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Original Research Article

Molecular Study of Two Virulence Genes of *Pseudomonas aeruginosa*, The *Oxa* 10 and *Tox* a with The Comparisons of The Relevant Sequences

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Abstract

Pseudomonas aeruginosa is one of the leading nosocomial pathogens worldwide. Fifty pre-identified local isolates of *P. aeruginosa* were collected from major hospitals in Duhok and Erbil during the period from April 2015 to September 2015. The isolates were identified by classical biochemical methods and antibiotic sensitivity profiles were also obtained. Molecular investigation started with DNA extraction, confirmatory identification by the detection of 16S rRNA; PCR amplifications were applied to detect the presence of *oxa* 10 and *tox*A genes and then sequencing of the resulted PCR products was also performed. The results showed that all the fifty isolates had biochemical profiles characteristic of *P. aeruginosa* as it was also confirmed by the amplification band of 956 bp relevant to 16S rRNA; and that 50 % of the isolates revealed resistance to imipenem while 98 % of the isolates were resistant to cefepime. Furthermore, *oxa*10 gene was successfully detected by PCR in 92 % of the isolates with products bands of 760 bp while *tox*A gene was detected in 84 % of the isolates with an amplification bands of 396 bp. Then the PCR products of randomly selected ten samples (five for *oxa* 10 gene (designated A1through A5) and another five for *tox* A gene (designated B1 through B5) were purified by using a commercial PCR product purification kit and sequenced. The results of sequencing were analyzed in the University of Duhok/Scientific Research Center using DNASTAR/Laser Gene software. Regarding *oxa*10 gene, the results revealed that strains A4 and A5 are much more related when compared with the other strains. While the result of sequencing *tox* A gene indicated that isolates B2 and B5 are much more related in comparison with the other three isolates and isolate B1 was much more related to the positive control when all the isolates were compared.

Key Words: *Pseudomonas aeruginosa*, *oxa* 10, *tox* A, sequencing, phylogenetic tree

دراسة جزيئية لاثنين من جينات الضراوة لبكتريا الذائفه الزنجاريه، *oxa* 10، *tox* A مع مقارنة تسلسل التتابعات الخاصة بهما

الخلاصة:

تعتبر بكتريا الزائفة الزنجارية من اهم مسببات العدوى المنتقلة عن طريق المستشفيات حول العالم . تم جمع خمسين عينه مشخصه مسبقا من هذه البكتريا من المستشفيات الكبرى في مدينتي دهوك واربيل في اقليم كردستان العراق خلال الفترة الممتدة من نيسان ٢٠١٥ ولغاية ايلول من نفس العام. تم تاكيد التشخيص المظهري للعزلات وكذلك تم عمل فحص الحساسية للمضادات الحيوية. تضمنت الدراسة الحالية استخلاص الدنا من العزلات والتشخيص التاكيدي للعزلات من خلال تفاعل البلمره المتسلسل لجين 16S rRNA وكذلك تم تشخيص وجود جيني عاملي الضراوة *oxa* 10 و *tox* A من خلال تفاعل البلمره المتسلسل ومن ثم اجراء تفاعل تسلسل التتابعات لناتجي تفاعل البلمرة المتسلسل لجيني الضراوه المذكورين. بينت نتائج الدراسة الحالية ان جميع العينات اظهرت صفات مظهريه بايوكيمياويه مماثله تماما لبكتريا الزائفة الزنجارية وهذا ما تم التاكد منه من خلال نتائج تفاعل البلمرة

