Molecular detection and Expression of some Virulence Genes in Pseudomonas aeruginosa Isolated from Wounds

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Abstract

Pseudomonas aeruginosa considered to be toxigenic and an invasive, that induces infections in abnormal host defenses patients. Besides, it is very important pathogenic nosocomial which produces infection in wounds, giving rise to blue-green pus. In this study 61 swabs were collected from wounds infection and cultured, the isolated bacteria have been identified by biochemical tests, and VITEK-2 compact System. Drug susceptibility tests for the 30 isolates of P. aeruginosa were done. Out of 30(49.2%) P. aeruginosa isolates were 20 (32.8%) Resistant isolates and 10 (16.4%) Sensitive isolates were found. The DNA has been isolated from 30 P. aeruginosa isolates and have been extracted by extraction kit of DNA. For 1µl of DNA ,199 µl of diluted Quanty Flour Dye was mixed. DNA concentration values has been estimated after 5min incubation at room temperature. In order to screening the virulence genes, Polymerase Chain Reaction has been utilized , (exoU, exoS, lasB) and resistance genes (ampC, oprM, oprN, rpoS). Result revealed that, isolates were positive for exoU gene 33.3%, exo S gene was 56%, lasB gene 93.3%, oprM and oprN genes were100%, ampC gene was 100% and *rpoS* gene were 100% in wound swabs. The technique of (RT-qPCR) was used for the transcription of the RNA to cDNA, followed by their amplification. The result showed that the exoU gene expression was increase (2.00,40.39) in resistance isolates when used Imipenem and Ciprofloxacin, respectively, compared with sensitive isolates. Expression of oprM gene increase (3.66) in resistance isolates while used Ciprofloxacin more than in sensitive isolates. Expression of lasB gene increase in resistance isolates when used Erythromycin (30.91) more than in sensitive isolates. While expression of oprM and rpoS genes, in sensitive isolates when used Gentamycin and Ciprofloxacin respectively, increased in able this bacterium to become resistant to those antibiotics compared with already resistance isolates to those antibiotics. In rpoS, oprM and ampC genes, fold change expression was reduced while treated isolates by Imipenem and Ceftazidime respectively. In addition, we found that tow genes (rpoS and exoS) have not contribution in Ciprofloxacin antibiotic resistance process.

Keywords: P. aeruginosa; wound infection; virulence genes (exoU, exoS, lasB), resistance genes (ampC, oprM, oprN, rpoS).

1. Introduction

pseudomonas aeruginosa continues to be one of the most virulent opportunistic pathogens (Akingbade et al., 2012). *P. aeruginosa* considered to be the main deriving cause of mortality and morbidity in immune-compromised patients being hospitalized (Moustafa et al., 2021)

One of health problems that are resulted from the invasion of pathogenic organisms in different origan of body is wound infection, and it threat life of large number of people in many countries (Akingbade et al., 2012). *P. aeruginosa* infection is difficult to be eradicated due to its highly resistance as well as their ability to acquire resistance to different antibiotics (Breidenstein et al., 2011)

In hospitals, *P. aeruginosa* is a matter of concern due to its frequently emerging of strains that classified as new strain having specific antibiotic resistant implicated in nosocomial infections. There are a complex network of gene expression regulatory mechanisms are contributing to lowered susceptibility of *P. aeruginosa* to many different classes of antibiotics. *P. aeruginosa* classified of having a variety of virulence factors contributing to its pathogenicity (Firme et al., 2010).

The *exoU* and *exoS* genes were type-III secretion system (T3SS). It is considered as to be an important factor leading to poor clinical outcome of *P. aeruginosa* infections. The *exoU* gene codifying for highly cytotoxic exoenzyme *exoU* (Holban et al., 2013a).

Exoenzyme *S* is important for the pathogenic activity of *P. arginase*(Habibollahi et al., 2015), it has antiphagocytic activities which have virulence factors that induce apoptosis in target host cells and its bleb niches occupation in the epithelial cells, but it is required for intracellular survival of *P. aeruginosa* (Kroken et al., 2018) .*P. aeruginosa* produces elastase *lasB* during biofilm formation,(Galle et al., 2013) which can influence properties of biofilm (Roshani-Asl et al., 2018).The *MexEF-OprN*, and *MexXY-OprM* are kinds of efflux pumps correlated with antimicrobial resistance *P. aeruginosa* (Fujiwara

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et al., 2022);(Horcajada et al., 2019); (Wang et al., 2010). The substrates of *MexXY*(*OprM*) include antibiotic classes fluoroquinolones, β-lactams, tetracyclines, erythromycin ,macrolides and chloramphenicol (Fujiwara et al., 2022) ; (Lister et al., 2009); (Pesingi et al., 2019); (Li et al., 2015) . *AmpC*-β-lactamases production of bacterial pathogens may lead to a major failure of therapy if not diagnosed and reported well advance in time, which could lead to uncontrolled spread of bacterial resistance and difficult to manage clinical concern. (Parveen R. et al., 2010); (Sageerabanoo et al., 2015)

The *rpoS* (RNA polymerase, sigma S) gene codes for sigma factor *RpoS* in P. aeruginosa strains. Sigma factors are the proteins regulating the transcription in bacteria and the central controller of the general stress response operating in both manners (retroactive and proactive) (Sedighi et al., 2015)

In order to detected the virulence factors among the isolates by PCR, it is required to discover the link between special virulence factors and manifestation of *P. aeruginosa* infections, (Aslani et al., 2012). Real-time PCR (quantitative PCR, qPCR) is considered as a well-established method to detect, quantification, and typing of wide range of microbial agents in the areas of clinical, veterinary diagnostics and safety of food.(Kralik and Ricchi, 2017).Real Time-PCR is utilized to detect qualitatively the expression of the gene by creation of complementary transcripts of DNA (cDNA) from RNA .(Maxwell Mackay, 2007).In Real Time -PCR, first convert RNA template into a complementary DNA (cDNA) by using a reverse transcriptase. Then, cDNA is used as a template for exponentially amplification by using PCR. currently the most sensitive method to detect RNA is known as real-time quantitative nucleic acid -based amplification- QT-NASBA (Schmittgen et al., 2000)

The present study aims to Isolate *P. aeruginosa* from wound infection and diagnosis by chemical tests and confirm by VITEK2 system. Detection the resistant isolates of *P. aeruginosa* for different antibiotics by using VITEK2 system. Extraction the DNA from the *P. aeruginosa* isolates. Detection of virulence (*exoU*, *exoS*, *lasB*) and resistance (*ampC, oprM, oprN, rpoS*) genes in *P. aeruginosa* isolates by PCR technique. Moreover, the gene expression of the resistant gens was detected using RT-qPCR technique.

