of group animal infected with *P. aeruginosa* intraperitoneal showed infiltration of inflammatory cells in liver parenchyma and sinusoids (red arrow) and hemorrhage in interstitial tissue (blue arrow) (H&EX400 ).



**Fig. 4 :** Histopathological section in intestine of group animal infected with *P. aeruginosa* intraperitoneal showed hyperplasia of goblet cells (blue arrow) (H&EX100 ).



**Fig. 5 :** Histopathological section in lung of group animal infected with *P. aeruginosa* intraperitoneal showed infiltration and aggregation of inflammatory cells (red arrow) and hemorrhage in interstitial tissue (blue arrow) in interstitial tissue (H&EX400).

**2- Molecular identification of *P. aeruginosa* isolates**

From such products, we can conclude that the three tested virulence factors were found in both human and dog the percentage were 100% from isolates (15 samples from human and 3samples from dog) found locally in Iraq as in table (5).

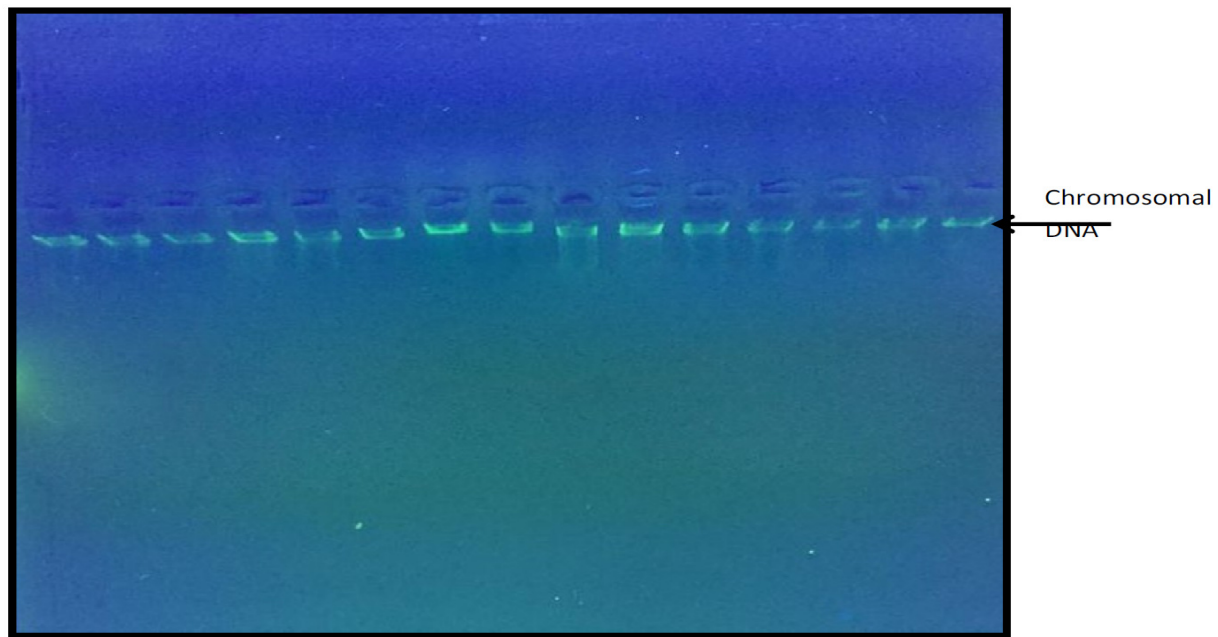
**Table 5 :** Percentage of presence of virulence factors of *P. aeruginosa* in human and dog isolates

Source	No. of total samples	No. of total isolates	No. of chosen isolates for study	No. of isolates carrying genes of virulence factors (PLC- H, Las B and algD)	Percentage %
Human	130	29	15	15	100%

**2.1 Extraction of genomic DNA**

Genomic DNA of *P. aeruginosa* was extracted using G-spin DNA extraction kit, and then the DNA was electrophoresed on 1% agarose gel to detect the chromosomal DNA bands, which will be used as a template in the amplification of DNA using PCR technique. The obtained results illustrated in figure (5) shows a clear sharp band of extracted genomic DNA with high integrity, which is suitable for use in PCR experiment.





