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APPLICATION OF MOLECULAR TECHNIQUES TO STUDY *PSEUDOMONAS AERUGINOSA* ISOLATED FROM BURN'S UNITS IN BAGDAD, IRAQ

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ABSTRACT

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) gene (OXA-10), (OXA-4) and (VEB-1). The results revealed that 15 strains were isolated from environment. All of 15 (100%) were positive OXA-10 and only one (6.6%) for OXA-4 and the final gene VEB-1 was found in 6 (40%) isolates.

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 (Branson et al., 2010). 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 (Tortora*et al.,* 1986; Kramer *et al.,* 2006).

Pathogens that have been linked to transmission via contaminated environmental surfaces and medical equipment include Gram positive such as Staphylococcus spp, and Gram negative such as Enterobacteraceaspp, and *Pseudomonas* spp. Which pose clinically important antimicrobial resistance problems and were among the most common causes of healthcare-associated infections (HAIs) (Tortora*et al.,* 1986; McGowan, 2006; Hidron*et al*., 2008). Burn patients were ideal hosts for opportunistic infections. The burn site remains relatively sterile during the first 48hr; thereafter, colonization of the wound by Gram positive and negative bacteria is common.

In Sweden, Appelgren*et al.* (2002) conducted a 3-year prospective study of all infections presented in the burns unit of a university hospital. Some 230 adult patients were included. Of these 83 patients had a total of 176 infections, giving an infection rate of 48 per 1000 patient, including both nosocomial and community-acquired infections. The most common microorganisms were coagulase-negative staphylococci and methicillin-sensitive *S. aureus*. The bacteria can infect the wound by the airborne route, direct contact from the hands of paramedics or contamination by non sterile equipment (Samy*et al*., 2003).

Pseudomonas aeruginosa was the number two causative bacteria which caused a change of the bacterial infectivity on day 5 and 10. These bacteria were always found when conducted bacterial

investigations from the water resource of the burns unit (Sudarmono and Wiwing, 2007).

Many research confirmed that *P. aeruginosa* has emerged as a predominant member of the burn wound flora (Pruitt *et al.,* 1998; Cochran et *al.*, 2002; Church *et al.,* 2006).

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 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 study included collected from burn's units environmental swabs (gloves, beds, floors, benches, walls and washing baths) in mentioned three hospitals.

Isolation and Identification of Bacteria

All swabs obtained were cultured directly on MacConkey agar and Blood agar, incubated aerobically at 37°C for 24 hr and citrimideagar at 42˚C. Identification by conventional biochemical methods and confirmed by API 20 E standardized identification system (Harly and Prescott, 2002; Forbes *et al.,* 2007 and Jawetz*et al*. (2007). The antimicrobial susceptibility test fifteen isolation of *P. aeruginosa* against (12) antibiotic was conducted by disc diffusions methods (DDM), as previously reported by Bauer *et al*. (1966).

Molecular Detection of ESBLs

Genomic DNA and Plasmid DNA were extracted from isolates of burn's unit's samples by using 2 Mini Kits extraction Genomic DNA and Plasmid DNA, Purification depending on instruction of manufacturing company (Geneaid, Thailand). Conventional PCR was used for 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).

PCR Reaction:

The primers sequence for ESBL genes showed in **Table (1).**

***Primer preparation*:**

Lyophilized forward and reverse primers were suspended with suitable volume of TE buffer as recommended by BioneerCorporation protocol. Lyophilized primers were dissolved in deionized water to give a final concentration of (100 pM/µl) (as stock solution); to prepare 10µM concentration as work primer re-suspended 10 pM/µl in 90µl of deionizer water to reach a final concentration 10µM These primers synthesized by Bioneercompany.

Table 1: The sequence 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 following: 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), (**Tables 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 (Tables -3,4and 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	
3.	**Annealing**	51	1 min	30
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	
3.	**Annealing**	55 ,58 ,60	45 sec	30
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	
3.	**Annealing**	50 – 64	1 min	30
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 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 *S. aureus*8(21.0%) while *K. pneumonia* came thirdly 6(15.7%), then *E. coli* 3(7.8%), *P. putida*, *E. aerogenes*recovered in similar rate 2(5.26%) ,the least isolated microorganism were *A.baumannii* and *P. mirabilis* as 1(2.63%) for each, (**Table -6**).