2. Materials and Methods

2.1. Isolation, Identification and Susceptibility Test of P. aeruginosa

In this study 61 swabs were collected from surgical wound infection patients from different ages, under sterile conditions. samples have been cultured all on nutrient agar, MacConkey agar, Blood agar, Cetrimide agar on HiFluore *Pseudomonas* Agar Base (used as a medium selection process of *P. aeruginosa* isolation from sputum, pus, and drains etc).Out of 61 30(49.2%) were *P. aeruginosa* isolated from wound infection swabs .The identification confirmed by biochemical tests (oxidase, catalase, motility, IMVIC) (Holt et al., 1994) and VITEK-2 System compact ID GNB cards was used to prove a final identification of *P. aeruginosa*. Antibiotic sensitivity done by AST-GN, No. 222 cards with VITEK-2 System compact. Antimicrobial susceptibility test for erythromycin done by using antibiotic disks on Mueller Hinton agar plates when using disk diffusion method

2.2. Extraction of DNA

The DNA of 30 isolates has been extracted from colonies identified as *P. aeruginosa* to provide a PCR templet, by using The Bacterial Genomic DNA Extraction kit provided by Alpha Company (USA). The concentration and purity were detected by Quantus Fluorometer. For 1 µl of DNA ,199 µl of diluted Quanty Flour Dye was mixed. The quantities and purity of DNA that have been already obtained were fair enough for PCR amplification . when the DNA samples analyzed by gel electrophoresis, results were observed, in which DNA bands have been detected to indicate the purified DNA samples.

2.2.1. Preparation of Primer and Detection of the Virulence Factors Genes

Primers were prepared according to the instructions of Manufacturer Company (Alpha DNA, USA) in lyophilized form. The primers choose (Table 1). Lyophilized primers first dissolved in a nuclease free water to get stock solution with final concentration of 100 mol/ µl. In order to prepare a working solution of these primers, 10 µl of primer stock solution (stored at freezer -20C) was added to 90 µl of nuclease free water to get a working primer solution of 10 pmol/ µl.

Table (1) The primer sequence and product of virulence and resistant genes

Genes	Primer sequence (5'–3')	product	Annealing temperature	Reference
exoU-F exoU-R	5-'GCCCTTTTGGCCTCAGGTAT-3' 5'-TGGATGCGTAGCGATCTGTC-3'	285	60 °C	This study
exoS-F exoS-R	5'-TTGAAGGGACTCGACAAGGC-3' 5'-GCCGATACTCTGCTGACCTC-3'	359		
lasB-F lasB-R	5'-ACCTACAAGCAGGTCAACGG-3' 5'-GTTCATTCCGCCTGATTGCC-3'	306		
ampC-F ampC-R	5'-CGATACCAGATTCCCCTGCC-3' 5'-GTGAAGGTCTTGCTCACCGA-3'	282		
oprM-F oprM-R	5'-TCAACCTGCCGATCTTCACC-3' 5'-GAGCTGGTAGTACTCGTCGC-3'	209		
oprN-F oprN-R	5'-TTCGAAAGCCTGTGGTGGAA-3' 5'-GAACAGGTCAAGCTCCCAGG-3'	276		
rpoS-F rpoS-R	5'-TCACCCGAAGAAATCGCCAA-3' 5'-CTTGTCGGTGAGTTCCGTCA-3'	228		

No. of reaction	21	rxn	Annealing Temp. of primer	30
Reaction Volume /run	20	µl	No. of primers	7
Safety Margin	5	%	No. of PCR Cycle	30

2.2.2. Detection of the virulence factors genes Reaction setup and Thermal Cycling protocol: Calculation of PCR-Component

Master mix component	Stock	Unit	Final	Unit	Volume	
					1 sample	21.05Samples
Master Mix	2	x	1	x	10	210.5
Forwarded primer	10	µM	1	µM	1	21.1
Reverse primer	10	µM	1	µM	1	21.1
Nuclease free water					7.5	157.9
DNA		ng /µl		ng /µl	0.5	
Total volume					20	
Aliquot per single rxn	19.5 µl of master mix per tube and add				0.5 µl of template	

Detection of virulence genes (*exoU*, *exoS*, *lasB*) and the resistant genes (*ampC*, *oprM*, *oprN*, *rpoS*)were performed by amplifying the genes by monoplex PCR, the PCR thermocycler program (Table 2).

Table 2. PCR thermocycler program for DNA amplification of *P. aeruginosa* genes.			
Steps	^{O}C	M:S	cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	10	10:00	

2.3. Gene expression

2.3.1. Preparation of control broth

Selected single colony of *P. aeruginosa* from certrimide agar and cultured on Luria Bertani (LB) broth and incubated at 37 °C for 24 h.

2.3.2. Preparation antibiotic broth

Single colony of *P. aeruginosa* was selected from cetrimide agar and cultured on L.B broth. The powder antibiotic was dissolved in1 ml normal saline. 0.1 ml of the dilution antibiotic was added to the cultured colony in the LB broth, for transformation. The Table (3) shows types of the antibiotics used in the gene expression. MIC can be given with drug having the lowest concentration, i.e., reduces, by more than 50% or 90% for MIC50 and MIC90, respectively.

