RESEARCH ARTICLE

Role of *higB-higA* Novel Genes in Antibiotics Resistance of *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a devious pathogen with the tendency to prompt many acute and serious chronic diseases. This study aims to detect novel genes (Toxins-Antitoxins II system), especially; *higB* and *higA* encoded from *P. aeruginosa* by PCR technique and the relation between these genes and antibiotic resistance of *P. aeruginosa*.

Methods: This study detected 50 isolates of *P. aeruginosa* from distinct clinical sources. The most common origin of isolates was (44%) burn swabs, (22%) urine culture, (12%) wound swabs, (14%) sputum, and (8%) ear swabs. The bacteria were isolated using implantation MacConkey agar and blood agar, as well as biochemical tests including oxidase test, catalase test then VITEK-2 System of *P. aeruginosa* isolates was improved a final identification. While the determination of sensitivity to antibiotics by using the ASST-VITEK2 compact system method. Genotypic detection was carried out using conventional polymerase chain reaction for *higB* and *higA*.Also sequencing of products for ***higB-higA*** genes were detected.

Results: The results revealed that 82% of isolates have novel genes *higB* in 823pb while only 30% have *higA* in 712pb have this gene. This study discovered correlations among toxins-antitoxins II (***higB-higA***) genes and resistance to antibiotics in *P. aeruginosa* with significant when ($p<0.05$) and highly significant differences when ($p<0.01$).

Conclusion: It was proposed that these genes have an important role in increasing resistant bacteria to antibiotics. As well as was introduced that antibiotic activity may be increased by the effecting of these genes and vice versa.

Keywords: Antibiotics, *higB-higA*, *Pseudomonas aeruginosa,* Toxin-Antitoxin.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which has the tendency to induce a wide variety of lethal, acute, and chronic infections, specifically in patients with feeble immune defense.[1] *P. aeruginosa* can render numerous antibiotic classes futile and evade host defenses through swiftly developing intricate resistance mechanisms.[2]

P. aeruginosa is a gram-negative microbial pathogen allied with copious infections, counting in with chronic wound infections, post-surgical infections, and cystic fibrosis (CF) related to lung disease.[3]

Pseudomonas has a large number of virulence factors that make it responsible for many human infections, such as forming biofilms and producing toxins and enzymes that cause widespread tissue damage and thus reach the bloodstream, causing the spread of these bacteria in the body tissues.[4]

In many strains, the activation of toxins of toxin/antitoxin (TA) systems is thought to be the cause of antibiotic resistance[5], which induces dormancy by inhibiting essential metabolic operations such as protein and ATP synthesis.[6,7]

P. aeruginosa has numerous phenotypes of antibiotic resistance and tolerance, allowing for the endurance of a bacterial population during an infection's antibiotic treatment. These phenotypes extensively vary from their developmental mechanism to the extent to which they can endure antibiotic incidence. Antibiotic tolerance also enables the developing complete antibiotic resistance.[8]

The antitoxin of the type II TA system from *P. aeruginosa* plays the role of a universal regulator which controls the expression of various genes by directly binding to their stimulators in order to regulate the procedures of carbon metabolism, iron uptake, and biofilm formation for responding to exterior stresses. This further specifies that with regard to pathogen treatment, a *HigB-HigA* module could be a latent antibacterial target.[9]

This study aims to detect novel genes (Toxin-Antitoxins II system), especially*; higB and higA* encoded from *P. aeruginosa* by PCR technique and the relation between these genes and antibiotic resistance of *P. aeruginosa*.

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MATERIALS AND METHODS

Isolation and Identification of Bacteria

A total of 100 clinical samples were gathered from different sources (wounds, burns, urine, sputum, ear). These sources involved Al-Yarmouk teaching hospital, AL-Mahmoodiya hospital and Child's central teaching hospital in Baghdad. All of the samples were cultured using a sterile loop. MacConkey agar appeared**,** blood agar and cetrimide agar media for identifying *P. aeruginosa* growth with fluorescent green color, elevated colonies, and grape-like odor. All isolates had the ability to grow on this media.[10] They were further identified and characterized by performing gram staining, traditional biochemical tests, including oxidase, catalase, and VITEK-2 system compact ID GNB (NO.222) cards were used to prove a final identification of *P. aeruginosa*.

