LINK BETWEEN SOME VIRULNCE FACTORS GENES AND ANTIBACTERIAL RESISTANCE OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The research was aimed to demonstrate the frequency of virulence factors genes (*exoenzyme S* and *exotoxin A*) and to express their relationship to antibacterial resistance among *Pseudomonas aeruginosa* isolated from patients suffering from otitis externa. The results were revealed that only twenty one (32%) out of 65 clinical ear swabs were *Pseudomonas aeruginosa*. According to PCR amplification, 18 (85.7%) bacterial isolates were expressed both toxins (exoenzyme S and exotoxin A). The data get by real time experiments were revealed that the isolates were give higher percentage of resistance were seen against Ceftazidime (90.5%) and Gentamicin (88.5%).Only thirteen isolates (61,90%) of Multidrug resistance (MDR) –*Pseudomonas aeruginosa* isolates were detected, and two (9.5%) isolates were resist to all 8 classes of antibiotics and considered as PDR, and three (14.2%) bacterial isolates resist seven classes of antibiotics which considered as XDR.

Keywords: *Pseudomonas aeruginosa,* virulence factors genes, antibacterial resistance.

العلاقة بين بعض جينات عوامل الضراوة ومقاومة المضادات البكتيرية للزائفة الزنجارية

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المستخلص

هدف البحث لتوضيح تواتر جينات عوامل الضراوة (***exoenzyme S*** و ***exotoxin A***) وللتعبير عن علاقتها بالمقاومة المضادة للبكتيريا للعزلات البكتيرية من ***Pseudomonas aeruginosa*** بين مرضى التهاب الأذن الخارجية. أظهرت النتائج أن 21 (32٪) فقط من أصل 65 مسحة أذن سريرية كانت ***Pseudomonas aeruginosa***. وفقًا لتضخيم تفاعل البوليميراز المتسلسل ، تم التعبير عن 18 (85.7٪) عزلة بكتيرية على حد سواء (السموم الخارجية S والسموم الخارجية A). أظهرت البيانات التي تم الحصول عليها من تفاعل البلمرة التسلسلي أن العزلات اعطت نسبة مقاومة أعلى ضد السيفتازيديم (90.5٪) والجنتاميسين (88.5٪) ، فقط ثلاثة عشر عزلة (61،90٪) تم الكشف عنها من بكتريا الزائفة الزنجارية ذات المقاومة المتعددة للادوية (MDR– ***Pseudomonas aeruginosa***) ، وعزلتان (9.5٪) قد قاومتا جميع فئات المضادات الحيوية الثمانية والتي تم اعتبارها PDR ، وثلاثة عزلات بكتيرية (14.2٪) يمكن أن تقاوم سبع فئات من المضادات الحيوية والتي تم اعتبارها XDR.

كلمات مفتاحية: الزائفة الزنجارية، جينات عوامل الضراوة، مقاومة المضادات البكتيرية.

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INTRODUCTION

Otitis externa is as an acute, chronic, and necrotizing inflammation of the external ear canal (1). Inflammation takes various types which includes: acute, chronic, and may be developed into tumor, circumscribed otitis externa (2). In contrast to acute otitis externa, chronic otitis externa is generally caused by allergies or underlying inflammatory dermatologi conditions (3) *Pseudomonas aeruginosa* (*PA*) is the most common cause of acute external otitis, accounting for more than 70 percent of cases in one series. It's (an opportunistic pathogen) can cause a higher percentage of acute or chronic infections with higher frequencies of mortality and morbidity (4,5). A wide range of infections caused by *PA* including: endocarditis, septicemia, pneumonia, wounds, burn, otitis and bacterial keratitis. It is also noticed in a cystic fibrosisfrequently, immunosuppressed and /or nosocomial infections (6,7).These infections are very difficult to eliminate due to the presence of (numerous virulence factors) such as flagella, pili, proteases, elastase, exopolysaccharides, iron chelators, lipases, and a variety of various toxins, including : exotoxin A, and the Type III Secretion System (T3SS) toxins ExoS, ExoT, ExoU, ExoY, in addition to chemical compound and pyocyanin (8). Two members of virulence factors genes namely (Exoenzyme S and Exotoxin A) catalyzes transporting of the Adenosine di phosphate (ADP) -ribose moiety of nicotinamide di phosphate (NAD) to the eukaryotic cells proteins (9,10)., several lines of studies were suggested that Exoenzyme S may be play a distinct role in the pathogenicity of *PA*. The role of exoenzyme in the past experiments were showed to be unselective in choice of substrate proteins, however, now has noticed that it preferentially ADP-ribosylates several of the low-molecular weight GTP-binding proteins. In addition to, there is higher similarity with cholera toxin, exoenzyme S also needed a protein of eukaryotic cells for their enzymic activity (11). Exotoxin A catalyzes the ADP-ribosylation of elongation factor 2 (EF-2), leading to inhibition and disruption of synthesis ofproteins (7).

