P- ISSN 1991-8941 E-ISSN 2706-6703 *Journal of University of Anbar for Pure Science (JUAPS)* Open Access
2012,(6), (3) :27-35

Molecular characterization of *Pseudomonas aeruginosa* Isolates Isolated from Clinical Patients by Using RAPD-PCR Technique.

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ARTICLE INFO

Received: 5 / 11 /2011
Accepted: 24 / 1 /2012
Available online: 29/8/2013
DOI: 10.37652/juaps.2012.78439

Keywords:
Molecular characterization,
P. aeruginosa,
RAPD-PCR.

ABSTRACT

The aim of the present study was the molecular characterization and the evaluation of variability and genetic relationship of six Pseudomonas aeruginosa isolates using PCR-based Randomly Amplified Polymorphic DNA (RAPD) technique. A total number of 86 samples were collected from patients that hospitalized in Tikrit Teaching Hospital in Tikrit city. These samples were taken from patients basing on the sources of infections, the isolates were taken from: wounds, ear, burns, urine, sputum, and eyes infections. Using enrichment, selective media, and biochemical tests, that characterized and identified as P. aeruginosa.

Genomic DNA was extracted from six P. aeruginosa isolates isolated from these different sources. These genomic DNA samples were found to have a suitable concentration and purity for RAPD-PCR analysis. RAPD-PCR technique was performed using 15 different Operon random primers. Eleven primers gave successful amplification results in repeated experiments. As a result, the total number of amplified bands was 270 and the total number of polymorphic bands was 234. The highest number of polymorphic bands (39 bands) was produced by primer OPX-01. The primer efficiency ranged from 3.70 (primer OPA-11) to 14.44 (primer OPX-01) and the discriminatory value ranged from 1.70% (primer OPA-11) to 16.66% (primer OPX-01). In addition, genetic distance and cluster analysis among different P. aeruginosa isolates were estimated by using UPGMA computer program basing on RAPD-PCR banding patterns that obtained in this study. These results suggesting that possible and frequent occurrence of mutations in DNA sequencing P. aeruginosa bacteria from different sources and locations. This study has proved existence genetic differences (DNA polymorphism) among the six P. aeruginosa isolates isolated from different sources. Therefore, we can say that RAPD technique could be an efficient technique for studying the molecular characterization and the epidemiology of P. aeruginosa bacteria.

Introduction

P. aeruginosa is an opportunistic pathogen that is a common cause of hospital- acquired infections, particularly infecting patients with predisposing factors, such as burn victim, immunocompromised hosts, or those with metabolic disorders. Also it is consider one of the most common nosocomial pathogens often causing major problems in intensive care units (1, 2).

In hospital, the bacterium is the leading cause of nosocomial lung infections, a common cause of wound infections, especially of thermal burns, nosocomial pneumonia infections, hospital acquired urinary tract infections, surgical wound infections, and blood stream infections (3,4). It is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce a variety of virulence factors (5). Multidrug-Resistant bacteria have commonly been reported as a cause of nosocomial infections (6).Molecular

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epidemiologic studies have an important role in determining the transmission routes of a pathogen (7).

Several molecular studies have been carried out to investigate diversity amongst *P. aeruginosa* isolates, polymorphism of certain of its genes and also genetic comparison of *P. aeruginosa* isolates from different hosts and environments (8,9,10,11). These were done to generate knowledge that would be useful in improving on the general management of infections due to *P. aeruginosa* (14). The molecular techniques widely used in these studies include RAPD-PCR (10, 11). Most of these studies reported high rate of polymorphism and genetic diversity among *P. aeruginosa* isolates.

Many studies have directed at *P.aeruginosa,* most studies are confined to the epidemiology and infections, concern outbreaks in patients. Only few articles are present in endemic situation on the genetic study of *P.aeruginosa* isolates from different infections. Therefore this study aimed to study the antimicrobial sensitivity profile, investigation and application of RAPD-PCR as an accessible molecular technique to detect polymorphism at DNA level among of six *P.aeruginosa* isolated. In addition, determine DNA fingerprinting, genetic distance and phylogenetic diversity of six *P.aeruginosa* isolated from different sources of infections wounds, ear, burns, urine, sputum, and eyes from patients that hospitalized in Tikrit Teaching Hospital.

Materials and Methods

Patients and Bacterial Isolates:

P. aeruginosa isolates were obtained from routine clinical specimens sent for bacterial culture from 86 patients admitted to different units of Tikrit Teaching Hospital from February to August 2011. Twenty-six cases were from intense care unit, and 60 cases from other locations including surgical unit, infectious diseases and different medicine wards. They were obtained from wound (n=19), ear (n=13), burn (n=8), urine (n=20), sputum (n=14), and eyes (n=12). All isolates were identified as *P. aeruginosa* on the basis of their typical colonial appearance, growth on Cetrimide agar, characteristic pigments, positive oxidase test and growth at 42ºC.