المتسلسل لجين 16S rRNA والتي كانت ٦٥٩ قاعده نتروجينيه .كما بينت النتائج ان ٥٠% من العزلات كانت مقاومه للاميبينيم في حين ان ٩٨% كانت مقاومه للسفيبيم. اظهرت نتائج تفاعل البلمره المتسلسل ان ٩٢% من العزلات كشفت عن وجود جين 10 *oxa* المسؤول عن المقاومة للمضادات الحيوية وذلك من خلال حزم التضاعف ذوات ال ٧٦٠ قاعده نتروجينيه في حين ان ٨٤% من العزلات اظهرت حزم تضاعف بحجم ٣٩٦ قاعده نتروجينيه والتي تؤكد وجود جين A *tox* المسوؤل عن انتاج احد اهم ذيفانات البكتريا قيد الدراسة. اجريت تجربة تسلسل التتابعات بعد اختيار عشرة عينات عشوائيا خمسه منها لجين 10 *oxa* (اعطيت الرموز A1–A5) وخمسه اخرى لجين A *tox*(اعطيت الرموز B1–B5) بعد اجراء عملية التنقية لناتجي عملية تضاعف البلمرة لجيني الضراوة المذكورين وتم تحليل النتائج في جامعة دهوك /مركز الابحاث العلمية وذلك باستخدام برنامج DNASTAR/Laser Gene واظهرت النتائج المتعلقه بجين 10 *oxa* ان العزلتين A4،A5 كانتا اكثر تقاربا مقارنة بباقي العزلات في حين بينت النتائج المتعلقه بجين A *tox* ان العزلتين B2،B5 كانتا متقاربتين بالمقارنة مع باقي العزلات وان العزلة B1 كانت اكثر قربا من جميع العزلات مع عزلة السيطرة الموجبة.

الكلمات المفتاحية: الزائفة الزنجارية. جينات A *tox* ,10 *oxa*، تفاعل تسلسل التتابعات، الشجره الوراثية.

Introduction

Pseudomonas aeruginosa is a widely distributed ubiquitous nosocomial pathogens [1]. It is widely recognized as an opportunistic pathogen, almost related to infections of immunosuppressed patients [2]. This bacteria is intrinsically resistant to multiple antibiotics including penicillins and cephalosporins, ciprofloxacins, chloramphenicol, tetracycline, erythromycin, trimethoprim–sulfamethoxazole and rifampin, this resistance might be explained by the ability to express efflux pumps and AmpC β-lactamase constitutively, in addition to a decreased permeability of the outer membrane [3]. The simultaneous expression of a combination of multiple antibiotic resistance mechanisms is a major factor of conferring multidrug resistance (MDR) phenotype specifically noticed in nosocomial strains of *P. aeruginosa* which stand for the large proportion of clinical strains [4].

The β-lactamases of the OXA-type are so termed because of their ability to hydrolyze oxacillin. These enzymes hydrolyze cloxacillin and oxacillin in a rate greater 50% than that for benzylpenicillin [5]. Oxacillinases (OXA type enzymes) grouped to molecular class D and functional category 2d. Classical OXA enzymes (OXA-1, OXA-2, OXA-10) specify resistance to ureidopenicillins and carboxypenicillins but not to ceftazidime. Moreover, the resistance to piperacillin and ticarcillin attributed to OXA-2 enzymes production is lower than the resistance shown when OXA-1 and OXA-10oxacillinasesare synthesized [6]. Five distinct categories of oxacillinases enzymes have been described in *Pseudomonas aeruginosa* [7].

Pseudomonas aeruginosa synthesize two different ADP-ribosyltransferase toxins: Exotoxin A (ExoA, toxA) andexoenzyme S. Exoenzyme S results in profound tissue destruction in lungs, burn and wounds infections. The extremely toxic ExoA is produced by almost every strain of *Pseudomonas aeruginosa*; it can impair cellular protein synthesis by inactivating polypeptide chain elongation factor 2 [8]. Exotoxin A is an important pathogenicity determinant of Pseudomonas spp., with a molecular weight of 66 kD; the action of which is to that of diphtheria toxin. The gene encoding for Exotoxin A is present in 90-95 % of *P. aeruginosa* strains whereas other *Pseudomonas* spp. and GC-rich microorganisms did not show evidence of *tox* A gene presence [9]. ETA contains two fragments or subunits; catalytic subunit A, and subunit B responsible for interacting with target cell surface receptors. Exotoxin Ahas considerable cytotoxicity to several mammalian cell types [10]. It is an extremely potent toxin, showing LD50 of 2.5 mg/kg in mice, and it is evidenced that mutants lacking *tox* A gene are less virulent than wild type variants, as well as immunization against

exotoxin A results in partial immunity to *P. aeruginosa* infection in animal models [11].