**Fig. 5 :** Electrophoresis pattern of extracted genomic DNA of *P. aeruginosa* isolates extraction, on 1% agarose gel at 5 vol /cm for 1:15 hours and stained by Red Safe Nucleic Acid Stain

## 2.2 PCR reaction

Polymerase chain reaction was used to detect genes of *P. aeruginosa* (PLC H, Las B and algD) which are considered as important virulence factor attributed to the bacterium pathogenicity, specific primers for each gene was used with specific length of (20 bp, 24bp and 20bp) respectively for each of them, amplification products were electrophoresed on 1.5% agarose at 5 volt/cm<sup>2</sup>. 1x TBE buffer for 1:30 hours.

### (a) plc H of gene specific amplification

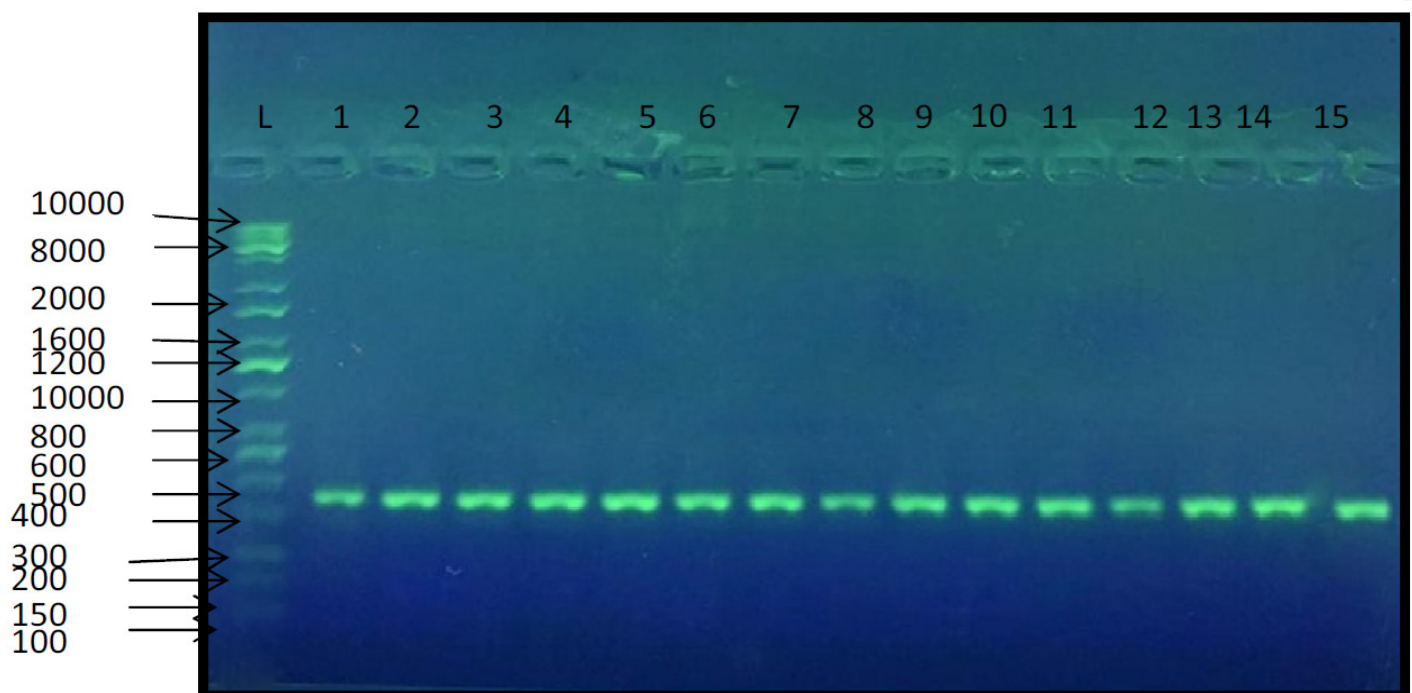
Amplification of this gene results in a product of size 608 bp as compared to the marker ladder. The product was electrophoresis on 1.5% agarose at 5 volt/cm<sup>2</sup>. 1x TBE buffer for 1:30 hours.