2.3.3.RNA extraction

According the instruction of the Promega kit, RNA of Four (2 sensitive and 2 resistant) *P. aeruginosa* isolates was extracted, obtained from wounds were chosen which contain the (4) resistance genes (*ampC*, *oprM*, *oprN*, *rpoS*) and (3) virulence genes (*exoU*, *exoS*, *lasB*),

Table 3. The antibiotic powder and solutions were used in the experimental study		
No.	Antibiotic Groups	The antibiotics
1	Cephalosporins	Ceftazidime
2	Carbapenem	Imipenem
3	Quinolone	Ciprofloxacin
4	aminoglycoside	Gentamycin
5	macrolide	Erythromycin

2.3.4. Gene expression analysis

Each virulence factors gene (*exoU*, *exoS*, *lasB*, *ampC*, *oprM*, *oprN*, *rpoS*) were tested for its ability to expressed using RT-qPCR technique. The expression values were calibrated with *fbp* gene which consisted of housekeeping gene. In this work, one step RT-qPCR had been used, RNA was transcript directly to cDNA before PCR beginning, material that had been used and their volumes and the Amplification steps of the gene expression were described in the Tables 4 and 5 Folding values were determined using ΔΔCt model, which known also as Livak equilibrium

Table 4. Materials and volumes of RT-qPCR technique.					
Master mix components	Stock	Unit	Final	Unit	Volume 1 Sample
qPCR Master Mix	2	X	1	X	5
RT mix	50	x	1	x	0.25
MgCl2					0.25
Forward primer	10	µM	1	µM	0.5
Reverse primer	10	µM	1	µM	0.5
Nuclease Free Water					1.5
RNA		ng/µl		ng/µl	2
Total volume					10
Aliquot per single rxn	8µl of Master mix per tube and add 2µl of Template				

Table 5. Amplification steps of the gene expression of RT-qPCR technique.			
Real Time PCR Program			
Steps	°C	m: s	Cycle
RT. Enzyme Activation	37	15:00	1
Initial Denaturation	95	05:00	
Denaturation	95	00:20	40
Annealing	58,60	00:20	
Extension	72	00:20	

Gene Expression Analysis using Pfaffi Method

Quantification (Relative)

Folding = (2-ΔΔCT)
ΔΔCT = (ΔCT Treated or Control- ΔCT Control)
ΔCT = (CT gene - CT House Keeping gene)

2.3.5. Results and Discussion

In present study, out of 61 wound infections swabs, 30(49.2%) *P. aeruginosa* isolated from swabs, it was 20(32.8%) resistance bacteria isolated from inpatient and 10(16.4%) sensitive bacteria isolated from outpatient were found. Study by (Ranjan et al., 2010) founded that *P. aeruginosa* was the most common isolated pathogens from postoperative wounds another study in Iraq by Alwan *et al.* (28) detected that *P. aeruginosa* the most common isolate from burns and wounds

P. aeruginosa colonies, on blood agar produce a clear zone due to produce β-hemolysis(MacFaddin, 2000). On MacCkonky agar medium the appearance of bacterial colonies was pale for it does not ferment lactose(Brook FG, Beutel SJ, Carroll CK, 2007) Cetrimide agar the bacteria produce pyocyanin and fluorescein pigments, so it selective for *P. aeruginosa*. The positive isolates produce pyocyanin pigment (blue or green) on Cetrimide agar (Tang & Stratton, 2014).On HiFluore *Pseudomonas* Agar Base, the fluorogenic compound would be broken by *P. aeruginosa* in order to release the fluorogen which produces a visible fluorescence, yellow-green and fluorescent pigment production under long wave UV light as show in instruction of (HiFluoro™ Pseudomonas Agar Base)*. All isolates of *P. aeruginosa* and samples contain *P. aeruginosa* grow on this media. The identification of *P. aeruginosa* was confirmed by using VITEK-2 compact System. Determination of Antibiotic sensitivity done by VITEK-2 compact System. The isolates of *P. aeruginosa* showed different percentage of resistance to each antibiotic of the Cephalosporin group: Ceftazidime 66.7% (20/30). Carbapenem group: Imipenem 53.3% (16/30). Aminoglycoside group; Gentamycin 53.3(16/30). Quinolone group: Ciprofloxacin 63.3 % (19/30). macrolide Groups; Erythromycin 100% all isolates were resist. According to the percentage of Ceftazidime (66.7%) our results closed to results of (Khudair & Mahmood, 2021)the percentage was 68% and (Safaei et al., 2017) the percentage was 60.4%. while in study by(Jawad, 2016) the percentage was100%. The percentage of sensitivity to Imipenem 53.3% was closed to results of Study in Iraq by(Jasim & Sweedan, 2022) percentage of resistance against Imipenem was 60%. In 2022 resistant percentage by(Saki et al., 2022) was 68.1%. While in anther result by (Maclean et al., 2022) the percentage was 100%. (49.5 %). The result of (Ra'oof, 2011) here the isolates were isolated from (burn, wound, otitis media, urinary tract infection), which rate was 75%, while another study by(Yolbaş, 2013) the bacteria isolated from burn wounds and the percentage rate was 45 %, Ciprofloxacin resistance percentages was 63.3 %. Study by(Kireçci, and Kareem2014.) came to a conclusion that resistant percentage was (73.3%). The (VinodKumar et al., 2011)[45] found out that, the *P. aeruginosa* percentage resistant of isolate from Pus samples of diabetic foot was (77.8%). while the percentage of (Jawad, 2016) [46] for Ciprofloxacin were 44.4% from Clinical isolates and 63.6% from Environmental isolates, also anther studies in Iraq by (AL-Shamaa, Noor F. K., Rasmia A. Abu- Risha, 2016a) the percentage was 39 %. Percentage of Erythromycin 100%, result was close with (Azeez & Bakr, 2019), percentage was 96%.