The aims of the present work were to identify pathogenic isolates of *P. aeruginosa*, PCR-amplify two important virulence factors encoding genes namely, *oxa* 10 and *tox* A genes responsible for antibiotic resistance and production of Exotoxin A, respectively; and to sequence the relevant PCR products of the two genes.

Materials and Methods

A total of fifty pre-identified clinical isolates of *P. aeruginosa* isolated from burn infection were collected from different age group, male and female patients who attended major hospitals in Duhok and Erbil/Iraq. Collection of specimens started from April 2015 to September 2015. Bacterial isolates were identified according to standard laboratory methods described in the literature [12].

Antibiotic Sensitivity Test

Disc diffusion method was performed to determine the antibiotic sensitivity profiles of the studied isolates. [13, 14] Briefly, the inoculums were prepared by culturing colonies on brain heart infusion broth, then it was spread evenly over a plate of Muller-Hinton agar, antibiotic discs were put on the streaked plates. The measurements of the inhibition zones diameters were determined and interpreted as sensitive or resistant according to the chart of interpretation of zone sizes. Table 1 describes the tested antibiotics and their relevant potency.

Table 1: Antibiotics used throughout the study and their relevant potency

Number	Antibiotic	Symbol	Disc Potency
1	Gentamicin	AK	10 µg
2	Tobramycin	TN	30 µg
3	Amikacin	CN	10 µg
4	Ticarcillin/clavulanic acid	TNC	10 µg
5	Ampcillin/Sulbactam	AMS	5 µg
6	Ampcillin	Am	30 µg
7	Piperacillin	PN	[20/10] µg
8	Meropenem	MP	10 µg
9	Imipenem	IMP	25 µg
10	Cefepime	CFM	30 µg

Extraction of genomic DNA

Genomic DNA of fifty *P. aeruginosa* isolates was extracted according to the phenol chloroform method described in the literature [15].

Electrophoresis [Agarose]

Different concentrations of agarose were prepared; the concentration and purity of DNA were determined using Nano-drop system [16].

PCR amplification for 16S rRNA, *tox* A, and *oxa* 10 genes

DNA fragment encoding for 16S rRNA specific for *Pseudomonas aeruginosa* was detected by PCR-amplification using specific primers (F-5"GGGGGATCTTCGGACCTCA3" and R-5"TCCTTAGAGTGCCCACCCG3") [17]. Master-mix was prepared for the whole fifty isolates enrolled in the study. The amplification reaction involved 25 cycles, the final volume was 25µl and it consisted of 12.5 µl master mix, 1µl of each forward and reverse primers (10 pmol/µl), 4µl of sample DNA (25-50 ng/µl) and 6.5µl of sterile deionized distilled water(D.D.W). The amplification conditions started with 2 minutes of initial denaturation under 95 ^{o}C followed by denaturation step for 20 seconds

under 94 ^{o}C, then annealing step at 54 ^{o}C for 20 seconds then extension step at 72 ^{o}C for 40 seconds, eventually final extension at 72 ^{o}C lasted for five minutes. The primers used for the amplification of *tox*A (encoding for exotoxin A) gene were F-5"GACAACGCCCTCAGCATCACCAGC3" and R-5"CGCTGGCCCATTCGCTCCAGCGCT3" [18] while those used for the amplification of *oxa* 10 gene were F-5" TATCGCGTGTCTTTCGAGTA3" and R-5"TTAGCCACCAATGATGCCC3"[19].