MATERIALS AND METHODS

Samples collection: During the period extended from February to August of the year of 2020. Sixty five of clinical ear swabs were collected from patients who suffering from ear infections and attended into out- patients at Teaching Hospitals in Baghdad city.

Bacterial isolation: All the clinical ear swabs were cultured on Blood, MacConkey, Brain heart infusion agar medium, and the selective media of *PA* Cetrimide agar, and all cultured media were incubated for 18 - 24 hrs. at 37 ºC. The suspected bacterial isolates which possibly belong to *PA* inoculated on Cetrimide agar to demonstrate the characteristic traits of this bacteria for ex: blue-greenish color, and the presence of fruity odor. The other diagnostic tests were included the detection of phenotypic and microscopic traits in cooperated with biochemical diagnostic tests. Which were done according to Forbes *et al* (12).

Bacterial identification

Colonial and phenotypic morphology on blood, MacConkey, Brain heart infusion, and Cetrimide agar medium were based principally to detect bacterial isolates of *PA*, and their colonial shape, texture, color and edges were noticed. The macroscopic examination were cooperated by microscopic examination of a Gram stained of bacterial smear which examined under a light microscope withspecial regard towards cell shape and arrangement. All suspected bacterial isolates of *PA* were subjected to many biochemical tests such as oxidase,catalase, and triple sugar Iron test etc.

Antibiotic sensitivity test

The disc diffusion method represented the gold standard for confirming and determining the susceptibility of the bacteria, in this test, the isolated bacterial pure colonies were selected and suspended into nutreient media, then were standardized by using a turbidity test and this point was done by using Macferland no. o.5. The standardized suspension is then cultured and disseminated onto Muller Hinton agar plate, and the different antibiotic discs were tapped and putted on the inoculated plates Seventeen different antibacterial disc were permitted to disseminate through the solidified agar which includes :

Ampicillin (AM), Amoxicillin (AML), Ceftazidime (CAZ), Imipenem (IPM), Colistin sulfate (CT), Mezlocillin(MEZ), Azithromycin

(AZM), Nalidixic acid (NA),Trimethoprim-sulfamethoxazol (STX),Tetracycline (TET), Aztreonam (ATM), Amikacin (AK),Levofloxacin(LVX),Ciprofloxaci n (CIP)Gentamycin (GEN), Piperacillin (PIP), and Chloramphenicol (C) (Table 1). All inoculated agar plates were incubated overnight at 37 °C. After that, the size of the inhibition zone formed around each disc was measured and recorded.

Table 1. Types and dosage of the seventieth different antibiotics discs used to performed antibiotic sensitivity test

Type of antibiotic	Conc. µg
Ampicillin	**10**
Amoxicillin	**25**
Ceftazidime	**30**
Colistin sulphate	**10**
Aztreonam	**30**
Ciprofloxacin	**5**
Tetracycline	**25**
Nalidixic acid	**30**
Azithromycin	**15**
Mezlocillin	**75**
Imipenem	**10**
Trimethoprim-sulfamethoxazol	**125/23.7**
Amikacin	**30**
Gentamycin	**10**
Piperacillin	**100**
Levofloxacin	**5**
Chloramphenicol	**30**

Primers of this genes

Table 2.The specific primer of *Exoenzym S* gene

Gene		Primers Sequence	Tm (°C)	GC (%)
Exo.S	**Forward**	**5'- ATGTCAGCGGGATATCGAAC- 3'**	**54.6**	**50.0**
	Reverse	**5'- CAGGCGTACATCCTGTTCCT- 3'**	**56.8**	**55.0**

Table 3. The specific primer of *Exotoxin A* gene

Gene		Primers Sequence	Tm (°C)	GC (%)
Exoto. A	**Forward**	**5'- GACAACGCCCTCAGCATCACCAGC- 3'**	**64.4**	**62.5**
	Reverse	**5'- CGCTGGCCCATTCGCTCCAGCGCT- 3'**	**70.1**	**70.8**

Molecular detection of *Exoenzyme S*

The extraction of RNA, measuring of RNA concentration and detection of *exoenzyme S* and *exotoxin A* genes by real time technique were performed according to the protocol of Promega QuantiFluor® Dye Systems.