### (b) lasB of gene specific amplification

Amplification of this gene resulted in products with size 284Base pair as compared to the marker ladder. The product was electrophoresis on 1.5% agarose at 5 volt/cm<sup>2</sup>. 1x TBE buffer for 1:30 hours

### (c) algD gene specific amplification

One and half µl of genomic DNA were used for each PCR reaction. A conventional PCR protocol was used to analyze simultaneously the presence of *Pseudomonas aeruginosa* AlgD gene. The presence of the *Pseudomonas aeruginosa* AlgD gene was identified by 313 bp, as shown in figure (6). Specific amplification of this gene for both human and dog isolates, resulted a products with 313 bp in size. when compared to the ladder marker. From such products, we can conclude that the three tested virulence factors was found in both human isolates found locally in Iraq.



**Fig. 6 :** Agarose gel electrophoresis for *Pseudomonas aeruginosa* AlgD gene (313bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel (1:5 h., 5V/cm, 1X TBE) and visualized under U.V. light after staining with red stain. Lane: 1 (M: 100bp ladder).

## Discussion

### Molecular identification of isolates of *Pseudomonas aeruginosa*

The PCR method was carried out for detection and distribution of 3 virulence genes *plcH*, *lasB* and *algD*, and among isolates, using specific primers the genotypic identification is complementary for the phenotypic identification methods (Hery-Arnaud *et al.*, 2017). Percentage of prescience results virulence factors of *P. aeruginosa* in human and dog was 100% for the three genes

### Detection of the *Las B* gene

*PlcH* from local isolates in human and dogs appeared 100%, and disagreement with (Jackson *et al.*, 2013) who revealed prevalences for the *plcH* gene 86.8% These prevalences showed that strains isolated were able of secreting hemolytic Exo-enzymes and phospholipase C, and thus, strains could be involved in pulmonary infections. Also not approve with (Benie *et al.*, 2017) that showed gene was detected on *Pseudomonas* showed importance *plcH* (72.1%) detected. (Jackson *et al.*, 2013) Showed Hemolytic phospholipase C (*PlcH*) is a secreted hydrolase that degrades host-associated phosphatidylcholine (PC) and sphingomyelin, these choline-containing phospholipids are abundant macromolecules in eukaryotic membranes and host lung surfactant. *PlcH* adversely affects the integrity of the lung and contributes to decreased lung function (Wargo *et al.*, 2009).

### Detection of the *LasB* gene

*Elastase B* is an important extracellular virulence factor that has been shown to play an essential role in the pathogenicity of this bacterium during host infection. It causes lysis of elastin, destroys the protein compounds, inactivates complement and cause degradation of clotting factors. *P. aeruginosa* is clearly multifactorial. *LasB* is one of the most important proteases of *P. aeruginosa* (Nikbin *et al.*, 2012). Mutation of *lasB* gene reduces markedly *P. aeruginosa* invasion, prevalence of the *lasB* gene in all the environmental and clinical isolates implies the importance of *LasB* factor to survival of *P. aeruginosa* in various settings (Lomholt *et al.*, 2001). The high prevalence of this factor among isolates from blood infections may show that the role of this gene in the blood infections is more important than burn and pulmonary tract infections, the differences in the distributions of virulence factor genes in the populations strengthen the probability that some *P. aeruginosa* strains are better adapted to the specific conditions found in specific infectious sites, and thus virulence genes expression differs according to site and severity of infection (AlKhattab *et al.*, 2015).

