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Molecular detection of *exo*U and *exo*S among *Pseudomonas aeruginosa* isolates from Baghdad and Wasit, Iraq.

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Abstract: *The present study* aimed to Detection of *exo*U,*exo*S genes *Pseudomonas aeruginosa* isolates in Baghdad and Wasit,Iraq . 150 samples of *P. aeruginosa* were collected and identified by biochemical tests, to identify the isolates of *P. aeruginosa* at the genus and species levels by means of primers targeting *oprI* and *oprL* genes. and were characterized for antibiotic resistance. and the presence of *exo* genes was evaluated by allele-specific PCR (polymerase chain reaction). The *exo*U (60.31%), *exo*S (90.47%) genes were detected *P. aeruginosa* in different clinical specimens. Among 63 isolates of *P.aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; *exo*U; *exo*S ; different clinical.

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Introduction:

Pseudomonas aeruginosa is a leading cause of nosocomial infections. Most of these are acute infections, including sepsis, ventilator associated pneumonia (VAP), and infections in post-operative wound and burn patient. (1).Many strains of *P.aeruginosa* were at first isolated based on their ability to produce pyocyanin, their typical colony structure when grown on agar media, which is due to the production of aminoacetophenone (2).Because these bacteria can grow on most surfaces, including medical devices such as catheters, they are an important cause of nosocomial infections (3).*P.aeruginosa* tends to form biofilms, which are complex bacterial communities that adhere to a variety of surfaces, including metals, plastics, and medical implant materials, and tissues. Growth in biofilms promotes bacterial survival(4). *P.aeruginosa* are an important cause of corneal ulcers worldwide, with *P.aeruginosa* infection resulting in severe corneal opacity, ocular pain and visual impairment. One of the main causes of encomia infections, including pneumonia,urinary tract infections, surgical wound infections and bloodstream in fictions. Understanding bacterial virulence ,the host-pathogen relationship at the cellular and molecular level is essential to identify new targets and develop new strategies to fight infection (5). *P.aeruginosa* harbors at least one or more *exo*S, *exo*T, *exo*U and *exoY* genes that translated into protein products related to type III secretion systems (TTSS). *exo*S and *exo*T are bio functional enzymes that 75% amino acid identity and encode Gtpase-activating protein (GAP) and ADP-

ribosyl transferase (ADP-RT) activities. In addition to its ability to grow in biofilms, *P.aeruginosa* possesses other important virulence factors. e*xo*S transfer the ADP-ribose moiety from NAD+ to many eukaryotic cellular proteins. *P.aeruginosa exo*S is an adenosine diphosphate ribosyl transferase that is distinct from Pseudomonas toxin A(6).*e xo*U has phospholipase/ lysophospholipase activity and disrupts eukaryotic cell membranes after translocation into the cell by type III secretion systems(7). The aim of present study was to characterize the presence of the *exo*S and *exo*U genes in clinically isolated *P.aerugino*sa strains in Iraq.

Materials and Methods:

A total of 150 sample were collected from clinical different clinical specimenses, (63) isolated were isolates from patients' C.S.F(n=3), ear (n= 15), wounds (n=4), sputum (n=4),burn. (n=37). samples were collected from hospitalized patients during the period from October 2016 the end of January 2017. From hospitals in Baghdad including, Al-Kindi, Al-Yarmuk, in wasit including Al-Zahraa hospitals, Al-Karama, hospitals and Al-Kut center lab.The isolates were identified by means of routine tests: colony morphology and pigment formation on selective medium (cetrimide agar), catalase test, oxidase reaction, non glucosefermentation,growthat42°C, and identify by Api 20 E, VITEK 2, (8) (9).

Antimicrobial susceptibility:

The drug susceptibility test was carried out for all the isolateson Mueller-Hinton method were used to measure zones of inhibition in accordance with the recommendations of clinical and laboratory standards institute (CLSI, 2013). the test antibiotics were: (TIC): Ticarcillin (75µg), (AK): Amikacin (30µg), (GM): Gentamicin (10µg), (CIP): Ciprofloxacin (5µg), (CAZ): Ceftazidime (30µg), (ATM): Aztreonam (30 µg), (IMP): Imipenem (10 µg), (CO): Colistin (25µg).

Molecular detection:

DNA was extracted from activated pure culture of *P.aeruginosa* bacteria using Presto™ Minig DNA Bacteria Kit (geneaid). Detection of DNA bands using Agarose gelelectrophoresis (1%) (12). primer selection.