PCR was used for screening the resistant genes (*ampC, oprM, oprN, rpoS*)) and the virulence genes (*exoU, exoS, lasB*) to the thirty of *P. aeruginosa* isolates, and the result showed in table(6) ,figures 1 (a-b-c-d-e-f-g)

Table (6): shows the distribution and percentage of resistance and virulence genes in *P.aerugsinoa* isolates		
Genes	Sources of isolates	
	Wound (30 isolates)	
	Positive	Negative
exoU	10(33.3%)	20(66.7%)
exoS	17(56%)	13(43%)
lasB	28(93%)	2(6.7%)
ampC	30(100%)	
oprM	30(100%)	
oprN	30(100%)	
rpoS	30(100%)	

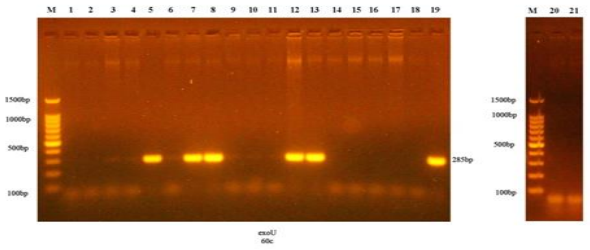

Figure (1-a): Amplification results of exoU primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 285bp

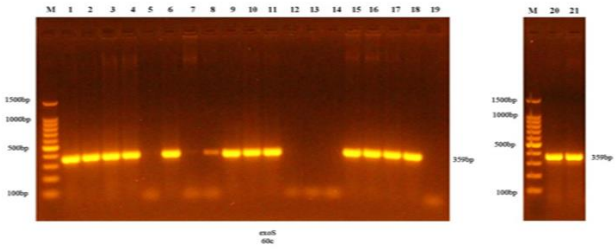

Figure (1-b): Amplification results of exoS primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 359bp

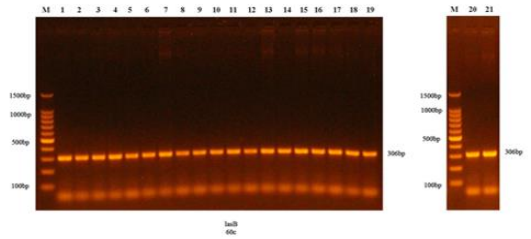

Figure (1-c): Amplification results of lasB primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 306bp

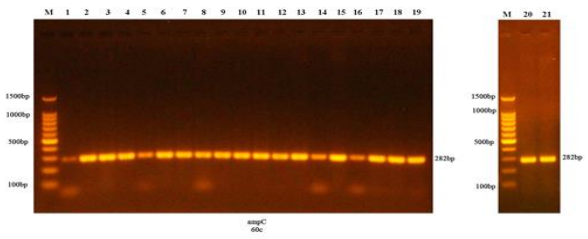

Figure (1-d): Amplification results of ampC primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 282bp

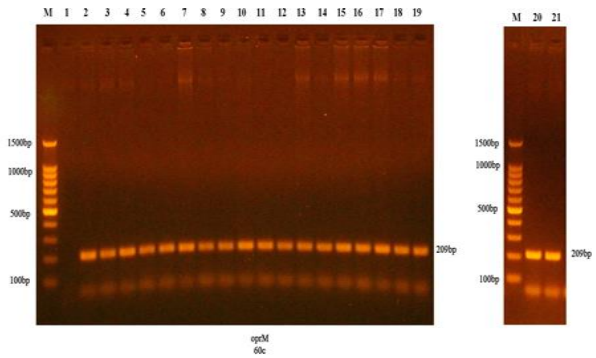

Figure (1-e): Amplification results of oprM primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 209bp

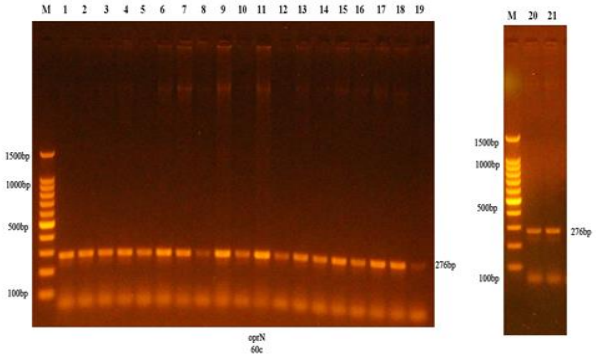

Figure (1-f): Amplification results of oprN primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 276bp

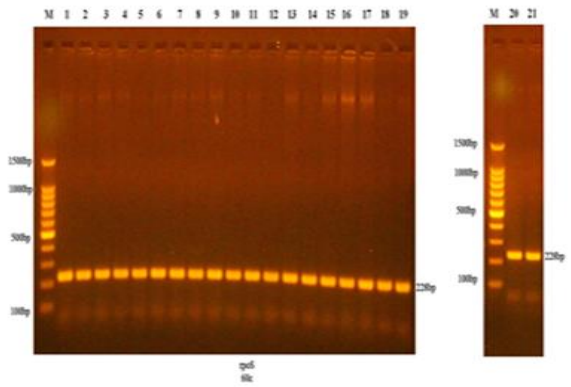

Figure (1-g): Amplification results of rpoS primers of Pseudomonas aeruginosa samples fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker, Lane 1-29 product size 228b

(Aslani et al., 2012)[49] reported in his study that the different levels of intrinsic virulence and pathogenicity of *P. aeruginosa* isolates determination of different virulence genes was associated with the distribution's differences of factor of virulence genes in the populations enhance the probability of *P. aeruginosa* strains to best adapted to the conditions specifically found in certain infectious sites.