The amplification reaction volume was 25µl prepared as follows: 12.5µl master mix, 1µl of each forward and reverse primers in a concentration of 0.5 pmol/ µl, 4µl (25-50ng) of sample DNA. The final volume was adjusted by adding 6.5 µl of D.D.W. The PCR programs are illustrated in table 2.Electrophoresis with 1.5 % agarose was done and molecular marker (100-1500bp) was added.

Table 2: PCR-amplification programs for *tox* A and *oxa* 10 genes

***tox* A**	94 ^{o}C 2 min	94 ^{o}C 2 min.	68 ^{o}C 1 min.	72 ^{o}C 1 min.	72 ^{o}C 7min.
	1 cycle	30 cycles			1 cycle
***oxa*10**	95 ^{o}C 4 min.	95 ^{o}C 45 sec.	56 ^{o}C 1 min.	72 ^{o}C 1 min	72 ^{o}C 5 min
	1 cycle	30 cycles			1 cycle

Sequencing of PCR products for randomly selected isolates

The PCR products were purified by using a commercial PCR product purification kit following the manufacturer's instructions. Purified PCR products were then sequenced by Bioneer® (South Korea) (http:// dna. macrogen.com).

DNASTAR/Laser Gene software war used to analyze the sequenced PCR products; analysis performed at the Scientific Research Center (SRC)/ University of Duhok. The results of sequencing were loaded in the Blast searching tool [http://blast.ncbi.nlm. nih. gov/Blast.cgi] to reveal similarity between GeneBank sequences and those of the present work.

MOLE-BLAST tools was applied to categorize numerous undetermined sequences and to reveal their relationship. The MOLE-BLAST service available in http://blast.ncbi.nlm.nih.gov/moleblast/ moleblast. cgi, then Megline (ClustalW) Laser Gene Software was used to align the sequences of the current study at the nucleotides level with the reference sequences retrieved from the Gene Bank. Then, a phylogenetic tree was constructed to address both the identity and the relatedness between sequences obtained from the present study with sequences from the Gene Bank.

Results

The results of the present study showed that all the selected isolates showed phenotypic criteria characteristics of *P. aeruginosa*. Antibiotic resistance profiles of the isolates ranged from the highest to

Cefepime shown by 98% of the isolates to the lowest to imipenem revealed by 50 % of the isolates (Table 3).

Table 3: Antibiotic sensitivity profile for the enrolled fifty isolates of *P. aeruginosa*

Antibiotic	Class	No Resistant isolates [%]
Amikacin	Aminoglycoside	46 [92]
Tobramycin	Aminoglycoside	48 [96]
Gentamicin	Aminoglycoside	46 [92]
Ticarcillin/clavulanate	Beta-Lactam	35 [70]
Ampcillin/Sulbactam	Beta-Lactam	40 [80]
Ampcillin	Penicillin	43 [86]
Piperacillin	Penicillin	44[88]
Meropenem	Carbapenem	47 [94]
Imipenem	Carbapenem	25 [50]
Cefepime	Cephalosporin	49 [98]

It is also shown that all the enrolled isolates of *Pseudomonas aeruginosa* had successfully revealed the presence of genomic DNA when subjected to DNA extraction procedures thus they were considered candidates for further molecular investigation [Figure 1].

