PCR Detection of Some ESBLs (*bla*) Genes in *Pseudomonas aeruginosa* Isolated from Burn's Units in Bagdad Hospitals

الكشف عن بعض جينات البيتا لاكتام الواسعة الطيف لبكتريا الزائفة الزنجارية المعزولة من وحدات الحروق لمستشفيات بغداد باستخدام تفاعل سلسلة البلمرة

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Abstract

Multi drug resistant (MDR) and Extended Spectrum Beta Lactamase (ESBLs) of *Pseudomonas aeruginosa* were detected. *Pseudomonas aeruginosa* is a bacterium responsible for severe infections in burn's units, plasmid DNA analysis and encoded many types of genes responsible for beta-lactamases. To determine the type of genes responsible for beta-lactam broad spectrum in *P. aeruginosa* strains isolated from 100 swabs of burn's units environment, using a molecular methods (PCR) by primers specific to ESBLs (*bla*) genes oxacillin hydrolyzing capabilities OXA-10, OXA-4 and Vietnam Extended-Spectrum β-Lactame VEB-1. The results revealed that 15 strains were isolated from burn units environment. All of 15 (100%) were positive OXA-10 and only one (6.6%) for OXA-4 while the other gene VEB-1 was found in 6 (40%) isolates.

Key words: Burn's units, *Pseudomonas aeruginosa*, PCR, Resistance genes.

الملخص

تم الكشف عن المضادات الحيوية المتعددة والواسعة الطيف لسلالات الزائفة الزنجارية، حيث ان هذه البكتريا تكون مسؤولة عن حصول التهابات حادة لدى المصابين الراقدين في وحدات الحروق، ومن خلال استخلاص وتحليل الدنا لبكتريا الزائفة الزنجارية تم الكشف عن تواجد العديد من الجينات المشفرة المسؤولة عن مقاومة البيتا لاكتام الواسعة الطيف. ولتحديد نوع الجينات المسؤولة عن البيتا لاكتام الواسعة الطيف لسلالات بكتريا الزائفة الزنجارية في 100 من المسحات المعزولة من بيئة وحدات الحروق باستخدام الطرق الجزيئية لتفاعل سلسلة البلمرة مع بادئات محددة لـــجينات البيتا لاكتام الواسعة الطيف خاصة للجينات (OXA-10)، (OXA-4) و(VEB-1). وكشفت النتائج أن 15 سلالة التي تم عزلها من بيئة الحروق كانت كلها 15 (100%) موجبة للجين OXA-10، وواحدة فقط (6.6%) للجين OXA-4 والجين الاخير VEB-1 ظهر في 6 (40%) من العزلات.

الكلمات المفتاحية: وحدات الحروق، الزائفة الزنجارية، تفاعل سلسلة البلمرة، جينات المقاومة

Introduction

Hospital environment is contaminate by a variety of pathogenic and nonpathogenic microorganisms that can persist on surfaces for prolonged periods, numerous studies showed that hospital surfaces and frequently used medical equipments become contaminated by a variety of these microorganisms. The acquisition of nosocomial pathogens by a patients and the resultant development of infection depend on a multifaceted interplay between the environment, a pathogen and a susceptible host [1]. Transmission can occur either indirectly when a healthcare worker's hands and/or gloves become contaminated by touching contaminated surfaces after which they touch patients, or when a patient comes in direct contact with a contaminated surface [2]. Antibiotic resistance in *P. aeruginosa* may be mediated via several distinct mechanisms including modification of site-targeted drugs or outer membranes, β-lactamase production, and efflux pumps. The increase in antibiotic resistance is mostly due to extensive abuse of antibiotics such as ciprofloxacin, β-lactamase and aminoglycosides in the burn centers as well as non-availability and high costs of other effective drugs [3]. Extended spectrum β- lactamases (ESBLs) are a group of enzymes produced by some microorganisms with the ability to hydrolyse and cause resistance to the antimicrobial agents as Oxymino - Cephalosporins (i.e.Cefotaxime, Ceftazidime, Ceftriaxone, Cefuroxime and Cefepime) Monobactams (i.e. Aztreonam), whereas cannot hydrolyze Carbapenems (i.e. Imipenem) efficiently [4]. Generally, ESBLs are not carried on the bacterial chromosome, rather they are found on an independent element of DNA called a plasmid. Plasmids can carry many different genes on them and have the ability to transfer a replica of themselves to other bacteria. This can be very serious for a number of reasons [5]. The molecular classification of b- lactamases is based on the nucleotide and amino acid sequences in these enzymes. To date, four classes are recognised (A-D), correlating with the functional classification. Classes A, C, and D act by a serine based mechanism, whereas class B or metallo b- lactamases need zinc for their action [6].