(Mitov *et al.*, 2010) who recorded the *Las B* gene available in 100% of isolates of *P. aeruginosa* from Bulgarian nosocomial that agreement with 100% of *Las B* gene in this study; while disagreement with (Al-Shafee, 2018) who concerning percentage of total isolates carrying *Las B* gene was 80% with percentage of 100% was from human samples, and also disagree with, (Kuang *et al.*, 2011) showed that 75% of the isolates of *P. aeruginosa* from clinical cystic fibrosis and other bronchiectasis patients in America that contained this gene.

### Detection of the gene *algD*

The pathogenicity of *P. aeruginosa* depends on many virulence factors. Alginate plays a major role in resistance to the immune system *algD* gene expression is dependent on environmental conditions such as limitation of carbon, nitrogen, phosphate, oxygen concentration and slow growth rate (Be atricea *et al.*, 2005). *algD* gene is expressed in response to environmental stress especially to nutrient deprivation, oxygen availability and slow growth rate and one of the system of regulation of the *Las* is dependant to cell density (Nikbin *et al.*, 2012).

(Fadhil *et al.*, 2016) Revealed Virulence factors associated with the membrane are generally involved in colonization and chronic infection, *P. aeruginosa* can also cause lung lesions by different mechanisms, the mutation responsible for the mucoid is associated with activation of the transcription of the alginate gene *algD*, the formation of mucoid colonies of *P. aeruginosa* composed of alginates, involving *algD* genes, protects the bacterium from the host's immune response and from antibiotics. As lactoperoxidase (LPS), alginates are involved in the adhesion of the bacterium to the respiratory epithelium. The high prevalence of *algD* gene could be at the origin of the conversion of *P. aeruginosa* strains to a mucoid phenol type overproducing alginates (Whitney *et al.*, 2015). This alginate prevalence indicates that isolated strains of animal products are involved in the formation of biofilms as alginates have been widely regarded as the major exopolysaccharides of the biofilm matrix (Benie *et al.*, 2017). The over production of alginates protects *P. aeruginosa* from antibiotics and also alleviates the immune response by inhibiting complement activation, reducing polymorphonuclear chemotaxis and decreasing phagocytosis (Whitney *et al.*, 2015). Microbiological culture methods resulted *P. aeruginosa* strains isolated from The results of the PCR assay using the newly designed *algD* primer sets were positive in 68, 70 and 69 clinical samples which represent 97.2%, 100% and 98% sensitivity for each primer set, respectively (Benie *et al.*, 2017) that nearly approved with our results. (Tae *et al.*, 2014) appeared almost identical to PCR results for *algD* gene were only positive 98.6% for *algD* gene, and disagree with (Lanotte *et al.*, 2004) who conducted of *algD* (70.1%).

### Histopathological study

Infection of *P. aeruginosa* using by intra peritoneal route was more effective and showed more pathological lesions on internal organs during 72 hours of infection. Majeed *et al.* (2014) and Al-Kafaji *et al.* (2016) appeared that Many pathological changes were observed in the histopathological section of intraperitoneal infection of *P. aeruginosa* in Liver pathological changes: these changes include congestion of blood vessels, hepatocytes degeneration and necrosis, kuffer cell infiltration. Severe ballooning degeneration in hepatocytes also was observed, lung pathological changes: the alveolar walls are thickened from the presence of lymphocytes and plasma cells, that approved of the lesions in liver and lung of group which infected intraperitoneally in the our results, and the lesion of kidney was infiltration of inflammatory cells and hemorrhage that agreement with (Tsuchimori *et al.*, 1994) that conducted the severity of renal pathology of kidney changes by using the following mild pyelitis with infiltration of a small amount of neutrophils in the renal pelvis; severe pyelitis with

infiltration of a moderate amount of neutrophils in the renal pelvis and renal cortex adjacent to the pelvis and thickening of the pelvic mucosa; pyelonephritis with infiltration of neutrophils in the renal pelvis and the renal medulla and thickening of pelvic mucosa; severe pyelonephritis with abscess formation.

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