Bacteriological and molecular study of *Pseudomonas aeruginosa* strains isolated from different clinical cases in Erbil and Kurkuk

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Summary

This study included isolates of bacteria from 125 clinical samples in Erbil and Kirkuk Hospital including (burns, wounds, urine and sputum); 38 isolates were identified as *P. aeruginosa* after conducting microscopic and biochemical tests. The results of antibiotic sensitivity test showed that all isolates of *P. aeruginosa* were different in resistance to Pipracillin, Erythromycin with rate of (100%) and to the Nalidixic acid (94.73%) while the lowest resistant antibiotics were to Co-trimoxazole, Ceftazidime and Ciprofloxacin, which amounted to (26.31%, 23.68 and 21.05%) respectively. For molecular diagnosis of *P. aeruginosa* some virulence genes the *alg D* and *exo A* were amplified through Polymerase Chain Reaction technique. The results showed that in 38 isolates cases only 22 (57.9%) were positive for *algD* gene by amplification of 520 bp band. While in urinary tract infection; 6 samples (60%) had *alg D* gene, and 8 (57.14%) isolates had *alg D* gene in wounds samples; also 7(70%) isolates from burns had that gene, while the sputum samples showed only one with *alg D* gene which was the lowest ratio; but in amplification of *exo A*, the results showed the presence of only one isolate from burns with molecular weight 396 bp with no appearance in others.

Keywords: Molecular diagnosis, *Pseudomonase aeruginosa*, Polymerase Chain Reaction.

Introduction

The *P. aeruginosa* is a ubiquitous environmental Gram-negative microorganism, which is one of the most important opportunistic bacteria in hospital-acquired infections and causes a wide variety of serious infections in individuals with thermal burn, mechanical extensive trauma, cancer, cystic fibrosis and surgical site infections (1).

The *P. aeruginosa* is the most common cause of infections and inflammations for many organs in human body especially after surgical operation in addition to infection of animals and plants (2). It causes community acquired (hospital-acquired) infections; therefore it is associated with pneumonia, cystic fibrosis and skin lesions (3). Despite considerable advances in antimicrobial therapy, effective treatment and control of *P. aeruginosa* infections remains as a persistent problem, primarily because of the natural resistance of the organism and its remarkable ability to acquire resistance to multiple antimicrobial agents by various mechanisms (4). Alginate production plays a central role in the pathogenesis of *P. aeruginosa* in which the expression is related to the presence of an operon composed by many genes with the activation processes being regulated by (*alg*R, *alg*P, *alg*B and *alg*U) (5); these genes are very important for the existence of the mucoid trait, the *alg*D being responsible for the expression of the alginate capsule (6). Alginate is one of the virulence factors; it is a mucoid exopolysaccharide, it like LPS functions as an adhesin, anchoring *P. aeruginosa* to the colonized respiratory epithelium (7). Exceptional research efforts during this time have revealed that *P. aeruginosa* is able to produce as many as three distinct exopolysaccharides, each of which is associated with specific types of biofilms and conditions under which they are formed.

Overexpression of the alginate exopolysaccharide was first identified as being associated with *P. aeruginosa* mucoid isolates recovered from the lungs of chronically infected cystic firosis patients, but rarely from other types of infections (8). Patients suffering from urinary and respiratory tract infections, *P. aeruginosa* is believed to be a major

contributory factor to chronic lung infections, which could form biofilm and adhere to human mucin in the lower respiratory tract and urinary system (9).

Many extracellular virulence factors have been shown to be controlled by a complex regulatory circuit involving cell-to-cell signaling (quorum sensing) systems that allow the bacteria to produce these factors in a coordinated, cell-density–dependent manner (10). Several studies showed that detection of bacteria by Polymerase Chain Reaction (PCR) assay using the newly designed primers. It is also shown that the results of the PCR assay on clinical samples of severe infections gives earlier results than that of conventional cultural method. Usually most laboratories use conventional microbiological methods such as culture and biochemical procedures for identification of *P. aeruginosa* in clinical samples. On the other hand, conventional microbiological methods are time-consuming and take several days for identification and confirmatory testing, which is a problem for controlling fatal infections (11). This study aimed to evaluate the presence of two selected virulence genes (namely *algD* and *exo A*) among *P. aeruginosa* strains isolated from different clinical cases.