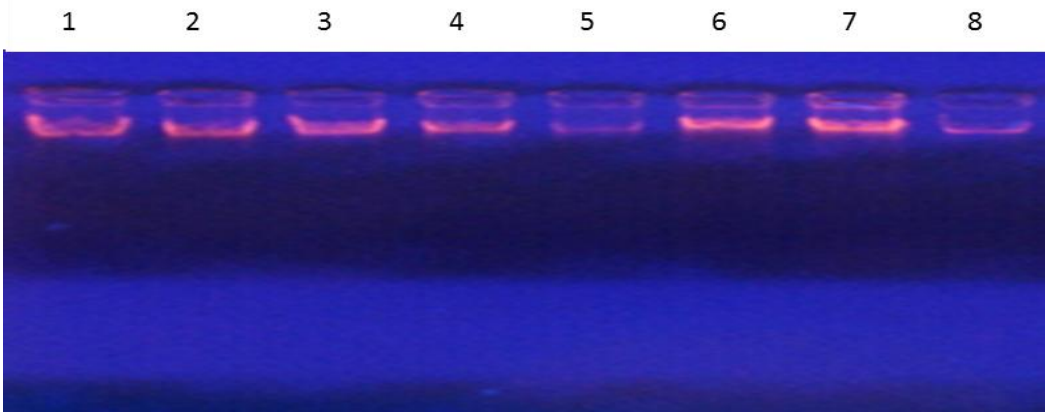


Figure 1: Genomic DNA bands of *P. aeruginosa* run on 1% agarose gel at 4V/cm for 45 minutes.

The results of the current study indicated that all the fifty isolates produced a single amplification band with a molecular weight of 956 bp characteristic of the 16S rRNA specific for *P. aeruginosa* thereby augmenting the primary phenotypic identification (Figure 2).

Figure 2: Species- Specific PCR amplification for selected isolates of *P. aeruginosa.* Amplicon size was 956 bp. Agarose gel 1%, 5v/cm, 1.45 hour conditions were adopted for electrophoresis. Molecular weight marker (1500-100bp) run through lane 1.

It is revealed that *oxa*10 gene was successfully amplified in forty six (92 %) isolates out of fifty; PCR products bands of 760bp molecular weight appeared by electrophoresis (Figure 3).

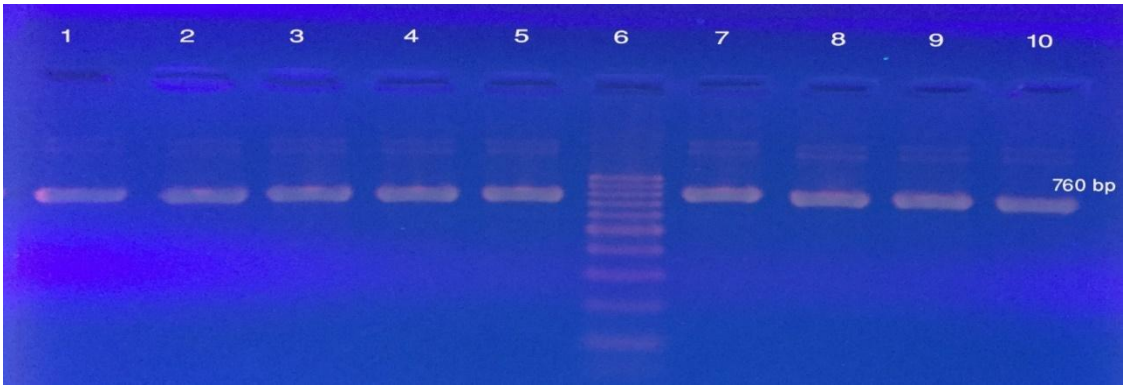


Figure 3: PCR amplification of oxa10gene and the resulted amplicon with molecular weight of 760 bp. Electrophoresis run on 2 % agarose gel with 5v/cm for 1 hour. Lane 6:DNA molecular weight marker (1500-100 bp).

The gene encoding for Tox A was properly amplified in forty two isolates out of fifty corresponding to 84 % of the isolates manifested by amplification bands of 396 bp molecular weight (Figure 4).