Our results of PCR show that the percentage of *exoU* gene 33.3%. The positive result of our study has same percentage with study in Iraq by(AL-Shamaa, Noor F. K., Rasmia A. Abu- Risha, 2016b) , percentage was 33.3%. Research by (Bahador et al., 2019) found close to our result, percentage was 38.6% which isolates from different clinical samples such as urine, wounds, respiratory tract, blood, sputum, eye. In Iraq study by (Ahmed Attiah et al., 2021)the percentage was 60% for burns and 40% for wound. The divergency of *exoU* presents in many clinical isolates may be because of the same source isolates were associated with patients having different clinical cases and hospitalization duration with many sources of infection, *exoU* has mutable trait and existing in different prevalence among P. arginase strain (Gawish, 2013)

Our result percentage of *exoS* gene was 56%, this percentage was close to (Tahmasebi et al., 2022) showed that the isolates from wound was 60%. Study of (Gawish, 2013) found different percentage of *exoS*, was 40% and the study by (Azimi et al., 2016) showed the isolates from wound was 26.3%. Since, exoenzyme S gene is not appears in all strains of P. aeruginosa, Thus, not all strains have the capablity to synthesize this virulence factor. The reduced virulence of multidrug-resistant P. aeruginosa strains is more likely caused by gene expression regulation, and not by the absence of virulence gene (Bogiel et al., 2017). Our results indicate that *exoS* gene higher then *exoU* gene in *P. aeruginosa* isolates and this agree with(AL-Mayyahi et al., 2018) and (Al-saadi, 2012), they found *exoS* gene detected in most isolates while *exoU* gene has been detected in minority of the isolates.

The PCR-Positive result of our study to las*B* gene was 93.3%, Study by (Elmouaden et al., 2019) showed that the isolates of *P. aeruginosa* harbored las*B* gene and the percentage was 98.7%. While in anther studies by (Al-Shwaikh & Al-Arnawtee, 2019)found the percentage was 100% close to our result. The PCR result for *lasB* gene suggested that the production of elastase is important in all types of *P. aeruginosa* infections as reported in study in Iraq by(Ra'oof, 2011) .(Tielen et al., 2010) mention that directly or indirectly, *lasB* can influence the formation and architecture of *P. aeruginosa* biofilms

Our results of positive -PCR for *oprM and oprN* genes showed different percentage from others studies, results of *oprM* gene was 30(100%) found in all isolates. Study by (Llanes et al., 2011)found different results, in case of *OprM* gene was 45.8% (39 out of 85) and *OprN* gene was 11.8% (10 out of 85). This difference in result may be attributed to size and source of samples, environment difference, random use of antibiotics which had a significant role in the development of resistant genes. The (Davies, 1996) mention that extreme microbes genetic capacities have taken the advantage of man's overuse of antibiotics exploiting every source of resistance gene and every means of horizontally transmission of gene in order to develop different resistance mechanisms for each and every antibiotic that has been introduced clinically and agriculturally into practice. The presence of gene does not always mean its expression but it depends on the environmental conditions (Rafiee et al., 2014)

Our positive-PCR result of *ampC* gene was 30(100%). these results agree with study that has been conducted in Iran by (Fazeli et al., 2015) [93], reported that, 100% *ampC* gene carriage among *P. aeruginosa* 72 isolates taken from many different clinical specimens. However, it did not show the *ampC* phenotype. Other studies, by (Zhu et al., 2013) found near our results, the percentage was 93.3%. Organisms having the potential for *ampC* production play an important role in management of the decisions. (Tamma et al., 2019).Resistance of *P. aeruginosa* to β-lactam antibiotics could be the result of resistance attained during over production and mutation of different antibiotic of inactivating enzymes (Rafiee et al., 2014)64

Current study shows results of positive- PCR to *rpoS* gene were 100%. According to literature survey, no much offer has been done in isolation of this gene from wound Most of researches has been conducted by the different methods. future work is required to isolate this gene from different sources and further analysis is required of those genes along with the effects of its expression on *P. aeruginosa* antibiotics resistance. There for the result of this study of this gene will be beneficial to future studies.

A comparative Study of presence the resistance and virulence genes between inpatients and outpatients' isolates in wound infection isolates were done and shows in Table (7), Figure (2)

Table (7) Shows comparison of presence the resistance and virulence genes between inpatients-vs- outpatients' isolates in case of wound infection isolates

% Of presence of resistance genes			
Gene	Inpatients	Outpatients	
ampC	100.00	100.00	0%
exoS	65.00	40.00	+25%
exoU	30.00	40.00	-10%
lasb	90.00	100.00	-10%
oprM	100.00	100.00	0%
oprN	100.00	100.00	0%
rpoS	100.00	100.00	0%

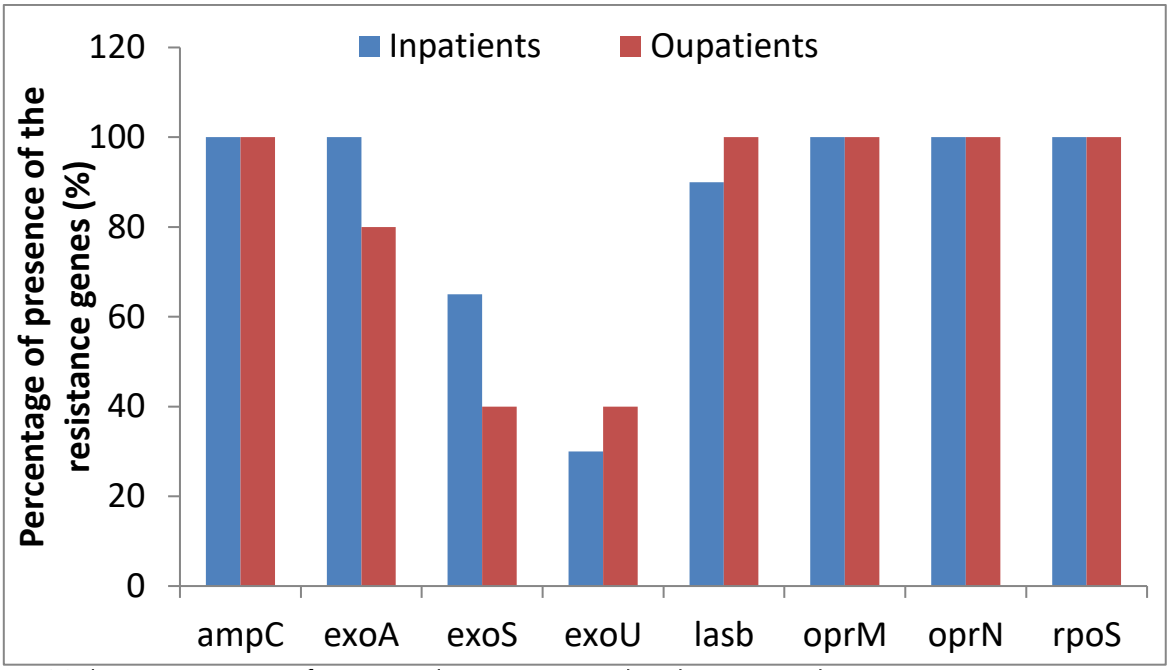

Figure (2) shows comparison of presence the resistance and virulence genes between inpatients-vs- outpatients' isolates in case of Wound isolates