Gram negative organisms were found to be more incidences, *P. aeruginosa* was found to be the most common isolate followed by *S. aureus*, *E.coli*, and *Klebsiella*spp. (Church *et al.*, 2006). These organisms do not have fastidious growth requirements and can grow at various temperatures and pH conditions prevalent in the hospital environment, and in addition, are 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 (Hart and Kariuki, 1998; El-Mahmood*et al.,* 2010).

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 have ESBLs enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and Monobactam (Aztreonam) but do not affect Carbapenem (meropenem or imipenem), (Lee *et al.*, 2005).

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 the disc diffusion method (DDM), as described by (Bauer *et al.*, 1966). 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 each for 86.6 against Cefotaxime, Ceftazidime respectively, 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 (Rajat*et al.*, 2012). This study found that Imipenem is the drug of chose in treatment of *P. aeruginosa*, because 66.6% of isolates were susceptible to it and only five isolate were exhibit resistance (**Table-7**).

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 environmental *P. aeruginosa* DNA clarified that almost all isolates were ESBLs producer.

The *bla* OXA-10 genes was detected in all isolates 15(100%) (Figure -1), and 6(40%) were positive for *bla*VEB-1 gene (**Figure -2**). While only one (6.6%) isolate were positive for OXA-4 ESBLs (**Figure -3**), as shown in **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 (Kulasekara and Lory, 2004).

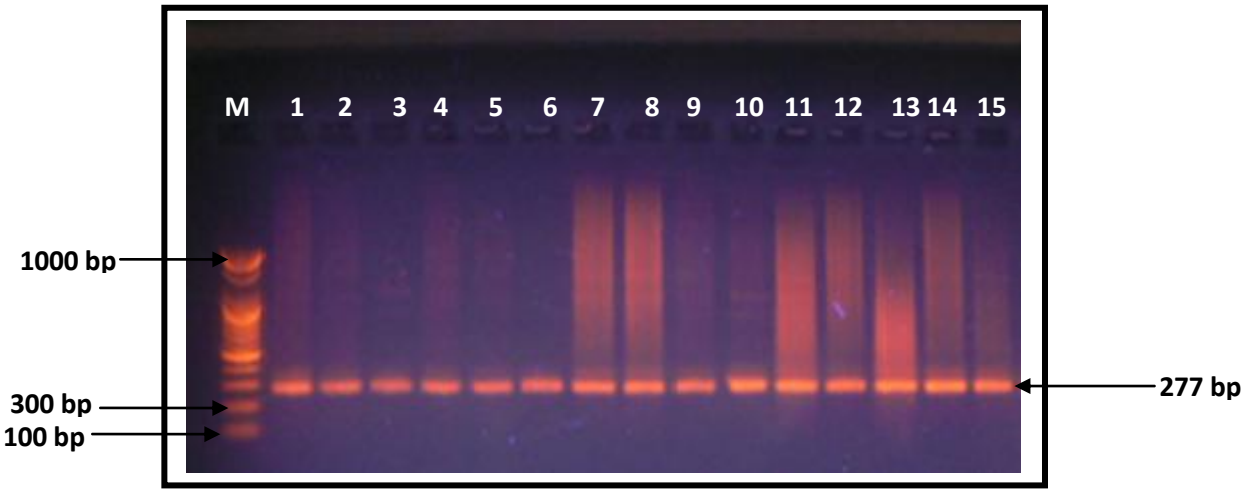

Figure 1: Gel electrophoresis of PCR product for detection of ESBL *bla*OXA-10 gene (277bp) using 1%agarose for 90 min at 70 volt

(1) M: Marker DNA ladder Size (100bp).

(2) Lanes (1-15) positive for *bla*OXA-10 (277 bp).