These enzyme are named OXA because they preferentially hydrolyze oxacillin and cloxacillin. These enzymes confer resistance to ceftazidime and are poorly inhibited by clavulanic acid [7]. The OXA-1 and OXA-4 enzymes are highly homologous: there are only two amino acid differences, with the OXA-4 enzyme having Aspartic acid 48 Valine and Aspartic acid 207 Glutamic acid substitutions relative to the OXA-1 sequence, OXA-4 *β*-lactamase is generally plasmid- mediated, the chromosomal DNA of these isolates, but not their plasmids, hybridized with the OXA-4 gene amplified by the PCR method [8]. OXA-10 β-lactamase also possesses the ability to hydrolyze Cephalosporins, hydrolyzing Cefotaxime, Ceftriaxone, and Aztreonam at low levels but sparing Ceftazidime, Cephamycins, and Carbapenems [9]. VEB-1 (for Vietnamese extended spectrum beta – lacctamase), VEB-1 has highest amino-acid identity with Pseudomonas Extended Resistance (PER-1) and (PER- 2) (38%), and confers high-level resistance to Ceftazidime, Cefotaxime and Aztreonam [10].

Aims of Study

Molecular detection of *bla* genes OXA-4, OXA-10 and VEB-1 which responsible for extended-spectrum β-lactamase (ESBL) in multidrug resistant *P. aeruginosa* strains.

Materials and Methods

Burn's Units Swabs Collection

This study last for nine months starting from beginning of August 2012, till the end of April 2013. One hundred environmental swaps were collected from burn's units in three hospitals in Baghdad, Al-Kindi General Teaching Hospital / Rusafa (40 swabs), Al-Yarmuk General Teaching Hospital / Karkh (35 swabs) and Al-Imam Ali Hospital / Rusafa (25 swabs). The swabs collected from burn's units environment included (gloves, beds, floors, benches, walls and washing baths) in the mentioned three hospitals.

Isolation and Identification of Bacteria

All swabs obtained were cultured directly on MacConkey agar and Blood agar media and, incubated aerobically at 37°C for 24 hr and citrimide agar at 42˚C. Identification by conventional biochemical methods and confirmed by API 20 E standardized identification system [11, 12]. Antimicrobial susceptibility test was conducted upon fifteen isolates of *P. aeruginosa* against 12 antibiotic was conducted by disc diffusions methods (DDM), as previously reported by Bauer**,** *et al* [13].

DNA extraction

Genomic DNA and Plasmid DNA were extracted from isolates of burn's units samples using 2 Mini Kits extraction Genomic DNA and Plasmid DNA, Purification depending on instruction of manufacturing company (Geneaid, Thailand).

PCR detection of ESBLs genes

Conventional PCR was used for the detection of ESBLs genes in *P. aeruginosa* isolated from burn's units (environmental isolates), which were (*bla* OXA-10), (*bla* OXA-4) and (*bla* VEB-1).

The primers sequence for ESBL genes are shown in Table (1).

Primer preparation: Lyophilized forward and reverse primers were suspended with suitable volume of TE buffer as recommended by Bioneer Corporation protocol. Lyophilized primers were dissolved in deionized water to give a final concentration of (100 pM/µl) (stock solution); to prepare 10µM concentration as work primer solution then 10 pM/µl was re-suspended in 90µl of deionized water to reach a final concentration of 10 µM.

Table (1): The sequence of forward and reverse primers of *bla*OXA-4, *bla*OXA-10 and *bla*VEB-1 genes.