In case of wound infection isolates the *ampC, oprM, oprN* and *rpoS* have same percentages (100%) in inpatients and outpatients, *exoS* was higher in Inpatients (65%) then Outpatients (40%), They spreading widely inside the hospital than the case outside the hospital. While *exoU* and *lasB* were higher in Outpatients (40%,100%) then inpatient (30%, 90%), respectively, this means that *exoU* and *lasB* are spreading widely outside the hospital than the case inside the hospital

There were differences in the virulence genes profiles of strains isolated from different clinical origins, correlating between clinical outcome of infection virulence patterns would be useful for hospitalized patients therapeutic procedures having positive cultures of *P. aeruginosa* (Holban et al., 2013). some virulence genes and source infections significant correlations indicate the implementation of infection control measures that helps in controlling the dissemination of virulence genes among isolates of *P. aeruginosa* as reported by study in Iraq of (Mona S Nour & ElSheshtawy, 2015)

For study the gene expression of the resistant genes (*ampC, oprM, oprN, rpoS*) and virulence genes (*exoU, exoS, lasB)*, the RNA of the Four of *P. aeruginosa* isolated (2 resistance and 2 sensitive isolates) obtained from wounds were chosen which contain the (4) resistance genes and (3) virulence genes ,then the RNA of this isolates was extracted by using (RNA extraction kit), the concentration of RNA samples has been measured while using the Quantus Florometer with concentration be in the range of 10-20 ng . Relative quantification expression ratios of virulence factors genes in RNA samples were measured in comparison with the housekeeping gene, *fbp* as a reference gene, using Reveres transcription-quantitative PCR (RT-q PCR) technique, Figure (3).Onther studies used same housekeeping gene *fbp*, the study by (Gifford et al., 2016) and (Chakravarty et al., 2022) . also used the *fbp* gene, mention that the Samples were normalized to the *fbp* transcript.

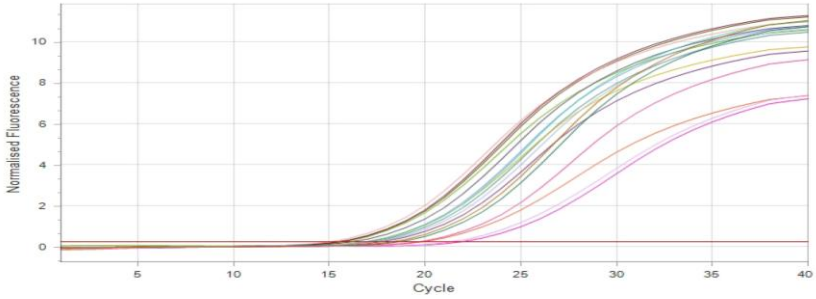

Figure (3): The amplified curve and Ct of housekeeping genes

The folding value (gene expression) for each of the resistance and virulence genes were determined as depending on the mathematical models are the (ΔΔCT model) by using Pfaffi Method.

Several procedures of data analysis have been developed as relative quantification is considered to be the goal for many RT-PCR experimentations. Mathematical models have been applied widely : the ΔΔCt model, (Livak & Schmittgen, 2001) . The (Ct) -threshold cycle values – have been determined automatically in each reaction. These (Ct)-values have been defined as the number of cycles at which the generation of fluorescence took place by the released reporter dye molecule that exceeds a fixed baseline threshold value (Michael WP, Graham WH, 2002). In current study we focused on using the most available antibiotic.: Ceftazidime, Imipenem, Ciprofloxacin, Gentamycin and Erythromycin in gene expression which show in Table (3). The reason to choosing these antimicrobials was their wide use as antipseudomonal agents(Tartor & El-Naenaeey, 2016)

Fold change of resistance genes expression challenged to antibiotics compared to non-challenged(control) to antibiotics among sensitive versus resistant isolates was show in figure (4).

Figure (4) shows fold change of resistance and virulence genes expression challenged to antibiotics compared to non-challenged to antibiotics among sensitive versus resistant isolates.

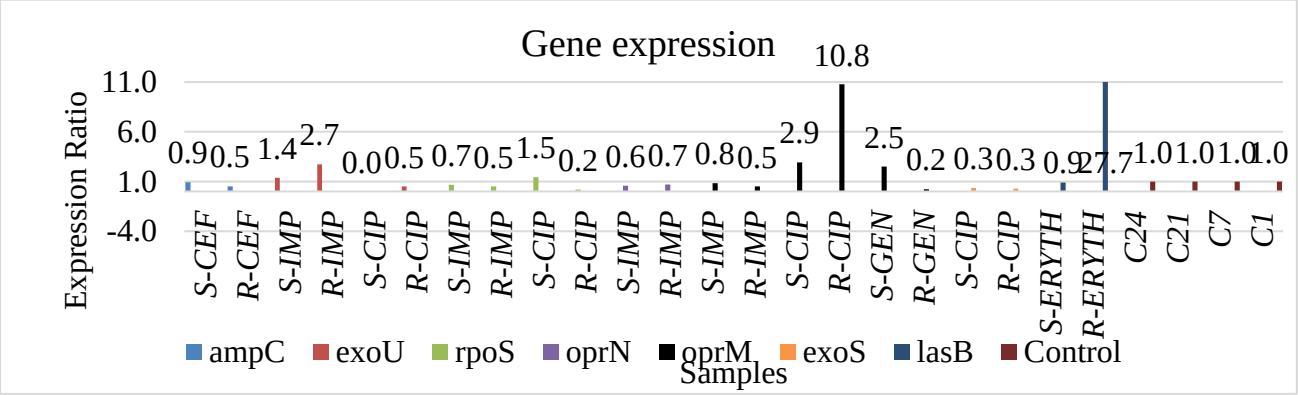

Figure 4: Show Fold change of resistance and virulence genes expression between resistant and sensitive isolates (S-sensitive and R- resistance isolated, C-control)