Isolation of Genomic DNA:

Total genomic DNA was extracted from only six isolates having six different sites (wounds, ear, burns, urine, sputum, and eyes) using a method described by (12). A single colony was inoculated on 5ml of brain heart infusion broth and incubated over night at 37ºC. Then 1.5 ml of a saturated culture was harvested with centrifugation for 5 min. at 14,000 rpm. The cell pellet was resuspended and lysed in 200µl of lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium-acetate, 1 mM EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µl of 5M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12,000 rpm for 10min. at 4ºC. After transferring the clear supernatant into a new eppendorf tube, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely formed. Following centrifugation at 14,000 rpm for 5min., the supernatant is then removed to another eppendorf tube and double volume of 100% ethanol was added. The tubes were inverted 5 to 6 times gently, then centrifuged at 10,000rpm for 5minutes. The supernatant was discarded and 1ml of ethanol (70%) was added to the pellet, and tubes centrifuged at 10,000 rpm for 5 minutes. Finally the supernatant discarded and the pellet was dried for 10 min at room temperature, The pellet was resuspended by 100µl H_2O. The stock was kept at -20ºC until use. The DNA concentration has been determined by measuring absorbance of the sample at 260 nm using spectrophotometer (13).

RAPD-PCR Amplification:

Fifty primers were used in this study, Eleven of these primers were give polymorphisms as listed in (Table 1).

Table 1: Primers and their sequences used in this study:

No.	Operon code	Nucleotide sequence (5'-3')	No.	Operon code	Nucleotide sequence (5'-3')
1	OPT-19	GTCCGT ATGG	7	OPD-20	ACCCGG TCAC
2	OPV-19	GGGTGT GCAG	8	OPE-20	AACGGT GACC
3	OPX-01	CTGGGC ACGA	9	OPF-16	GGAGTA CTGG

4	OPA-11	CAATCG CCGT	10	OPG-13	CTCTCCG CCA
5	OPB-17	AGGGAA CGG	11	OPI-06	AAGGCG GCAG
6	OPC-05	GATGAC CGCC			

Amplifications were performed in 25µl consist of 10x reaction buffer with $MgCl_2$ (Promega), 200µm dNTPs, 10 pmol of primer , 1 unit of *Taq* DNA polymerase and 25 ng of genomic DNA. The amplification were performed in thermocycler programmed as follow: 1 cycle 94ºC for 2 minutes, 40 cycles (92 ºC for 1 minutes; 37 ºC for 1 minutes; 72 ºC for 1 minutes) and 1 cycles 72 ºC for 7 minutes. The amplification products were resolved by electrophoresis in a 1.5% agarose gel in 1X TBE buffer at 75 volts for 1.5 hr (5 volt/cm)(14). Gels stained with Ethidium bromide, visualized under UV light and photographed using a high resolution digital camera (12.1 mega pixel), standard molecular weight markers also used in each electrophoresis run (15).

RAPD Data Scoring and Analysis:

The PCR-based DNA marker RAPD was used in this study as an accessible tool for studding the polymorphism, genetic variation and fingerprinting of *P. aeruginosa* isolates collected from different sites of infections**.** RAPD results were analyzed by using all information obtained from tables and figures. This information included; the presence or absence of amplified DNA bands, total number of amplified bands across all isolates of *P. aeruginosa*, the number of polymorphic bands, which can be detected horizontally.

The Numerical Taxonomy System (NTSYS) 1.8 software, using the Jaccard coefficient of similarity (16), and Unweight pair group method arithmetic (UPGMA) averages cluster analysis were used to calculate genetic distance and obtaining phylogenetic tree (17). Primer efficiency and discriminatory power were calculated for each primer using two equations as described by (18).

RESULTS and DISCUSSION:

Eighty six specimens were collected from different sources in Tikrit Teaching Hospital. After diagnosis these isolates as *P. aeruginosa* according to morphological and biochemical testes. Total genomic DNA was extracted from only six isolates having six different sites (wounds, ear, burns, urine, sputum, and eyes) for carry out RAPD-PCR markers.

Table (2): Sources of isolates subjected to RAPD analysis:

No. of isolates	Sources	No. of isolates	Sources
1	Wound	4	Urine
2	Ear	5	sputum
3	Burn	6	Eye

This study could be considered the first one accomplished in Iraq, Tikrit city that used Random Amplified Polymorphic DNA (RAPD) markers in the characterization of *P. aeruginosa* isolates. RAPD-PCR technique used in this study as a PCR-based molecular technique in an attempt to detect detection genetic variation and determine DNA fingerprinting of six *P. aeruginosa* isolates isolated from different sources.