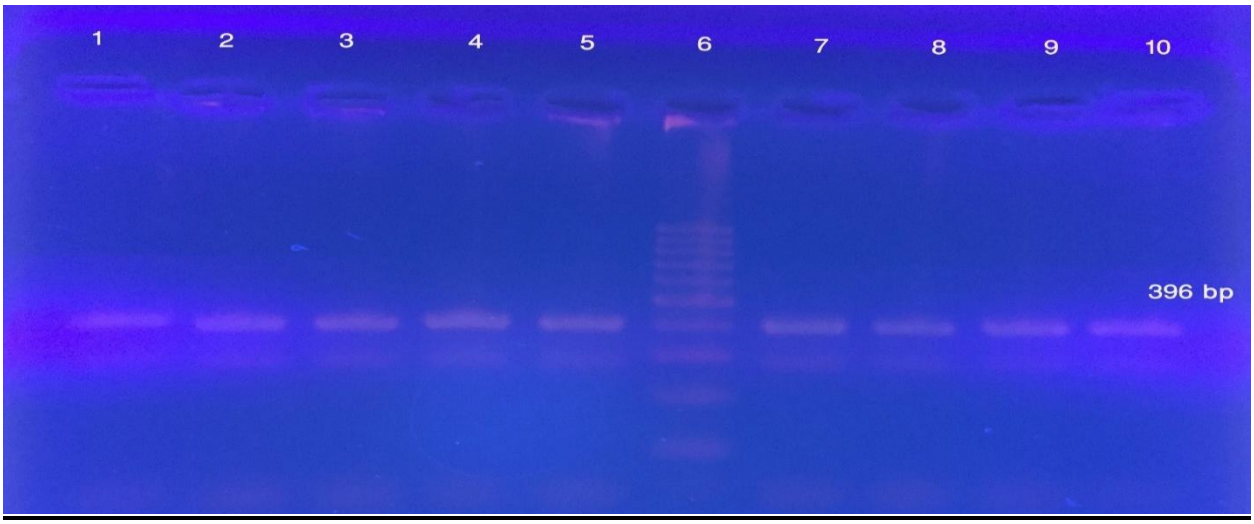


Figure 4: PCR amplification of *tox* A gene and the resulted amplicon with molecular weight of 396 bp. Electrophoresis run on 2% agarose gel with 5v/cm for 1.0 hour. Lane 6: DNA molecular weight marker (1500-100 bp).

Sequencing and comparison results of five DNA samples of *P. aeruginosa* expressing *oxa* 10 gene with two positive controls (NZ_KB894767.1 and NZ_ CDBY01000029.1) and one negative control (KX039965.1) had shown the diversity among these groups at the nucleotide level (Figure 5) and it was clear that isolates A4 and A5 are much more related when compared to the others, moreover, all the studied isolates were related when compared with positive controls and a distinctive diversity appeared when comparisons extends to involve the negative control.