The folding rate (the gene expression) of the *exoU* gene in resistance (2.7) and sensitive (1.4) isolates in case of used imipenem, expression of *exoU* was increase in both isolates and consider as a risk factors. While in case of Ciprofloxacin was not significant (reduce), in case of *exoU* and *exoS* gene expression, The reason that could not provide the condition in vitro like in vivo. Study by (Ferguson et al., 2001) mentioned that in certain clinical settings, the lower frequency of expression of *exoS* ,related to the loss of the expression of gene rather than to its limited acquisition .In addition to that, the differences in *exoS* production by these isolates has been occurred at the level of gene expression rather than secretion. The *exoS* production is independently regulated from the rate of bacteria growth or protein levels of secretion during the growth of bacteria in exoS induction. *ExoS* expression is under the control of a transcriptional activator (Hovey & Frank, 1995).

Expression of *rpoS* gene in resistance isolates was reduced (0.2) when used Ciprofloxacin, while increase with sensitive isolates (1.5) trying to resists this antibiotic but failed to resist and consider as a risk factor. In case of used imipenem was not significant (reduced).

The gene expression of *oprM* increase while used Ciprofloxacin in resistance (10.8) and sensitive (2.9) isolates. And when used Gentamycin the sensitive isolates increase expression of *oprM* gene (2.5), bacteria trying to resist antibiotic and consider as a risk factors. while in case of used imipenem was not significant (reduced) in case of *oprM* and *oprN* expression.

Expression of *lasB* gene in resistance isolates was increased (27.7) when used Erythromycin, while in case of sensitive isolates reduce expression, *lasB* has no great import adjective in sensitive isolates. Study in Iraq (Ra'oof, 2011) showed that, *lasB* expression may be related to infection site which depends on the availability of their substrates.

There was no great import adjective in expression of *ampC* when used Ceftazidime. However, under normal circumstances, that may be, only a small amount of *ampC* would be produced if no inducer effect exists. However, the use of a large amount of third-generation cephalosporin, as an inducer effect, can greatly increase its expression level, which gives an indication that the abuse useage of antibiotics in clinical practice will result in the widespread prevalence of the AmpC enzyme *P. aeruginosa* strain. (Drawz & Bonomo, 2010)

Fold change of resist genes expression in resistance isolates compared to sensitive isolates in challenge to the antibiotics under investigation in this study were shown in Table (8) and Figure (5)

Table (8): Fold change in resistance genes expression in resistant isolates compared to sensitive isolates in challenge to the studied antibiotics

resistance and virulence genes from isolated treated with antibiotics	Fold change
CEF-ampC	0.56
IMP-exoU	2.00
CIP-exoU	40.39
IMP-rpoS	0.75
CIP-rpoS	0.12
IMP-oprN	1.21
IMP-oprM	0.62
CIP-oprM	3.66
GEN-oprM	0.09
CIP-exoS	1.00
ERYTH-lasB	30.91

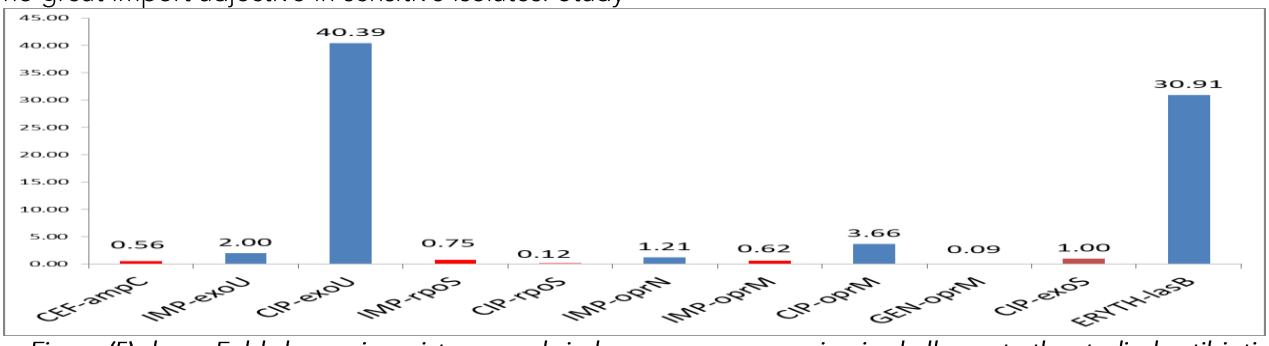

Figure (5) shows Fold change in resistance and virulence genes expression in challenge to the studied antibiotics

exoU gene was increase expression (2.00,40.39) in resistance isolates when used Imipenem and Ciprofloxacin, respectively, compared with sensitive isolates. That means increase expiration of *exoU* while used this antibiotic.

Increase expression of *oprM* gene (3.66) in in resistance isolates while used Ciprofloxacin more than expiration of this gene in sensitive isolates.

Expression of *lasB* gene increase in resistance isolates when used Erythromycin (30.91) more than expression of this gene in sensitive isolates.

While fold change expression of *oprM* and *rpoS* genes, in sensitive isolates when used Gentamycin and Ciprofloxacin respectively, increased in able this bacterium to become resistant to those antibiotics compared with already resistance isolates to those antibiotics.

In *rpoS, oprM* and *ampC* genes, fold change expression was reduced while treated isolates by Imipenem and Ceftazidime respectively.

In addition, we found that tow genes (*rpoS* and *exoS*) have not contribution in Ciprofloxacin antibiotic resistance process.