REASULTS AND DISSCUSION

Swimmer's ear it is the synonym of acute otitis externa (AOE), it considered as one of the most common infection of adult, children and even adolescents. Principally it's an infection of children who up to 2 years old, it is known to be related with swimming. Prolonged ear canal wetness may be leading to impairing of local defense mechanisms (13)

Isolation and identification of *PA*

After doing and following of conventional analyzing and diagnostic steps which includes both microscopic and macroscopic demonstration, and in addition to biochemical tests to detect the presence of *PA*. The results were revealed that only twenty one (32%) isolates of *PA* among 65 clinical ear swabs were detected.

Distribution and percentage of *PA* among infected patients

Table (4) was showed that the most commoncausative isolated pathogens are *PA* 21 (around 33%), followed by *Staphylococcus aureus* 15 (23%) and *S. epidermidis* 9 (14%) respectively. Furthermore, the remaining 10 specimens (15 %) don't exhibited any bacterial grow, this probability can be due to a fungal infection (typically *Aspergillus spp*.) or viral infection.

Table 4. Distribution and percentage of *PA* and other species among infectedpatients

Species of the bacteria	NO. and percentage of isolates
Pseudomonas aeruginosa	**21(33%)**
Staphylococcus aureus	**19 (29 %)**
Staohylococuus epidermedis	**15 (23 %)**
No growth	**10 (15. %)**
Total	**65 (100%)**

The present result comes in fit with the previous studies which revealed that both of *PA* and *Staphylococcus aureus* were the most commonly isolated organisms (14). The isolates are (poly- microbial) in a significant number of cases. Other Gram-negative bacteria are uncommon. *Aspergillus* species and *Candida* species are rare fungal infections (15) . Swabs taken from the external canal should be swabbed with caution because (they may be reflect normal flora or colonizing organisms). Ear swabs should be taken only in severe cases or unresponsive. The previous researches revealed that the bacterial flora or bacterial commensals of the External Auditory Canal (EAC) is commonly composed of Gram - positive bacteria. The most prevalent recognized bacteria are *Staphylococcus epidermidis* (38%) fallowed by *Diptheroid* (22.4%). Gram - negative bacteria are less common, isolated from (< 5%) of the external auditory canal (AOE) specimens. Prolonged exposure to the water may leads to the changes of flora of the EAC, becoming dominated by Gram - negative organisms for example *PA* which constitutes the mostcommon pathogenic bacteria in AOE, detected in (22-62%) of cases in series on AOE. *Staphylococcus aureus* (11-34% of cases) is the most important Gram - positive bacteria (16,17,18).

Antibiotics Resistance in *PA*

Sensitivity and resistance phenotypic pattern were done on each of diagnosed 21 *PA* isolates by using agar disc diffusion method and by using seventieth different antibiotic disc. The results were interpreted according to recommendation of the National Committee for Clinical Laboratory Standard (NCCLS) (19). The *Pseudomonal* isolates that were found to belong to different interpretive categories, including susceptible (S), and resistant ®. Approximately 95% of *Pseudomonas* strains were susceptible to colistin, Imipenem, and pipracillin followed by 90.4 % that were susceptible ciprofloxacin and 80.9 % of all isolates give sensitivity against ceftazidime, amikacin, gentamycin and levofloxacin. tetracycline were (71.4%), aztreonam and trimethoprim-sulfamethoxazole were (66.6%), However, a high percentage of *Pseudomonas* strains were resistant to Amoxicillin (95%) followed by ampicillin and nalidixic acid (90%), Azithromycin and chloramphenicol (57.1%), the result of antibiotic sensitivity test of pseudomonal isolates against Mezlocillin was (52.3%).The frequencies of resistance phenotypic patterns to the 17 tested antibiotics were arranged in the following table 5.Totaly 21 (60 %) bacterial isolates were (resistant to three or more drug classes) were defined as MDR strains. Two (9.5%) isolates were resistant to all eight classes of antibiotics and considered as Pan drug resistance (PDR), and three (14.2%) bacterial isolate could resist seven classes of antibiotics and considered as extensive drug resistance (XDR).