Primer Name	5' – Sequence - 3'	Detected gene	Product size
OXA-4 (F)	TCA ACA GAT ATC TCT ACT GTT	*bla*OXA-4	216bp
OXA-4 (R)	TTT ATC CCA TTT GAA TAT GGT		
OXA-10 (F)	TCA ACA AAT CGC CAG AGA AG	*bla*OXA-10	277bp
OXA-10 (R)	TCC CAC ACC AGA AAA ACC A		
VEB-1 (F)	CGA CTT CCA TTT CCC GAT GC	*bla*VEB-1	643bp
VEB-1 (R)	GGA CTC TGC AAC AAA TAC GC		

The PCR mixtures were performed in a total volume of 20µl consisting of the followings : 15µl of distilled water, lyophilized of PCR master mix (Bioneer Corporation) was dissolved by vortexing , and 2µl of each primer forward and reverse (10 pM each), final 3µl of DNA (total volume, 20µl) as illustrated in Table (2).

Table (2): The mixture of conventional PCR working solution for detection of OXA-4, OXA-10 and VEB-1 genes in *Pseudomonas aeruginosa*.

Component	Concentration	Volume (µl)
Deionizer water	-	15
Primer F.	10 picomol	1
Primer R.	10 picomol	1
DNA	5 – 50 ng	3
Total Volume		20 µl

Amplification was included in every set of PCR reactions, the reaction mixtures following a "hot start" were subjected to empirically optimized thermal cycling program which represented in Tables (3, 4 and 5).

Table (3): PCR program for OXA-4 gene amplification by conventional methods.

No.	Steps	Temperature (°C)	Time	cycles
1.	Initial Denaturation	95	4 min	1
2.	Denaturation	94	1 min	30
3.	Annealing	51	1 min	
4.	Extension	72	1 min	
5.	Final extension	72	5 min	1

Table (4): PCR program for OXA-10 gene amplification by conventional methods.

No.	Steps	Temperature (°C)	Time	cycles
1.	Initial Denaturation	96	5 min	1
2.	Denaturation	96	30 sec	30
3.	Annealing	55 ,58 ,60	45 sec	
4.	Extension	72	1 min	
5.	Final extension	72	5 min	1

Table (5): PCR program for VEB-1 gene amplification by conventional methods.

No.	Steps	Temperature (°C)	Time	cycles
1.	Initial Denaturation	94	5 min	1
2.	Denaturation	94	45 sec	30
3.	Annealing	50 – 64	1 min	
4.	Extension	72	1 min	
5.	Final extension	72	7 min	1

Results and Discussion

The cultural result of 100 environmental swabs collected from burn's units (gloves, beds, floors, benches, walls and washing baths) of the three hospitals, revealed that 38 (38%) of swabs gave positive result for bacterial growth and the rest 62 (62%) were negative. The predominant bacteria was *P. aeruginosa* 15 (39.50%), followed by *Staphylococcus aureus* 8 (21.0%) while *Klebsiella pneumonia* came thirdly 6 (15.7%), then *Escherichia coli* 3 (7.8%), *Pseudomonas putida*, *Enterobacteraerogenes* recovered in similar percentage 2 (5.26%) , the least isolated microorganisms were *Acinetobacterbaumannii* and *Proteus mirabilis* as 1 (2.63%) for each, as illustrated inTable-6.

Gram negative organisms a client the highest incidence, *P. aeruginosa* was found to be the most common isolate followed by *E.coli*, and *Klebsiella* spp [14]. These organisms did not have fastidious growth requirements and can grow at various temperatures and pH conditions prevalent in the hospital environment, and in addition, were able to exploit varieties of carbon and energy sources. These properties explain the ability of these pathogens to persist for a reasonable time in either dry or moist conditions in the hospital environment, thereby causing disease. These hard line posture combined with their intrinsic resistance to many antimicrobial agents, contribute to the organisms fitness and enable them to spread in the hospital environment [15]. One of the most worrisome characteristics of *P. aeruginosa* is its low antibiotic susceptibility, which is attributable to a concerted action of multidrug. *Pseudomonas aeruginosa* is naturally resistant to a large range of antibiotics and may demonstrate additional resistance after unsuccessful treatment, also it has ESBLs enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and Monobactam (Aztreonam) but did not affect Carbapenems (meropenem or imipenem), [16].

Table (6): Types of environmental isolates from burn's units.