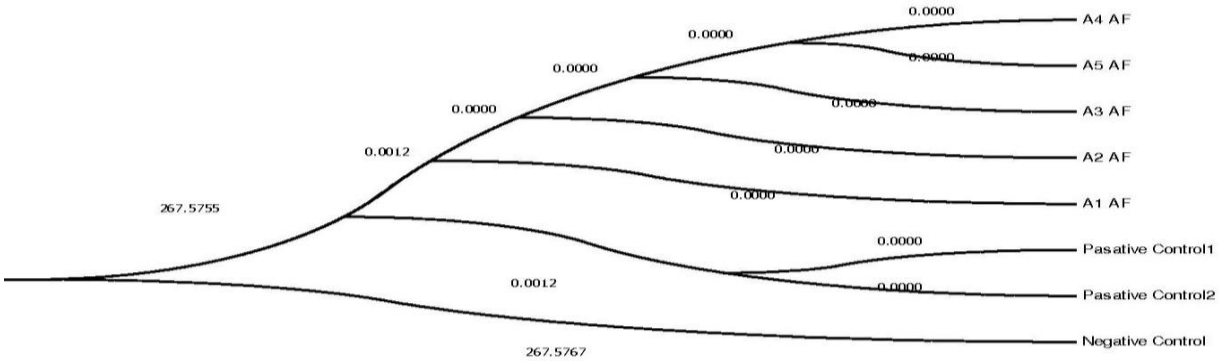
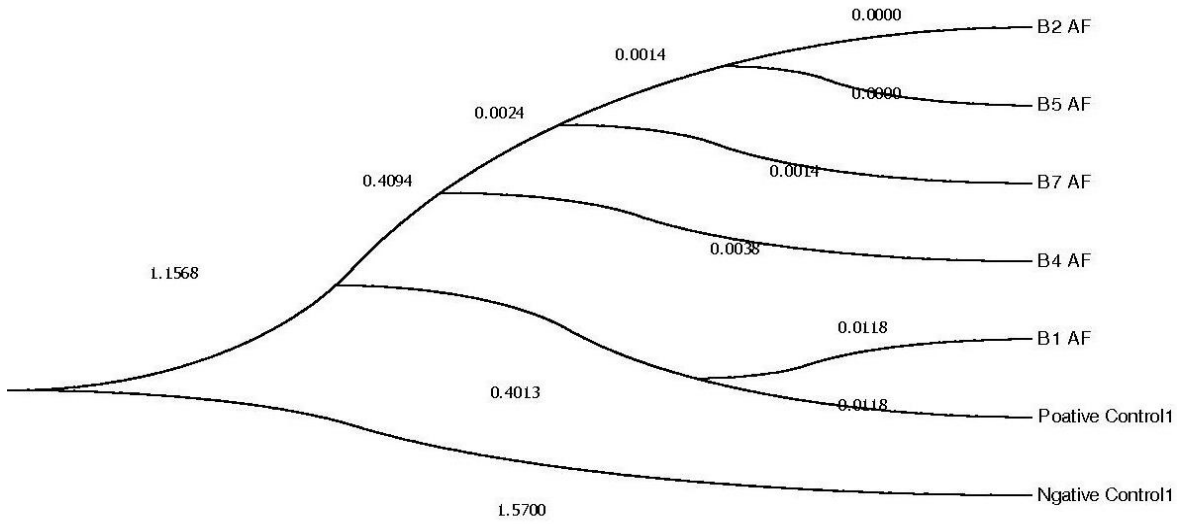


Figure 5: The relatedness and diversity shown by *P. aeruginosa* strains when sequences of the PCR products of *oxa* 10 gene were compared.

Diversity at the nucleotide level was also investigated when the sequencing of PCR-amplification product of *tox*A gene of *P. aeruginosa*was compared with the positive control (NC_002516.2) and the negative control (KX039965.1). It was evident that isolates B2 and B5 are much more related in comparison with the other isolate, furthermore, isolate B1 revealed striking relatedness to the positive control while all strains clearly diverged from the negative control (Figure 6).



Posative Control 1: Pseudomonas aeruginosa with NCBI Code NC_002516.2
Negative Control : Echinococcus granulosus with NCBI Code KX039965.1

Figure 6: The relatedness and diversity shown by *P. aeruginosa* strains when sequences of the PCR products of *tox*A gene were compared**.**

Discussion

Although considerable improvement in the survival of burn victims has been attained, complications after primary infection still represent the primary etiology of morbidity and mortality [20].

Burn wound infection due to *Pseudomonas aeruginosa* creates an important danger in terms of sepsis, graft loss, prolonged durations of hospital admission, and increased mortality [21].

Infections by *P. aeruginosa* are hardly treated because of the intrinsic ability of the bacteria to resist many classes of antibiotics and the capability to acquire resistance trait through several mechanisms. All the documented antibiotic resistance strategies might be noticed in this pathogen[adaptive, acquired, and intrinsic]; many times all within an individual isolate and the resistance rates are exaggerating despite the usage of combination drug therapies [22]. As few recent medications are now in use to counteract infections caused *P. aeruginosa*, there has been an approach to re-use older agents such as polymyxins that had originally rendered out of use due to extensive information of deleterious drawbacks [23].