Genotyping Detection of (Toxin –Antitoxins II system) HigB-HigA Genes

DNA extraction from bacteria

Genomic DNA was isolated from bacterial growth under the Wizard Genomic DNA Purification Kit protocol, Promega (Promega, USA). Quantus Fluorometer was used to detect the concentration of the extracted DNA to detect the quality of samples for downstream applications. For 1-µL of DNA, 199 µL of diluted QuantiFlour Dye was mixed. DNA concentration values were assessed after incubating it for five minutes at room temperature. DNA concentration rang 15 to 30 ng/µL, accurately followed by the genotyping detection to *higB* and *higA* genes using PCR technique and conventional procedures in identifying gram-negative bacteria.[11]

PCR amplification

PCR amplifications were performed with 100 ng of DNA bacteria plus 12.5 µL of master mix (Bioneer/ Korea) and 1.5 µL of each primer. Macrogen Company supplied these primers in a lyophilized form as shown in Table 1 were prepared to 10 pmol/µL concentration as work primer and distal water to reach the final volume of 25 µL. In the PCR program, as shown in Table 2, each PCR augmented product was planned with 1.5% (w/v) agarose gel in Tris-acetate-EDTA buffer. The products were run for 120 minutes at 90 V. The bands were observed after staining with ethidium bromide using ultraviolet light.

Sequencing of PCR product of P. aeruginosa

The samples were sent to Microgen/Korea for gene sequencing. Using genetic analyzer (ApplieBio system) and homology search was performed using (BLAST) program online blast n and blast x algorithms at NCBI. According to Savari *et al.*[12], Primers were planned, as illustrated in Table 1. According to the amplify gene program, denaturation was for 95°C at 30 minutes. While the annealing tempareture for *higB* was 65°C, for *higA* was 48°C at half hour for both genes. The final extention was for 72°C at 7 minutes.

Table 1: The primers of genes and annealing temperature, and product size

Primers Name	*Seq.*	*Annealing temp (°C)*	*Product size (bp)*
higB-F	5`-CAGGTGGAGAGCG-CAGGTC-3`	65	712
higB-R	5`-CAATTGTCCCAACG-CCTCCTTCG-3`		
higA-F	5`-GTTTGCCACGTTTG-CATGCAG-3`	48	823
higA-R	5`-CGCTCAGTTCTGGA-TGAATCTCC-3`		

Antibiotic susceptibility assay

AST-GN (No. 222) cards did the resistance to antibiotics with VITEK-2 System compact performed to determine antimicrobial susceptibility patterns under the manufacturer's directions (bioMerieux, France), as well as to detect the MIC of antibiotics to *P. aeruginosa*. The antibiotics used are ciprofloxacin, ceftazidime, ticracillin, ticarcillin/ clavulanic acid, piperacillin, meropenem, cefepime, imipenem, tobramycin, amikacin, gentamicin and colistin.

Statistical Analysis

The mean ± SE of the mean was calculated using the IBM SPSS version 28.0. The probability was also examined by using the ANOVA table (Duncan test). Pearson's chi-square test was used to compute the probability for non-parametric data.[13]

RESULTS AND DISCUSSION

Isolation and Identification of *Pseudomonas aeruginosa*

Total 100 samples were collected and cultured on blood agar, cetrimide agar, and MacConkey agar. However, after analysis, only 50 isolates were found to be associated with *P. aeruginosa.* Microscopically the isolated bacteria are gram-negative bacteria. Therefore, they occurred as pale colonies on MacConkey agar. When the beta type of hemolysis was detected on blood agar, and various strains of *P. aeruginosa* produced several species of pyocyanin on cetrimide agar, the *P. aeruginosa* isolates were positive for the oxidase test. And the recognition was established by VITEK 2 System.[14]

The prevalence of bacteria according to samples was (burn 44%, urine 22%, wound 12%, sputum 14%, and ear 8%), as shown in Figure 1. These percentages are close to other studies that isolate the same bacteria in Baghdad, and the results were (burn 36.67%, urine 23.33%, wound 18.33%, sputum 6.67%, ear 6.67%).[15]

Genotyping detection of (Toxin -Antitoxin II system) *HigB-HigA* genes

The *higA* and *higB* gene were detected with PCR for 50 isolates, using the *higA* and *higB* primers to amplify constitutional gene bands confirmed with gel electrophoresis. This step found that 82% of isolates have *higB* with product size 712 bp, and 30% have *higA* with product size 823 bp, as shown in Figure 2.