Table 5. Sensitivity and resistance phenotypic pattern of twenty one *P. aeruginosa* isolates

Type of antibiotic	R	S
	No. and %	No. and %
Ampicillin	18 (90%)	3 (10%)
Amoxicillin	20 (95%)	1 (5%)
Ceftazidime	4 (10.1%)	17 (80.9%)
Colistin	1 (5%)	20 (95%)
Aztreonam	14 (66.6%)	7 (34.4%)
Ciprofloxacin	2 (9.6%)	19 (90.4%)
Tetracycline	15 (71.4%)	6 (28.6%)
Nalidixic acid	18 (90%)	3 (10%)
Azithromycin	15 (71.4%)	6 (28.6%)
Mezlocillin	11 (52.3%)	10 (47.7%)
Imipenem	1(5%)	20(95%)
Trimethoprim-sulfamethoxazol	14 (66.6%)	7 (34.4%)
Amikacin	4 (10.1%)	17 (80.9%)
Gentamycin	4 (10.1%)	17 (80.9%)
Piperacillin	1 (5%)	20 (95%)
Levofloxacin	4 (10.1%)	17 (80.9%)
Chloramphenicol	12 (57.1%)	9 (42.9%)

In the last years, the prevalence of higher risk strains of *PA* such as (MDR; XDR; and PDR), has constitute a major public health problem in the worldwide. Also recently there is a significant increasing in the percentage of MDR ,XDR, and PDR *of PA* bacterium, with a percentage between (16% and 31%) in many countries (20,21.22). Many Europian countries reported frequency of antibiotics resistance exceeded(10%) for all antibiotics classes under control and notification (23). Combination of resistance was also noticed in *PA* In the last seven years (the European Centers for Disease Prevention and Control) revealed that (14%) of *PA* strains were expressed antibiotic resistant to more than two antibacterial classes and (6.5%) to morethan four antibacterial different classes under control (EARS-Net)[23]. The observation of United States, revealed that the MDR - *PA* is the cause of (14%) of severe health care associated infections (24). There are different and a big cause why the prevalence of (MDR), (XDR), and (PDR) *PA* bacteria have in recent years become issues of public health concern. The first reason, *PA* responsible for highly severe infections, especially in the health care units and in immunosuppressed persons. Second reasons, it has an outstanding capacity for being selected and for disseminationof antibacterial resistance in the human body (25,26,27). Third, the successful worldwide spread of the so-called "high-risk" clones of *PA* poses a threat to global public health that needs to be studied and managed with urgency and determination (28,29,). Quantitative analysis of *Exoenzyme S* and *exotoxin A* virulence gene The expression of *Exoenzyme S* and *Exotoxin A* genes were detected successfully by using new molecular technique which is Real time PCR (qRT-PCR) with used specific primer. The amplification accuracy of gene product was noticed by the value of cycle threshold (Ct) for the triplicate reactions and the results revealed that 18 (85.7%) of the isolates exhibited both the *Exoenzyme S and Exotoxin A* genes and only three bacterial isolates don't exhibited exotoxin gene (Table 6,7 and Figure 1).

Table 6. PCR analysis result of *Exoenzyme S* virulence genes of 21 *PA* isolatesamong otitis externa patients

Result	FAM average Ct. values	No. of the sample
-	-	Sample 1
+	24.9	Sample 2
+	24.9	Sample 3
+	10.9	Sample 4
+	23.9	Sample 5
+	21.9	Sample 6
+	9.9	Sample 7
+	15.8	Sample 8
+	23.9	Sample_9
-	-	Sample 10
-	-	Sample 11
+	8.9	Sample_12
+	11.9	Sample 13
+	10.9	Sample 14
+	22.8	Sample 15
+	24.9	Sample_16
+	25.9	Sample 17
+	12.9	Sample 18
+	11.8	Sample_19
+	11.9	Sample 20
+	20.8	Sample 21