The primers used in this study are shown in Table (I). PCR amplification of I lipoprotein(*Opr*I) for the detection of *Pseudomonas* genus and L lipoprotein (*Opr*L) for the detection of *P. aeruginosa* species was performed on all phenotypically tested strains of *P. aeruginosa* and primers for *exo* U, *exo* S genes.

Table (1). The Sequence of Forward and Reverse Primers of *0pr*I,*opr*L, *exo*U and *exo*S Genes

Primer Name	Sequence 5' 3'	Product Size (bp)	Reference
*opr*I (F) oprI (R)	ATG AAC AAC GTT CTG AAA TTC TCT GCT CTT GCG GCT GGC TTT TTC CAG	249	13
*opr*L (F) *opr*L (R)	ATG GAA ATG CTG AAA TTC GGC CTT CTT CAG CTC GAC GCG ACG	504	13
*exo*U(F) *exo*U(R)	AGC GTT AGT GAC GTG CG GCG CAT GGC ATC GAG TAA CTG	1572	14
exoS (F) *exoS* (R)	TCA GGT ACC CGG CAT TCA CTA CGC GG TCA CTG CAG GTT CGT GAC GTC TTT TTA	565	14

PCR amplification:

All reaction mixtures were set up in a PCR room separate from that used for DNA extraction and amplification PCR was completedin adapted PCR micro centrifuge tubes according to the thermocycler used. Following optimization, reaction mixtures (20 µl) were set up as follows: 11µl deionizer water. Table(2). PCR master mix (Bioneer Corporation) ,(2µl F+R) of each set of primers (*Opr*L *orOpr*I) and 5 µl of DNA template .The reaction mixtures were subjected to the following empirically optimized thermal cycling parameters in a thermocycler (Senso-Quest Labcycler, Germany): 94°C for 5 min, followedby 35 cycles of 94°C for 40 sec, 57°C for 40 sec, 72°Cfor 50sec, and a final extension at 72°C for 4 min. Table(3).and reaction mixtures (20 µl) for *exo*U,exo S (Bioneer Corporation) and primer (1+1) and 5 µl of DNA template and 13 µl deionizer water.Table(4). PCR Program included initial denaturation at 94° C for 10 min, followed by 35 three-five step cycles, including denaturation at 94° C for 40sec, annealing at 62°C for 50sec, extension at 72°C for 55 secand a final extension at 72°C for 10 min.Table(5).Following amplification, aliquots (5µl) were removed from each reaction mixture and examined by means of electrophoresis (75 V,1.40 min) in gels composed of 2% agarose in TBE buffer (40 mM Tris, 20 mM boric acid,1 mM EDTA, pH 8.3). Gels were visualized under UV illumination by using a gel image analysis system (UVitec, Cambridge, United Kingdom) and all images were archived. oprI and *opr*L. and *exo*U,*e xo*S correct expected size.

Table (2): The mixture of conventional PCR working solution for detection of, *OPrI, OPrL* in genes in *Pseudomonas aeruginosa*

Component	Concentration	Volume (µl)
Deionizer water	-	11
Primer(F and R) *OPr*I	10 picomol	(1+1)
Primer (F and R) *OPr*L	10 picomol	(1+1)
DNA	ng	5
Total Volume		20 µl

Table (3): PCR program for *OPrI,OPrL* gene amplification by conventional methods

No.	Steps	Temperature (°C)	Time	Cycles
1.	Initial Denaturation	94	5min	1
2.	Denaturation	94	40 sec	35
3.	Annealing	57	40sec	
4.	Extension	72	50s	
5.	Final extension	72	4min	1

Table (4): The mixture of conventional PCR working solution for detection of,*exo*U,*exo*S genes in *Pseudomonas aeruginosa*

Component	Concentration	Volume (µl)
Deionizer water	-	13
Primer F.	10 picomol	1
Primer R.	10 picomol	1
DNA	ng	5
Total Volume		20 µl

Table (5): PCR program for *exo*U,*exo*S gene amplification

	Steps	Temperature (°C)	Time	Cycles
1.	Initial Denaturation	94	10min	1
2.	Denaturation	94	40s	35
3.	Annealing	62	50s	
4.	Extension	72	55 s	
5.	Final	72	10min	1

Results and Discussion:

A total of 150 sample of different clinical from October \ 2016 to January 2017, 63 isolates were collected from different specimens include wounds, burns, ear swabs, sputum and C.S.F. that admitted in five hospitals in Baghdad and Wasit .From both gender with age ranged from 3 year to 60 year. All colonies appear mucoid, smooth in shape with flat edges and elevated center, creamy color and have a fruity odor on cetrimide figure 1. In another hand all 63 isolates gave positive result for oxidase, catalase and did not produce H_2S.