Nosocomial outbreaks due to multidrug resistant bacteria (MDR) had been almost recognized as a result of infections caused by bacterial colonization of burn lesions. [24]. Multidrug resistant strains of *P. aeruginosa* (resistant to at least three of the following antibiotics cefotaxime, imipenem, gentamicin and ciprofloxacin) are usually isolated from persons affected by nosocomial infections [4, 25]. The major source of this type of resistance is the release of β-lactamase enzyme. The synthesis of the enzyme might be elicited to an elevated expression rate resulting in a quantity high enough to confer resistance [26].

In the current research, antibiotic sensitivity study revealed resistance trait to the generally prescribed antibacterialssuch as gentamycin, cefepime and meropenem which are usually indiscriminately given as primary therapeutic approach for prolonged duration. Other researchers also noted this higher rate of resistance to the above mentioned agents [27]. Also, resistance to

tobromycin was higher than that reported by others [25]. The results of the present study showed that imipenem was the most effective antibacterial against *Pseudomonas aeruginosa* was with 50 % of the studied isolates being sensitive while the higher resistance (98%) was displayed by cefipime which in turn can be justified by the prolonged and continuous prescription of this antibiotic for the treatment of pseudomonas infection in our locality.

P. aeruginosa has multiple pathogenicity determinants that may participate to its disease causing ability [28]. Exotoxin A is an extracellular toxin that is released by the majority of clinical strains of *Pseudomonas aeruginosa*. It contributes to the pathogenicity of the bacterium by inhibiting the synthesis of proteins, direct cellular effects, and alteration of the immune functions of the targets [29]. As this toxin is usually produced by this pathogen, it can be a reliable candidate for rapid identification and differentiation of *P. aeruginosa* in clinical settings [30]. The results of the present work were not correlated with these of other investigators [31]who claimed that *tox* A gene was successfully amplified in 100 % of their isolates, however, when the high expression of the gene is taken into consideration, both studies confirmed the importance of *exo* A as far as invasion in concerned. In a study conducted by a group of researchers [32], they stated that exo A gene was properly identified in 26% of their isolates and this markedly disagreed with the results obtained in the current study, the discrepancy might be explained by the fact that different isolation source of the microbe dictate different genetic ability to express a gene of interest, as invasion in cases of otitis is not a priority for the pathogen compared to burn infections [33].

Among *P. aeruginosa* isolates, research indicated that there is a conserved set of 5021 genes, but between two different isolates of *P. aeruginosa*, a substantial variation can be detected [34]. It is generally accepted that, the greater the number of the sequenced genomes the higher the variation among pseudomonas isolates [35]. It was postulated that the huge and complex genome of *P. aeruginosa* reflect evolutionary adaptations allowing it to survive in different ecological sites.

P. aeruginosa has well known abilities to transport, metabolize and grow on organic materials, variety of iron-siderophore intake systems, and the improved characteristic to produce molecules [e.x., enzymes]. *P. aeruginosa* also has the highest number of genes devoted to command and control systems noticed within microorganism. The proposed role of theses regulatory genes is the alteration of the transcriptional activity and biochemical characteristics in response to a change in environmental settings. Infections due to pseudomonas spp. are difficult to manage because of intrinsic antibiotic resistance. Indeed, the unusual regulatory features may offer greater flexibility for manipulative drug resistance by gene activity modulation than found in other bacterial spp. collectively, the divergent metabolic ability, transport mechanisms and regulatory adaptations empower this bacterium to survive and compete with other microorganisms. Therefore, sequencing of genes provides an interesting data for the discovery and usage of new antibacterial agents, and an avenue for the invention of efficient approaches to combat the serious nosocomial conditions attributed to this pathogen [36].

Conclusion

The majority of the studied isolates constitutively expressed *oxa* 10 and *tox*A genes and the relevant sequences of the two genes revealed striking similarity indicating that these genes are conserved among isolates and their diversity is a matter of simple change that implies no functional alterations.

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