Materials and Methods

A total of 125 clinical cases samples of burns, wounds, urine which were taken from patients suffering from urinary tract infection (UTI) and the sputum from patients of pneumonias. These samples were taken between March and December 2015, from patients of general government hospitals of Kirkuk and Erbil.

Identification of *P. aeruginosa*: According to (12), the swabs were enriched in brain-heart infusion broth (Himedia, India), and plated on to MacConkey agar (Himedia, India). A single colony was selected and inoculated on brain-heart Agar medium; then morphological characteristics of *P. aeruginosa* was investigated after Gram staining, including pigments production after incubation at 37ºC for 1-2 days in cetrimide agar (Himedia, India). The biochemical tests included: Growth at 42ºC in trypticase soya agar, Indole production, Methyl red, Voges-proskauer (VP), Citrate utilization, urease activity, Oxidase, gelatin liquefaction and the catalase were done according to (13).

Antimicrobial susceptibility tests of the isolates to antibiotics (Bioanalyse, Turkey), were determined by the disc diffusion technique according to (14). Sterile swabs were used to inoculate the suspension by streaking on prepared and dried Mueller Hinton agar (Himedia, India) plate evenly, then allowed to stay for 3-5 min., sterile forceps were used to place the antimicrobial discs on the inoculated plates. The plate was incubated at 37°C for 18-24 hrs. The diameter of each zone of inhibition was estimated in millimeters using a meter ruler on the underside of the plate. The zone diameter of each isolate was compared with National Committee of Clinical Laboratory Standards NCCLS 2010 (15). Results were recorded as susceptible, intermediate susceptible or resistant, based on the zone size of each antimicrobial disc used.

DNA Extraction: Clinical samples were processed as described by (16), which included the following steps: One µl of an overnight culture was added to a 1.5 ml microcentrifuge tube. Centrifuged at 13000-16000 xg for 2 minutes to pellete the cell, then the supernatant was removed. The cells were resuspended gently by 600 µl of nuclei lysis solution. The suspension was incubated at 80˚C for 5 min., then cooled at room temperature. A quantity of 3 µl of RNase solution was added to the cell lysate then the tubes were inverted 2-5 times for mix it. The tubes were incubated at 37˚C for 15-60 min. then cooled at room temperature. 200 µl of protein precipitation was added to the lytic cells then vortex it vigorously at high speed for 20 seconds, then Incubated in ice for 5 min. The samples were Centrifuged at 13000-16000 xg for 3 minutes, then the supernatant was transferred which contain the DNA to a clean 1.5 ml microcentrifuge tube containing 600µl of isopropanol and mixed gently until the thread –like strands of DNA form a visible mass. The samples were Centrifuged at 13000-16000 xg for 2 minutes. The supernatant was poured off carefully and the tubes were drained on clean absorbent paper then added 600 µl of 70% enthanol and the tubes were

inverted several times to wash the DNA pellet. Then the tubes were centrifuged at 13000-16000 xg for 2 min. and the ethanol was aspirated carefully. The tubes were drained on clean absorbent paper and the pellet was allowed to air-dry for 10-15 minutes. 100 µl of DNA solution was rehydrated and incubated at 65˚C for 1 hr., the solution was gently mixed periodically, the rehydrated DNA was kept at room temperature at 4 ˚C. The DNA stored at -2˚C. The two primers pairs were purchased from Integrated DNA Technologies Inc. from Canadian. The two primers pairs were previously published by (17) (Table, 1).

Table, 1: Biological materials for DNA primers.

Primer	Primer sequence (5'-3')		Product Size
alg D	Forward	TTC CCT CGC AGA GAA AAC ATC	520 bp
	Reverse	CCT GGT TGA TCA GGT CGA TCT	
exo A	Forward	GAC AAC GCC CTC AGC ATC ACC AGC	396 bp
	Reverse	CGC TGG CCC ATT CGC TCC AGC GCT	

PCR conditions: The primers were prepared by melting of *alg* D and *exo* A with distilled water; the size of 636 microliter, then the initiators were blended well and stored on ice to complete the interaction; PCR master mix reaction was prepared by using (AccuPower PCR PreMix Kit)*and (GoTaq® Green Master Mix from Promega, USA)* this master mix was carried out according to the instructions of the company as shown in (Table, 2). Dried material was found in the standard equipment pipeline for the purpose of conducting the PCR reaction, which contains all the necessary elements for interaction such as Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2, stabilizer, and tracking dye.