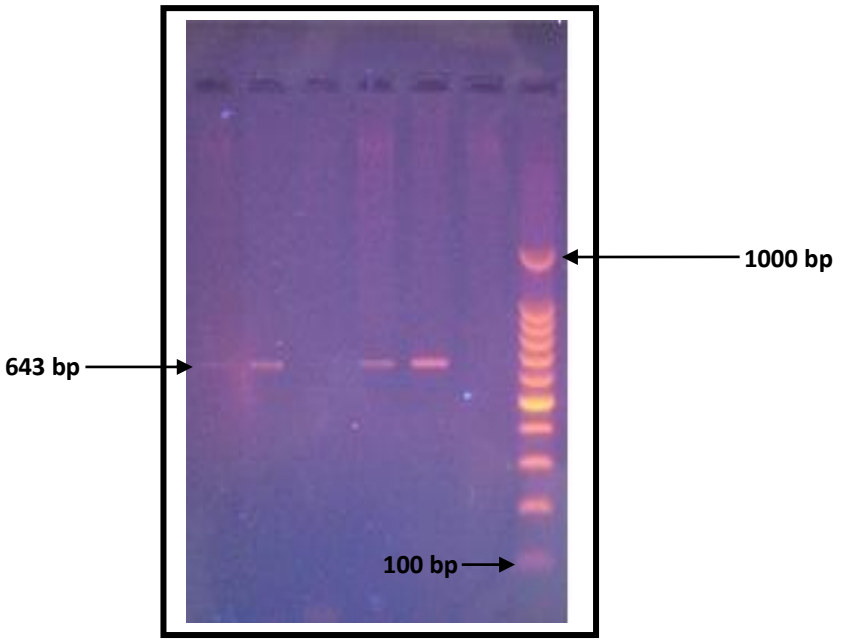

Figure -2: Gel electrophoresis of PCR product for detection of ESBL *bla*VEB-1 gene (643bp) using 1% agarose for 90 min at 70 volt

(1) M: Marker DNA lader Size (100bp).

(2) Lanes (1,2,4,5) positive for *bla*VEB-1 (643 bp)

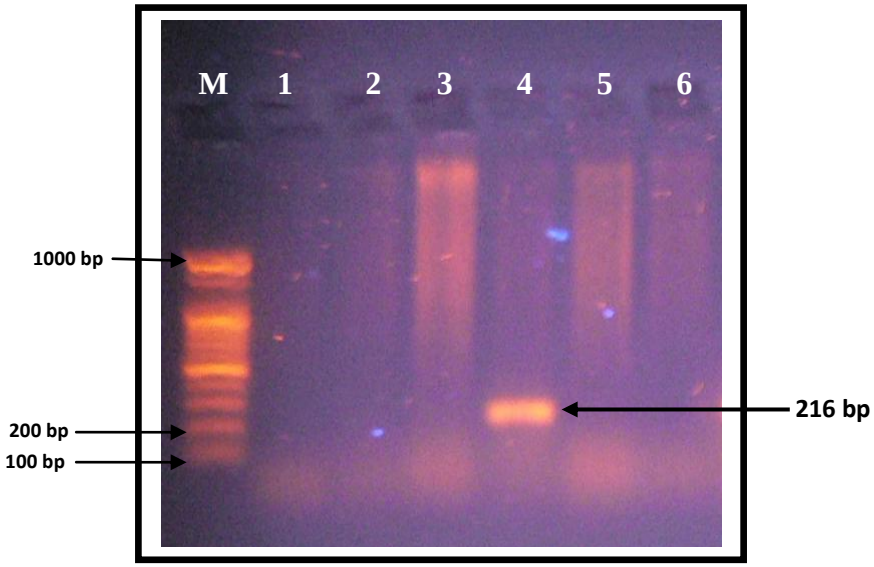

Figure -3: Gel electrophoresis of PCR product for detection of ESBL *bla*OXA-4 gene (216bp) using 1%agarose for 90 min at 70 volt

(1) M: Marker DNA lader Size (100bp).

(2) Lanes (4) positive for *bla*OXA-4 (216 bp).

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 (+)

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

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

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%)

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

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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