Isolate	Number & frequency		Total No.	Percentage (%)
	Single Isolates	Mixed Isolates		
Pseudomonas aeruginosa	**11**	**4**	**15**	**39.50**
Staphylococcus aureus	**6**	**2**	**8**	**21.0**
Klebsiella pneumonia	**4**	**2**	**6**	**15.7**
Escherichia coli	**3**	**-**	**3**	**7.8**
Pseudomonas putida	**2**	**-**	**2**	**5.26**
Enterobacteraerogenes	**2**	**-**	**2**	**5.26**
Acinetobacterbaumannii	**1**	**-**	**1**	**2.63**
Proteus mirabilis	**-**	**1**	**1**	**2.63**
Total No.	**29**	**9**	**38**	**100**

Antimicrobial susceptibility was performed on 15 *P. aeruginosa* isolates against 12 antibiotics; 7 of them were ESBLs represented by Cefotaxime, Ceftriaxone, Ceftazidime, Imipenem, Aztreonam, Piperacillin and Cefepime, and to 5 antibiotics were non ESBLs represented by Aminoglycoside (Amikacin, Gentamicin and Tobramycin), Chloramphenicol and Fluoroquinolone (Ciprofloxacin), by DDM [13]. The antibiogram for studied isolates was revealed that all isolates 100% resist to Ceftrixone, Cefepime, Chloramphenicol and Tobramycin,and this resistance became 93.3 against Gentamicin, while 86.6 against each Cefotaxime and Ceftazidime, followed by 80% for Piperacillin, and lower resistance 60% for Aztreonam, Amikacin respectively and 46.5% for Ciprofloxacin. *Pseudomonas aeruginosa* are becoming resistant to commonly used antibiotics and gaining more and more resistance to newer antibiotics [17]. This study found that Imipenem is the drug of choice in treatment of *P. aeruginosa* , because 66.6% of isolates were susceptible to it and only five isolate were exhibit resistance, Table (7) represent these results.

Table (7): Antibiotic susceptibility of *Pseudomonas aeruginosa* isolates.

Antibiotics	Disk content	Resistant		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Cefotaxime	**30µg**	**13**	**86.6**	**-**	**-**	**2**	**13.3**
Ceftrixone	**30µg**	**15**	**100.0**	**-**	**-**	**-**	**-**
Ceftazidime	**30µg**	**13**	**86.6**	**1**	**6.6**	**1**	**6.6**
Imipenem	**10µg**	**5**	**33.3**	**-**	**-**	**10**	**66.6**
Aztreonam	**30µg**	**9**	**60**	**-**	**-**	**6**	**40**
Amikacin	**30µg**	**9**	**60**	**1**	**6.6**	**5**	**33.3**
Gentamicin	**10µg**	**14**	**93.3**	**-**	**-**	**1**	**6.6**
Ciprofloxacin	**5µg**	**6**	**40**	**-**	**-**	**9**	**60**
Piperacillin	**100µg**	**12**	**80**	**1**	**6.6**	**2**	**13.3**
Cefepime	**30µg**	**15**	**100.0**	**-**	**-**	**-**	**-**
Chloramphenicol	**30µg**	**15**	**100.0**	**-**	**-**	**-**	**-**
Tobramycin	**10µg**	**15**	**100.0**	**-**	**-**	**-**	**-**

The outcome of PCR amplification of ESBLs (*bla*) genes in the hospital environment clarified that *P. aeruginosa* noticed in almost all fifteen (39.50%) isolates were ESBLs producer. The *bla* OXA-10 gene was detected in all

isolates 15(100%) Figure (1), and in Figure (2), only 6 (40%) were positive for *bla*VEB-1 gene. While only one (6.6%) isolate was positive for OXA-4 ESBLs as shown in Figure (3), and Table (8). The structure of the *P. aeruginosa* genome is a mosaic to be the result of multiple acquisitions from different donors during its evolution, to horizontal gene transfer includes the presence of genes or remnants of genes associated with mobile elements (i.e., insertion sequences, bacteriophages or plasmids) and the presence of numerous genomic islands [18]. There is no phenotypic confirmatory method for the presence of OXA gene and VEB-1 in *P. aeruginosa*, the current study provides precise and the only reliable genotypic method for detecting OXA, VEB resistance genes in *P. aeruginosa.* In a similar study conducted by Bert *et al*. [19], they reported that PCR detecting OXA-10 gene in *P. aeruginosa* isolates was positive in 68 (26.3%) isolates; 31 carried *bla*OXA-10, one carried *bla*OXA-14 and 36 carried a new variant intermediate between *bla*OXA-13 and *bla*OXA-19, levels of antimicrobial susceptibility and mechanisms of resistance to anti pseudomonal agents of *P.aeruginosa* were evaluated by a European team, [20] who showed close results to our study except for Impinem.