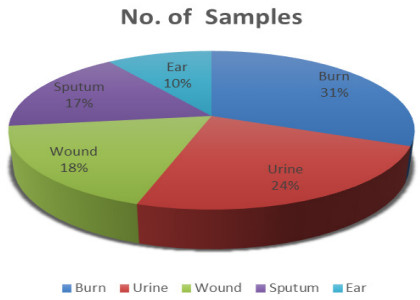


Figure 1: Prevalence of *P. aeruginosa* in different clinical samples

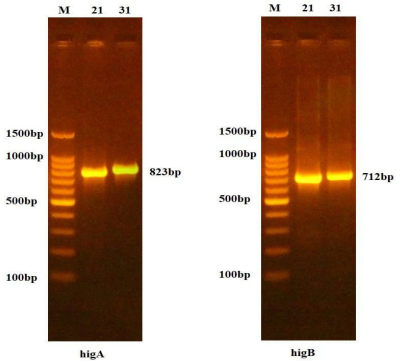


Figure 2: Results of the amplification of *higB and higA* of *Pseudomonas aeruginosa* samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 21,31 bands in712bp. and 823bp

In study of 174 *P. aeruginosa* isolates, *relBE* and *higBA* were universal, but p*arDE* was less prevalent.[12] Another study demonstrated that *higBA* and *relBE* TA systems in *Staphylococcus aureus and P. aeruginosa* were found in 100% of the corresponding strains.[16]

PCR Products Sequencing Analysis

In the current study, the PCR product of (2) was isolated, examined and compared with the reference strains existing in the GenBank database of the NCBI database. With the usage of the Basic Local Alignment Search Tool (BLAST), available at the NCBI, the sequencing results unveiled 99.96 and 100% compatibility with reference strains and alignment with references from NCBI, as specified in Figure 3.

Antibiotic Susceptibility Assay

The susceptibility of fifty isolates was tested using a VITEK2 compact system by kit (GN222). This study detected the resistance rate of *P. aeruginosa* isolated from different specimens to different antibiotics, as illustrated in Figure 4. The highest resistance was noted in *P. aeruginosa* colistin with 100%, which disagreed with another study of 7.2% resistant percent,[17] ticarcillin showed 98% resistance. Only one specimen from the wound was sensitive to ticarcillin. These results contradict the outcomes reported by Akinloye *et al.*[18], who mentioned the resistance in 100% of isolates. The third most antibiotic resistance was shared between two antibiotics, ticarcillin/clavulanic acid and piperacillin. They shared the same percentage of 88%, and these results are almost similar to another study with 70%.[19] The lowest resistance rate was noted

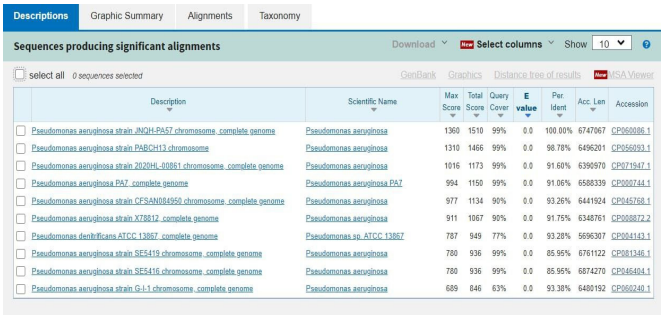
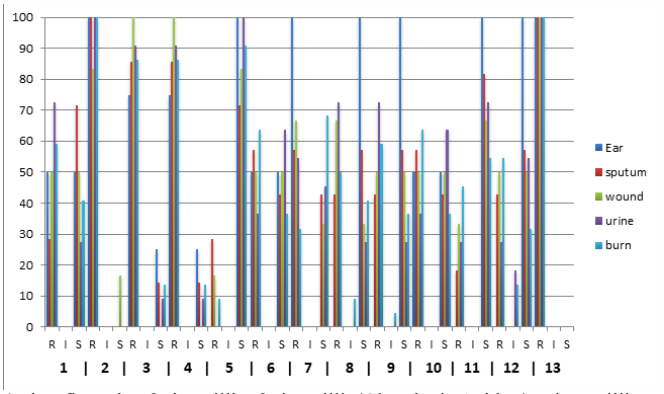


Figure 3: The sequencing results compatibility with reference strains of two genes



1:ciprofloxacin , 2:ticracillin ,3:ticracillin/Clavulanic Acid , 4 : piperacillin 5:Piperacillin/Tazobactam , 6:meropenem , 7 :tobramycin , 8 : Ceftazidime 9:Cefepime , 10 : Imipenem , 11:Amikacin , 12: Gentamicin , 13 : colistin

Figure 4: Results of antibiotics sensitivity test according sources of P. aeruginosa

in *P. aeruginosa* to piperacillin/tazobactam. Only 10% resisted then amikacin was shown to be 34% resist to this antibiotic. When the resistance rate to other antibiotics showed a 40 to 60% range resistance, the outcomes are consistent with another study with resistance percentages of gentamycin and tobramycin as 51.39 and 45.83%, respectively. At the same time, the isolates also revealed a moderate-high resistance to imipenem and meropenem as 40.28 and 44.44%, correspondingly.[20]