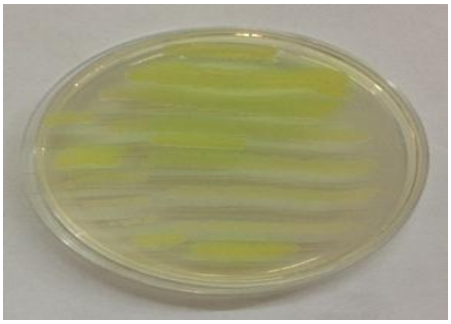

Figure (1): *Pseudomonas aeruginosa* culture on Cetrimide Agar total DNA was extracted using Presto Mini g DNA bacteria Kits with concentration 53ng and purity between 1.7- 1.9 then detected by gel electrophoresis as show in figure 2.

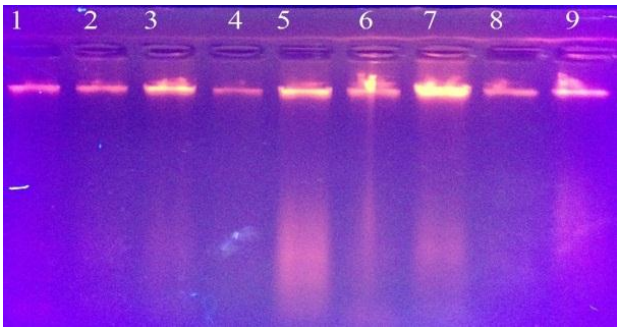

Figure (2): Gel electrophoresis of DNA extraction of *P. aeruginosa* isolates using 1% Agarose for 30 min. at 75 volt.

Multiplex PCR techniques were used for further conformational diagnosis of all (63) isolates of *Pseudomonas* bacteria, depending on species specific primers for the *Opr*I, *OPr*L genes, which are specific for diagnosis of *Pseudomonas aeruginosa* (249bp,504bp) respectively, which give same result when compare with biochemical test (API 20 system,vitek 2 system) which is specific for diagnosis of *P.aerugenosa*, product of multiplex

PCR detect by using gel electrophoresis as show in figure 3. Clear correlation was appeared between PCR techniques and culturing method, when all positive samples with culturing method give appositive amplification results with multiplex PCR. Detection of *P. aeruginosa* by PCR of *opr*I and *opr*L genes has a high sensitivity and provides results within few hours (15).

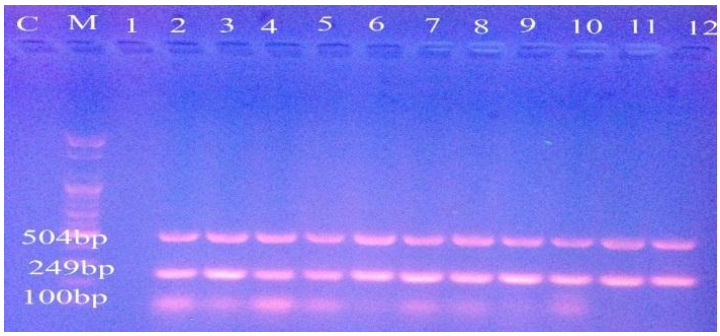

Figure (3): Agarose gel electrophoresis (2% agarose, 75 Vol / 1.45 hour) of multiplex PCR for identification *Pseudomonas* species, M: marker (100bp ladder), lanes (1, 2, 3, 5, 7,8,9,10,11,12) positive amplification of *Opr*I gene (249bp) and *OPr*L gene (504bp).

The resistance pattern to the 8 antimicrobials tested is shown in Table(6). According to the results, isolates had the lowest rate resistant to colistin. In the present study 1.08 % of the isolates were resistant to colistin, which shows that this antibiotic the last choice of therapy for these infections (16). The antibiotic resistant profile of isolates showed increasing resistance, especially in wound and ear isolates. *P. aeruginosa* isolates while sensitive for colistin (polymyxin E). This indicates the importance of antibiotic superintendence development and control of infection in hospital settings.(17).

Table (6): Percentages of antimicrobial susceptibility rate of 63 *P. aeruginosa* isolates against 8 antimicrobial agents.