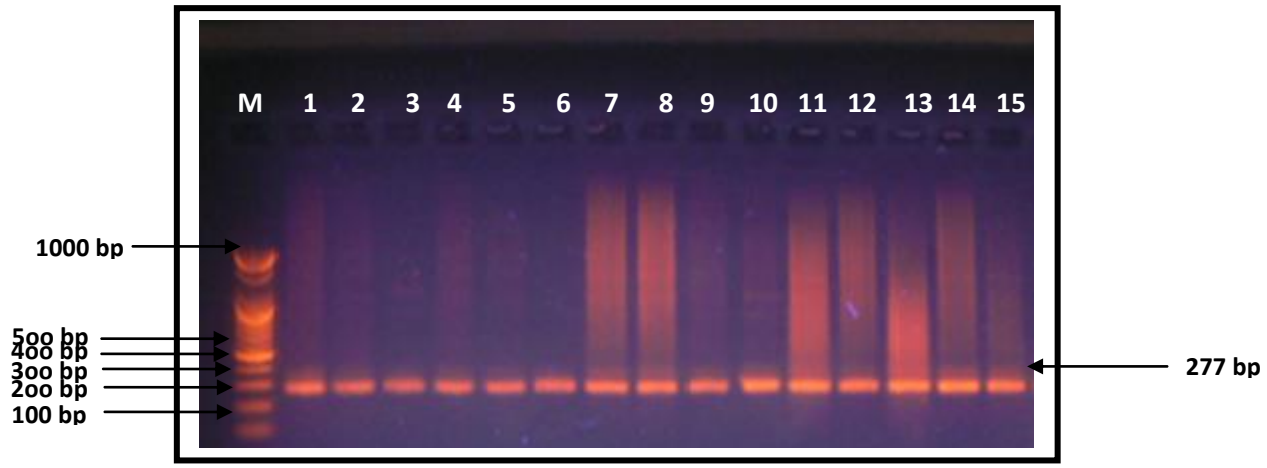

Fig. (1): Agarose Gel electrophoresis of PCR product for the detection of ESBL *bla*OXA-10 gene (277bp) using 1%agarose for 90 min at 70 volt , stained with ethidium bromide, M: Marker DNA ladder Size (1000bp); Lanes 1-15 positive for *bla*OXA-10 (277 bp)