Role of *higB-higA* in Resistant to Antibiotics

Toxin–antitoxin (TA) systems, comprising cognate antitoxin and toxin, have a decisive part in persisting bacterial cells.[21] The persisting bacteria are intermittent cells in a bacterial population tolerant to lethal antibiotics. The persisting bacterial cell formation was observed in nearly each bacterial specie investigated[22] result shown in Tables 2, and 3 results were referred to as the relation between antibiotic-resistant and the presence of *higA* and *higB* when $p<0.05$ the outcome was considered significant when $p<0.001$ highly significant. *P. aeruginosa* is famous for its high resistance against antibiotics. *P. aeruginosa* makes many antimicrobics useless, and aminoglycosides and polymyxins as the last choice of antibiotics. Even though various resistance mechanisms have already been familiarized for these drugs, numerous researchers have found factors and ways for developing novel strategies with the purpose of increasing their effectiveness towards antibiotics.[23] However, the study by Coskun[24] revealed that

Table 2: Role of *higA* gene resistant to antibiotics

Antibiotics	*higA gene*		*Probability*
	Positive	*Negative*	
Ceftazidime			
R	6	20	
I	1	1	0.005*
S	6	16	
Ticarcillin			
R	13	36	0.04*
S	0	1	
Ticarcillin/Clavulanic Acid			
R	11	33	0.0002**
S	2	4	
Piperacillin			
R	12	32	0.0002**
S	1	5	
Piperacillin/Tazobactam			
R	0	5	0.153^{NS}
S	13	32	
Meropenem			
R	9	18	0.001*
S	4	19	
Cefepime			
R	7	20	
I	1	0	0.006*
S	5	17	
Imipenem			
R	9	18	0.005*
S	4	19	
Tobramycin			
R	5	20	0.008*
S	8	17	
Amikacin			
R	3	14	0.002*
S	10	23	
Gentamicin			
R	4	17	
I	1	4	0.001*
S	8	16	
Colistin			
R	13	37	0.0001**
S	0	0	
Ciprofloxacin			
R	8	20	0.001*
S	5	17	

p > 0.05 *significant, *p > 0.0001* ** highly significant, *p < 0.05*NS Non significant

Table 3: Role of *higB* gene resistance to antibiotics

Antibiotics	*higB* gene		Probability
	Positive	Negative	
Ceftazidime			
R	21	5	0.0006**
I	2	0	
S	18	4	
Ticarcillin			
R	40	9	0.00004**
S	1	0	
Ticarcillin/Clavulanic Acid			
R	36	8	0.00006**
S	5	1	
Piperacillin			
R	35	9	0.00001**
S	6	0	
Piperacillin/Tazobactam			
R	5	0	0.0123*
S	36	9	
Meropenem			
R	21	6	0.0007**
S	20	3	
Cefepime			
R	22	5	0.0004**
I	1	0	
S	18	4	
Imipenem			
R	0	0	0.0004**
I	20	3	
S	23	2	
Tobramycin			
R	23	2	0.00008**
S	18	7	
Amikacin			
R	15	2	0.00009**
S	26	7	
Gentamicin			
R	18	3	0.0007**
I	4	1	
S	19	9	
Colistin			
R	41	9	0.00001**
S	0	0	
Ciprofloxacin			
R	23	5	0.001*
S	18	4	

p>0.05 *significant, *p< 0.0001* ** highly significant, *p < 0.05*NSNon significant

antibiotics activity might be increased by affecting these genes and *vice-versa.*

CONCLUSION

This study found novel genes of the toxin-antitoxins system in *P. aeruginosa,* such as *higA and higB* genes. Furthermore, the resistance of these bacteria to antibiotics was elevated, especially in hospitalized patients in Iraq. This study also proposed that these genes have a profound role in increasing the resistance of bacteria to antibiotics. Moreover, it was also comprehended that antibiotics activity might be increased by affecting these genes and vice-versa. The results of NCBI reported that two genes are novel in *P. aeruginosa*. For that, the researchers register these genes in NCBI according to the following sites https://www.ncbi.nlm.nih.gov/protein/2186803491, https://www.ncbi.nlm.nih.gov/protein/2186803497

Ethical Disclosure

The Ethics Committee of Baghdad University of College of Sciences approved the current study protocol (CSEV/0921/0099).

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