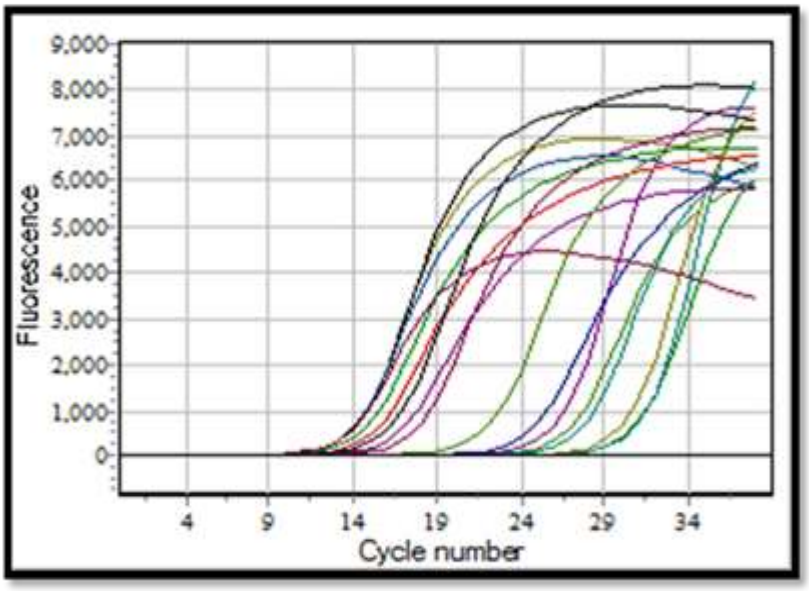

Figure 1. Quantitative detection of *Exoenzyme S* virulence gene of 21 *PA* isolatesamong otitis externa patients

Table 7. PCR analysis result of *Exotoxin A* virulence genes of 21 *PA* isolatesamong otitis externa patients

Result	FAM average Ct. values	No. of samples
-	-	1
+	26.9	2
-	-	3
+	10.1	4
+	28.0	5
+	22.9	6
+	10.9	7
+	13.1	8
+	21.0	9
-	-	10
-	-	11
+	10.1	12
+	11.0	13
+	8.9	14
+	19.9	15
+	27.0	16
+	23.0	17
+	12.0	18
+	11.9	19
+	11.0	20
+	18.9	21

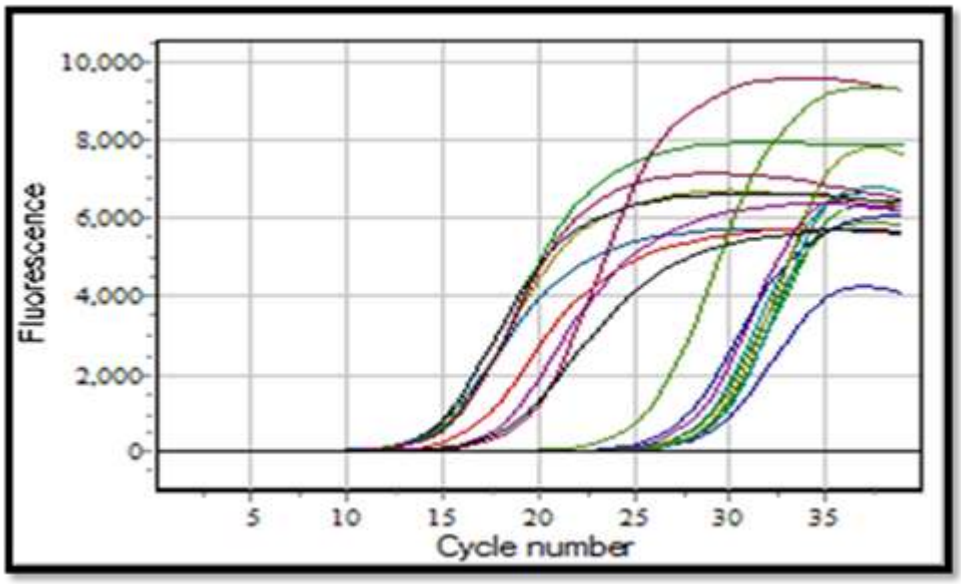

Figure 2. Quantitative detection of *Exotoxin virulence* gene of 21 *PA* isolatesamong otitis externa patients

Also the results revealed that. The frequencies of genes among MDR strains were 18 (85.7%) for both toxA and exoS which included MDR and XDR pseudomonal isolates. The non-MDR strains 3(14,28%), harbored lower prevalence of simultaneous toxA and toxS genes compared to MDR strains of *pseudomonas aeruginosa*.

CONCLUSION

The recent study proved that by using very specificity and sensitivity technique (real time PCR), the bacterial toxins genes of *PA* which is *Exoenzyme S* and *Exotoxin A* they were and still are the most potential virulence factors of *PA* and the most related to initiation of various types of antibacterial resistance.

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