Studies mention that, the prevalence of the *exoU*

gene among carbapenem and fluoroquinolone non-susceptible strains was significantly higher than that among susceptible strains. Although this analysis was limited to the carbapenem and fluoroquinolone non-susceptible strains.(Agnello & Wong-Beringer, 2012);(Sullivan et al., 2014)

The *rpoS* gene which is a genes of stress response global regulator that pertaining to growth stationary phase, tolerance of antibiotic and osmotic stress, that have been significantly enriched in genes sets that were induced in the biofilm. It is considered the heat shock protein that has been involved in peptide folding and, it is ,therefore, a potential contributor to the resistance of antibiotic (Firme et al., 2010) ;(Stewart et al., 2015) . Study by (Firme et al. 2010) suggests that *RpoS*-regulated genes (i.e. *dnaK*) were participated in the tolerance of antibiotic, but the global resistance to antibiotics was mediated by gene regulatory complex networks that may not be dependent entirely on *RpoS* and they mention that no significant antibiotic resistance to ciprofloxacin

The genome of *P. aeruginosa* encodes efflux pumps of several multidrug. The efflux system which requires *OprM* was shown its contribution to its resistance to the cephalosporins and quinolones, but not β-lactams, such as carbenicillin cefoperazone and, in the *P. aeruginosa* PAO1 background (Zhao et al., 2020). *MexXY-OprM* is the only pump that has been described to export aminoglycosides . the most common mechanism of aminoglycoside resistance was the upregulation of this pump (Pagès et al., 2005); (Lister et al., 2009); (Morita et al., 2012).Aminoglycosides remain important in the treatment of P. aeruginosa infections, despite their known toxicity (Seupt et al., 2021).

Study by (Goli et al., 2018) mention that , in non-susceptible isolates ,some ICU and burn isolates that has been considered to be sensitive to the antibiotics showing an overexpression of the efflux pumps, indicates that the efflux genes overexpression may not be the key reason of resistance . the role of MDR efflux pumps in the emergence of cross-resistance to different classes of antibiotics can be utilized to explained this outcomes (Van Bambeke et al., 2013)

It has been showed by results of this study that there is difference in the percentage of gene expression of the seven resistance and virulence genes between the (4) different isolates. The genes expression virulence can vary based on the infection site and severity (Aslani et al., 2012). Increase in factors of virulence expression could be associated to the reduced susceptibility to the antimicrobial agents (Bratu et al., 2006). The low expression case of the resistant gene may be presence of another type of the resistant gene responsible for the resistant of the antibiotic. (Vaisvila et al., 2001) showed that the *P. aeruginosa* genome large size, its versatility along with its distribution in aquatic habitats, which can form a reservoir for bacteria that carrying other resistant genes. Another mechanism that affecting the resistant.

The resistance mechanisms identification that is constructed based on gene expression analysis could be become more complex when several mechanisms affecting antibiotics are at work of the same class . (AL-Shamaa, Noor F. K., Rasmia A. Abu-Risha, 2016b)

It provided evidence showing that *p. aeruginosa* as a population exhibiting a specific repertoire of highly expressed genes related to virulence, drug resistance, and nutrient utilization in vivo (Son et al., 2007)

From a clinical point of view, detection of *exoU* and *exoS* genes in hospital isolates of P. aeruginosa could be useful, because these genes were strongly correlated with different TTSS types. The real time PCR method provide to be rapid, specific, and effective for such a detection. Once validated, this technique could be used to detect TTSS genes of P. aeruginosa directly in clinical samples (Berthelot et al., 2003)

P. aeruginosa genes virulence factors expression, that is under comprehensive regulation and expression in vitro, does not necessarily reflect the expression during infection. Certain P. aeruginosa strains could require some extra signals or interactions that may not be present in vitro for expression of these genes. since bacterial isolates laboratory analysis does not require to fully reflect the true in vivo conditions. Featuring of studies should, also, include the virulence determinations factors in situ. (Andrejko et al., 2013)

Conclusion

From current study it can conclude that *P. aeruginosa was* the most common isolated organism from postoperative wounds. The less antibiotics resist were imipenem and gentamycin in case of wound infection and the detected of seven virulence factors genes (*exoU, exoS, lasB ,ampC, oprM, oprN* and *rpoS*)of *P. aeruginosa* showed high positively rate of virulence factors genes in isolates from wounds infection except *exoU* and *exoS* genes not all isolates harboring this tow genes. Our results indicate that *exoS* gene higher then *exoU* gene. The *ampC, oprM, oprN* and *rpoS* genes have same percentages (100%) in inpatients and outpatients, *exoS* was higher in Inpatients (65%) then Outpatients (40%), They spreading widely inside the hospital than the case outside the hospital. While *exoU* and *lasB* were higher in Outpatients (40%,100%) then inpatient (30%, 90%), respectively, this means that *exoU* and *lasB* are spreading widely outside the hospital than the case inside the hospital. The folding rate (the gene expression) of the seven virulence factors genes (*exoU, exoS, lasB ,ampC, oprM, oprN* and *rpoS*) was very important ,because the gene expiration of resistant genes detection the risk factors of the drug resistant *P. aeruginosa*. The expression of *exoU* gene was increase (2.00,40.39) in resistance isolates when used Imipenem and Ciprofloxacin, respectively, compared with sensitive isolates. That means increase expiration of *exoU*

while used this antibiotic. Expression of *oprM* gene increase (3.66) in resistance isolates while used Ciprofloxacin more than expiration of this gene in sensitive isolates.Expression of *lasB* gene increase in resistance isolates when used Erythromycin (30.91) more than expression of this gene in sensitive isolates. While expression of *oprM* and *rpoS* genes, in sensitive isolates when used Gentamycin and Ciprofloxacin respectively, increased in able this bacterium to become resistant to those antibiotics compared with already resistance isolates to those antibiotics. In *rpoS*, *oprM* and *ampC* genes, fold change expression was reduced while treated isolates by Imipenem and Ceftazidime respectively. In addition, we found that tow genes (*rpoS* and *exoS*) have not contribution in Ciprofloxacin antibiotic resistance process.

Instruction of (HiFluoro™ Pseudomonas Agar Base – HiMedia. Found on http://himedialabs.com)*

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