Table, 2: Reaction mixture of DNA amplification.

Reagent		Final concentration
DNA template		1.5 µl
Primers	F. primer	1 µl
	R. primer	1 µl
PCR water		16.5 µl
Total reaction mixture		20 µl

The amplification of *alg D* gene was performed in a programmed thermal cycler. The reaction conditions show in (Table, 3).

Table, 3: Showed thermal cycler.

Cycle	Step	Temperature (°C)	Time
Initial 1 cycle	Denature	94 °C	3 min
30 cycles	Denature	94 °C	1 min
	Annealing	60 °C	30 sec
	Extension	72 °C	1 min
Final 1 cycle	Extension	72 °C	7 min

The amplification of *exo* A gene was performed in a programmed thermal cycler. The reaction conditions show in (Table, 4).

Table, 4: Showed thermal cycler.

Cycle	Step	Temperature (°C)	Time
Initial 1 cycle	Denature	96 °C	3 min
40 cycles	Denature	96 °C	1 min
	Annealing	55 °C	1 min
	Extension	72 °C	1 min
Final 1 cycle	Extension	72 °C	10 min

Results and Discussion

Bacteriological findings showed that 38(30.4%) isolates of *P. aeruginosa* were recovered from suspected patients. The highest isolation rate was among burns patients (40%), wound and UTI isolates were (35 and 25%) respectively, while the lowest rate was isolated from sputum, which amounted to 20% (Table, 5). The results agreed with the findings of the researcher (18 and 19), which reported higher isolate of *P. aeruginosa* isolates in the Hillah teaching hospital samples as 37% of wounds. Also the results agreed with the researcher (20), toward the isolation and clinical cases of bacterial isolates. Their findings were 34.35% from the wounds, while the sputum sample showed 14.87% availability.

Table, 5: Distribution of *P. aeruginosa* isolates grown on different media according to their source of isolation.

Samples	Total No. of specimens	Positive specimens	Prevalence (%)
wound	40	14	35%
Burn	25	10	40%
UTI	40	10	25%
sputum	20	4	20%
Total	125	38	30.4%

The results of microscopic identification showed that *P. earuginosa* isolates were rod-shaped bacteria, gram-negative characterized

by the movement, for they contain flagella unipolar this agreed with (21), while the results of biochemical identification showed these isolates were positive for Gelatin test, Oxidase, Catalase, urea, positive for utilization of Citraite, and production of pigment, while they were negative for Methyl –red, indole, Vogas–Prakaur and grows in temperature (42°C), these tests were done according to (13) for diagnosis of *P. aeruginosa* and the results agreed with (22 and 19).

The antibiotics susceptibility test showed that all isolates of bacteria were resistant to antibiotic Piperacillin (PRL), Erythromycin (E) rate of (100%) and the second highest resistance to the Nalidixic acid (NA) and amounted (94.73%) while the lowest resistant antibiotics were to Trimethoprim-sulpha methoxazole (SXT), Ceftazidime (CAZ) and Ciprofloxacin (CIP), which amounted (26.31%, 23.68 and 21.05%), respectively as mentioned in (Table, 6).

Table, 6: Showed antibiotics susceptibility test.

	PRL	E	NA
Wound	14(100)	14(100)	13(92.8)
Burn	10(100)	10(100)	10(100)
UTI	10(100)	10(100)	9(90)
Sputum	4(100)	4(100	4(100)
Total	100	100	94.73
	SXT	CIP	CAZ
Wound	5(35.71)	3(21.4)	3(21.42)
Burn	2(20)	2(20)	4(40)
UTI	3(30)	3(30)	2(20)
Sputum	0	0	0
Total	26.31	21.05	23.68