Antibiotics	Resistant		Intermediate		Sensitive	
	N	%	N	%	N	%
Ticarcillin (Tic)	41	66	1	1.08	21	33.33
Gentamicin (GM)	30	48	2	30	31	49.20
Imipenem (Ipm)	24	38.09	3	5	36	57.14
Amikacin (AK)	22	35	3	5	38	60.31
Ceftazidime(CAZ)	20	32	1	1.08	42	67
Aztreonam (AZT)	18	29	24	38.09	21	33.33
Ciprofloxcin(CIP)	17	27	2	3.1	44	70
Colistin (CO)	1	1.08	-	-	62	98.41

Conventional PCR amplification were performed for *pseudomonas* in order to consolidate the presence of extracellular surface protein,*exo*U and *exo*S genes coding for different virulence factors by using specific primers for each one genes, the product of PCR detect by using gel electrophoresis as show in figure 4, 5 . The type III secretion toxin encoding gene patterns are shown in Table (7) 90.47 % of isolate carried *exoS* and 60.31% carried *exoU*, respectively.

Table (7): The number and percentage of *exo*U and *exo*S genes in *Pseudomonas.*

Genes	Total	percentage
***exo*S**	57	90.47%
***exo*U**	38	60.31%

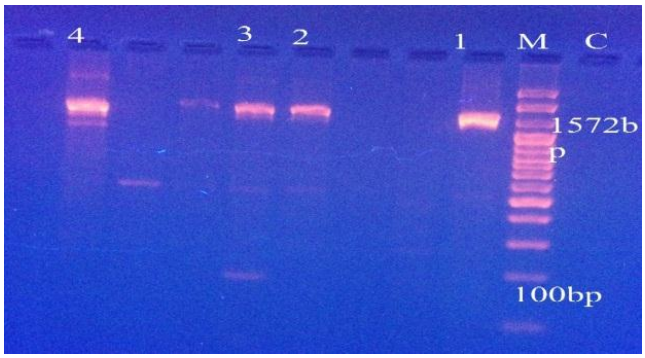

Figure (4): Agarose gel electrophoresis (2% agarose, 75 Vol / 1.45 hour) of conventional PCR amplification products of *Pseudomonas aeruginosa exo*Ugene (157bp).M: marker (100bp ladder).

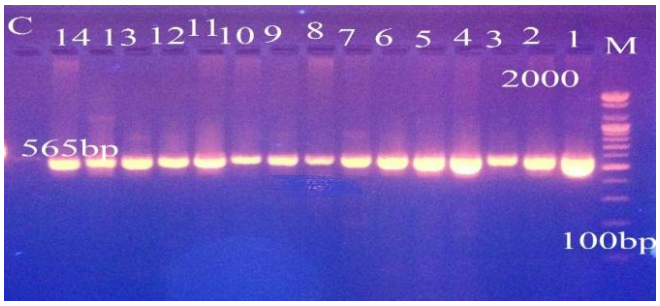

Figure (5): Agarose gel electrophoresis (2% agarose, 75 Vol / 1.45 hour) of conventional PCR amplification products of *Pseudomonas aeruginosa exo*S gene (565bp).M: marker (100bp ladder) .

P. aeruginosa secretes four known effector proteins via the type III secretion system: *exo*S, *exo*T, *exo*U, and *exo*Y (18). These proteins overrun host cell functions which are important in cytoskeletal organization and signal transduction. *Exo*S and *exo*T are bifunctional toxins exhibiting ADP-ribosyltransferase and GTPase-activating. (19). The plurality of *P. aeruginosa* strains carry *exo*U and *exo*S genes. Different frequencies of cytotoxin encoding genes, however, have been reported in different studies (20). Unlike other studies, that show high prevalence of *exo*U 60.31%.*exo*U but is found less than in other studies done in Romania (45%) (21).In a similar study in Egypt the rate of *exo*U was (61%). (22).in a study on isolates from Babylon, Iraq hospital infections, report the rate of *exo*U 86.6% (23).The results of the study of were different from our study, this difference may be due to the dissimilarity strains of bacterium or the disparity years. We findings vertify an association between secretion of *exo*U and high levels of cytotoxicity. These results indicate that *exo*U is a victorious cytotoxin of *P.aeruginosa*. Heterogeneity in the type III secretion among the clinical isolates of *P. aeruginosa*, all analyzed strains contain the genes for at least one effector proteins. The existence of type III secretion genes in clinical isolates is consistent with its important role in the virulence of *P. aeruginosa* and the

understanding of the specific benevolence of *exo*S,*e xo*U to the clinical score of the infectious process may have important implications for the therapeutic management of patients infected with *P. aeruginosa* (24).

In conclusion, findings of the present study showed different distribution of *exo* genes in clinical isolates of *P. aeruginosa* in Baghdad and Wasit,Iraq**.**Due to the advantages of molecular

Methods in the diagnosis of opportunistic pathogenic bacteria,it could be convenient and swift technique to prevent the progress of infections and mortality among these patients.

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