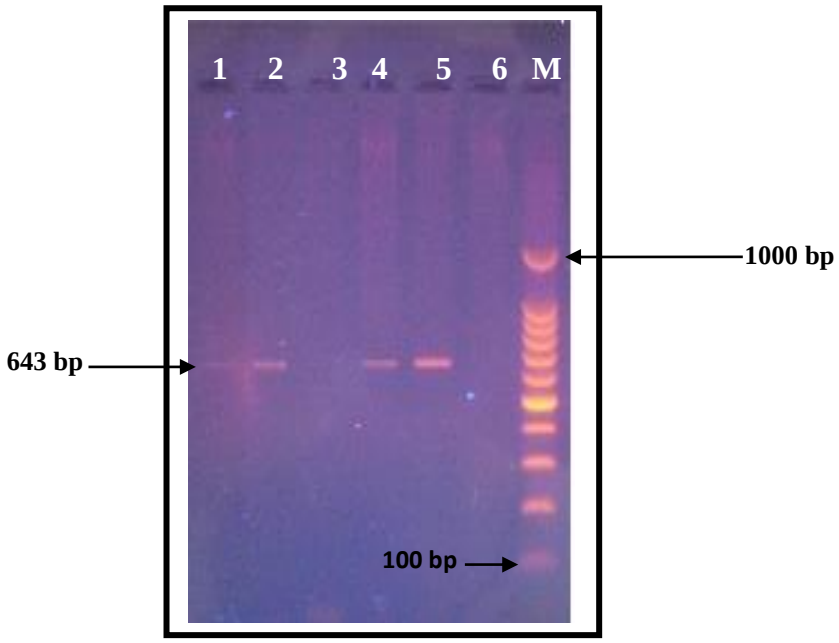

Fig. (2): Agarose Gel electrophoresis of PCR product for detection of ESBL *bla*VEB-1 gene (643bp) using 1% agarose for 90 min at 70 volt , stained with ethidium bromide, M: Marker DNA lader Size (1000bp) ; Lanes 1,2,4,5 positive for *bla*VEB-1 (643 bp).

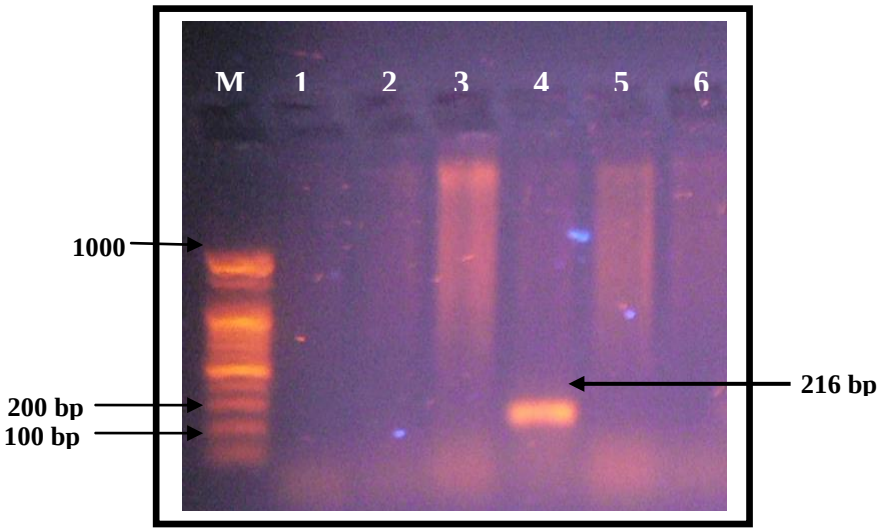

Fig. (3): Agarose Gel electrophoresis of PCR product for detection of ESBL *bla*OXA-4 gene (216bp) using 1%agarose for 90 min at 70 volt , stained with ethidium bromide, M: Marker DNA lader Size (100bp); Lanes (4) positive for *bla*OXA-4 (216 bp).

Table (8): Distribution of *bla* genes in environmental *P. aeruginosa* isolates

Samples Collected	No. *P. aeruginosa* isolates	*bla*OXA-10 (277 bp)		*bla*OXA-4 (216 bp)		*bla*VEB-1(643bp)	
		Positive	Negative	Positive	Negative	Positive	Negative
Burn's Units	15	15 100%	0 0.0%	1 6.6 %	14 93.3 %	6 40%	9 60%

Six isolates from total 15 carried more than one kind of gene on their plasmids, one isolate hold triple genes (*bla*OXA-10, *bla*OXA-4 and VEB-1), five isolates carried two gene with different kind of frequency for each genes, and 9 isolates carried only one gene *bla*OXA-10 as demonstrated inTable (9).

Table (9): Distribution of resistance ESBLs (*bla*) genes in environmental isolates.

No. *P. aeruginosa* Isolates	OXA-10 Positive	OXA-4 Positive	VEB-1 Positive
1. E.I.	+		+
2. E.I.	+		+
3. E.I.	+		
4. E.I.	+		
5. E.I.	+		
6. E.I.	+		
7. E.I.	+		
8. E.I.	+		
9. E.I.	+		
10. E.I.	+		
11. E.I.	+		
12. E.I.	+	+	+
13. E.I.	+		+
14. E.I.	+		+
15. E.I.	+		+
Total (15) Isolate from Burn's Units	15 (+)	1 (+)	6 (+)

In the current study, testing for ESBLs production using antibiotics agents isolates resistance and molecular detection of OXA-10, OXA-4 and VEB-1 genes in *P. aeruginosa* isolates revealed that there is a harmony between result of antibiotic resistance and positive molecular detection of these genes.

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