These results agreed with the findings of the (19), and also agreed with (23), who attributed the resistance of antibiotics to the ability of bacteria to form biofilm where available basic materials within the surrounding environment makes creating biofilm for granted to them, which is the first building block in the beginning of the resistance of antibiotics, through the mechanisms of transport and communication between Cell-to-Cell and mechanisms of the sensor (QS), which has proved effective in the resistance of antibiotics; physiological factors are Efflux System (Efflux Multidrug Pumps) which works to reduce the anti-concentration in the cell where it makes stream systems to remove anti-macrolides, Fluoroquinolones, β–lactams, aminoglycosides (24) and to reduce significantly the permeability of the outer membrane through modulating or reduce the number of channels porins; both attempts reduce the antibiotic pressure (25), as well as various ferocity that characterizes *P. aeruginosa* to increase resistance to various antibiotics, on the other hand resistance be relevant to the viability of the bacteria to secrete enzymes inhibitor to antibiotics such as β–*lactamase* which is characterized as grants resistance to a wide variety of β–lactam antibiotics, β–lactam that is a broad-spectrum and produced by the most gram negative bacteria coded by chromosomal DNA which could be transferred from chromosome to plasmids that leads to increase risk of resistance to this type of antibiotics (26 and 27).

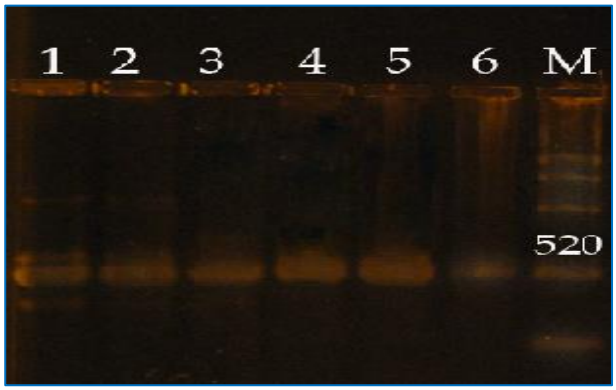
DNA PCR technique was used to identify and diagnose *P.aeruginosa* of clinical and sub-clinical cases through the amplification of *alg D* and *exo A* genes region. A total of 38 clinical isolates were collected and tested by the PCR method, there were 22 positive isolates were confirmed by PCR as *P. aeruginosa*. As observed in (Fig. 1) the size of *alg D* gene was 520 bp. The results of UTI showed that 6 samples were positive (60%) for having *alg D* gene and 8 isolates (57.14%) in wounds, while from were isolates 7 (70%) from burns, while the sputum samples showed only one positive case (25%) which was lowest ratio (Table, 7).

Our study agreed with (28), as in positive test ratio in determining gene *algD* in burns samples in Hillah teaching hospital (53.3%) where the findings were consistent with (29), being among the highest in the percentage of positive test to identify the *algD* gene in samples of UTI, which amounted to (95%). Other studies indicated that there were some limitations that had a role in regulating gene (*algD*). Alginate is the main factor in the development process shortly after the settlement of the bacteria *P. aeruginosa* from mucosal and non-mucosal strains; in addition to the increase in the copying process even under anaerobic conditions and may be considered one of determinant catalysts for the produce Alginate, which is one of the genes of

virulence factors which are responsible for the production of important enzymes in the production of (GDP-mannuronic acid), as well as the secretion of foreign multi sugars. Exopolysaccharaid is considered one of the important ingredients for the production of Alginate contributes to building a bio-membrane which in turn is one of the limitations to trimming of entering and mechanisms of action of a lot of antibiotics (30).

Table, 7: PCR results for *alg D* gene amplification in different clinical samples.

Samples	Positive specimens	No. of positive isolates for *algD* gene	Prevalence (%)
wound	14	8	57.14%
Burn	10	7	70%
UTI	10	6	60%
sputum	4	1	25%
Total	38	22	

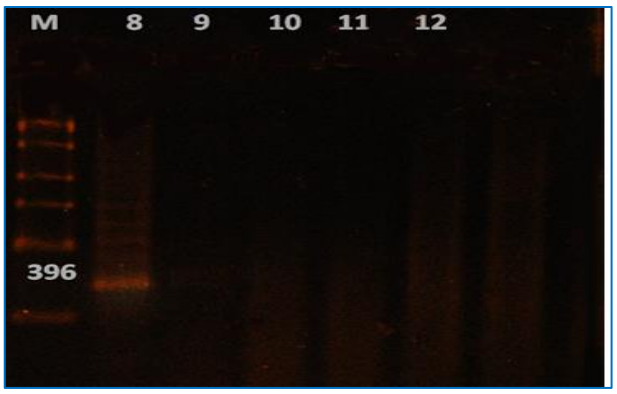


Figure, 1: Agarose gel electrophoresis of PCR products for *alg D* gene. Lanes M: marker ladder 100-bp. Lanes 1, 2, 3, 4, 5 and 6: *alg D* gene with 520 bp. Lane 9: negative control.

Results of amplification *exo A* gene for identification of *P. aeruginosa* showed only one isolate among the burn samples having a band with molecular weight 396 bp with no appearance of any bands in other clinical isolates (Table, 8) and (Fig. 2). *exo A* gene encodes for the production of exotoxins in *P.aeruginosa* which is one of the important virulence factors produced by that bacteria, these toxins inhibited biosynthesis of eukaryotic proteins by stopping of elongation factor for poly peptide and thus the effect is similar to the effect of diphtheria toxin (31). The results of this study agreed with the findings of the researcher (32), who found that there was only one isolate (2%) from 50 isolates of *P. aeruginosa* encodes the *exo A* gene for the exotoxins.

Table, 8: PCR results for *exo A* gene amplification in different clinical samples.

Samples	Positive specimens	No. of positive isolates for *exo A* gene	Prevalence (%)
wound	14	0	0%
Burn	10	1	10%
UTI	10	0	0%
sputum	4	0	0%
Total	38	1	



Figure, 2: Agarose gel electrophoresis of PCR products for exo A gene. Lanes M: marker ladder 100-bp. Lanes 8: exo A gene with 396 bp.

The reason for these results could be due to rearrangement of the regions upstream and downstream of *exo A* gene; this coincided with (33) who found that the *exo A* gene sequences rearranged between the strains of *P. aeruginosa*. The above diagnosis by PCR is better than diagnosis by conventional methods; as regards that sometimes on time and human life, as well as the presence of more virulent isolates and opportunism may threaten human life dramatically and influentially.

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دراسة بيولوجية وجزيئية لعترات من جراثيم الزائفة الزنجارية عزلت من حالات سريرية مختلفة في أربيل وكركوك

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الخلاصة

شملت هذه الدراسة عزل ١٢٥ من الجراثيم والتي عُزلت من حالات سريرية تضمنت (حروق، جروح، ادرار وقشع) في مستشفيات أربيل وكركوك، وبعد العزل شُخّصت الجراثيم بإجراء الاختبارات المجهرية والبايوكيميائية حيث عُزل ٣٨ عترة من جراثيم الزوائف الزنجارية. وأظهرت نتائج اختبار حساسية المضادات الحيوية أن جميع عزلات الزائفة الزنجارية كانت مقاومة للـ Erythromycin، Pipracillin بمعدل (١٠٠٪) والحمض Nalidixic بلغ (٩٤,٧٣٪) في حين كانت أدنى مقاومة للمضادات الحيوية Co-trimoxazole، Ceftazidime، Ciprofloxacin والتي بلغت (٢٦,٣١ و ٢٣,٦٨ و ٢١,٠٥٪) على التوالي. التشخيص الجزيئي لبعض جينات الضراوة في جراثيم الزائفة الزنجارية كان بتضخيم الجينات *alg D* و *exo A* بتطبيق تقنية تفاعل انزيم البلمرة المتسلسل. وأظهرت النتائج ان من بين ٣٨ عزلة فقط كان ٢٢ عزلة إيجابية لتلك الجراثيم، حيث شخص فيها الجين *alg D* والذي كان بوزن جزيئي ٥٢٠ bp. وأما في عينات عدوى المسالك البولية تبين أن ٦ (٦٠٪) تحتوي على الجين *alg D* و ٨ (٥٧,١٤٪) من الجروح، وأيضاً ٧ (٧٠٪) عزلة من الحروق، في حين أظهرت عينات القشع ان هناك عزلة واحدة من جراثيم الزائفة الزنجارية لها جين *alg D* والتي شكلت النسبة الأقل، ولكن في التضخيم من *exo A* ، أظهرت النتائج وجود عزلة واحدة من عينات الحروق تحتوي على هذا الجين وبوزن جزيئي ٣٩٦ bp التي لم تظهر أي حزمة في العينات السريرية الأخرى.

الكلمات المفتاحية: التشخيص الجزيئي، الزائفة الزنجارية، تفاعل انزيم البلمرة المتسلسل.