Although various methods are variable for genetic characterization of bacterial isolates, RAPD technique was used successfully for *P. aeruginosa* and is less costly and time consuming than other techniques. The RAPD technique is straightforward and does not require previous knowledge of nucleotide sequence of target organism. Furthermore, it is very quick and convenient to perform, since a strain can be typed within 48 hours of harvesting the cells from the agar medium. It is highly sensitive, requires minimum amount of template DNA. Furthermore, RAPD-PCR is discriminatory because it analyzes the whole genome. However due to its sensitivity and reproducibility, can be affected by small variations in reaction mixture and temperature cycles. Thus, care is needed to standardize the procedure if it is to be used for routine analysis. Positive (type strain template DNA) and negative (no template DNA) should always be included (19,20). RAPD-PCR yield different information, since they analyze different sequences and detect different types of variations in the bacterial DNA. RAPD-PCR detects differences along the entire bacterial genome, not only in particular sequences. Thus, this system is helpful in characterizing bacterial isolates over long periods (21).

This study could be considered the first one accomplished in Iraq, Tikrit city that used RAPD-PCR technique in the characterization of six *P. aeruginosa*

isolates isolated from different sources of infections from patients that hospitalized in Tikrit Teaching Hospital. Eleven primers of 15 random primers (Table 3) amplified their targets across all the strains. The products of RAPD were analyzed as shown in (Table 3).

Table (3) :Shows the number of bands, number of polymorphic bands, efficiency & discriminatory value of each RAPD primer gave amplification product in this study:

No.	1	2	3	4	5	6	7	8	9	10
Primer code	OPT-19	OPV-19	OPX-01	OPA-11	OPB-17	OPC-05	OPD-20	OPE-20	OPF-16	OPG-13
Nucleotide sequence 5 to 3	GTCCG TATGG	GGGTG TGCAG	CTGGG CACGA	CAATC GCCGT	AGGGAA CGG	GATGA CCGCC	ACCCG GTCAC	AACGGT GACC	GGAGT ACTGG	CTCTCC GCCA
No. of bands	20	34	39	10	27	23	31	23	19	23
No. of polymorphic bands	14	34	39	4	27	23	25	23	13	11
Primer efficiency	7.40	12.59	14.44	3.70	10	8.51	11.48	8.51	7.03	8.51
discriminatory power %	5.98	14.52	16.66	1.70	11.53	9.82	10.68	9.82	5.55	4.70

11	Total
OPI-06	
AAGGC GGCAG	
21	270
21	234
7.77	
8.97	

The use of Eleven RAPD primers generated a total of 270 amplified bands and 234 polymorphic bands, this indicate that there is a significant genetic diversity among the isolates. Based on the number of revealed bands per primer, they varied between (10 and 39) (Table 3). The high number of RAPD patterns was shown in primer OPX-01 (39 bands), while the lowest number was shown with primer OPA-11 (only ten bands).The high number of bands may be attributed to the presence of high number of primer annealing sites on the template DNA of the tested isolates. The highest number of bands usually gives a better chance for detecting polymorphisms among individuals (22).

High level of polymorphism was presented with primer OPX-01 (39 bands), while the lower level of polymorphism was shown with primer OPB-17 (18 bands) (Table 3). The differences in molecular weights of polymorphic bands reflect the number of targets for each primer site locus within the DNA in question (23, 24). Polymorphisms at DNA level may occur as a result of several types of mutations, such as single base change in the primer-annealing site in the genome that prevents amplification by introducing a mismatch at 3‘ end of a DNA segment (25). Other sources of polymorphisms may includes deletion of a priming site, insertion that render priming sites to be too distant to support amplification, or they may change the size of DNA segment without preventing its amplification (26). The failure of many primers to amplify DNA may be due to their need to special requirements for amplifications in terms of PCR-reagents or temperature profile, since all of the reaction parameters were identical for all primers. Moreover, differences in banding patterns are likely due to specific requirements of a given primer. The G+C content of the primer may further interfere with PCR yield (22, 27).

Furthermore, the current study focused on determining the efficiency of each primer (Table 3). The primer OPX-01had the highest calculated efficiency (14.44), while primer OPA-11demonstrated the lowest efficiency of (3.70).The discriminatory

power of the various primers varied greatly. The highest discriminatory power showed with primers OPX-01 (16.66), which produced distinct fingerprints. However, primers OPA-11 revealed the minimal discriminatory power of (1.70) (Table 3). This can be explained by primer capacity to reflect variations in the genomic of microorganisms to the total number of variations. The primer with high discriminatory power has capacity to obtain high number of polymorphic bands to the total number of polymorphisms(28). Determining the efficiency and discriminatory power of any primer is important, since it can be used to reduce the extent of blind screening of the primer used to analyze the molecular bases of other individuals of the same species. This will eliminate the possibility of producing monomorphic patterns between genetically different individuals. It will also increase the possibility of obtaining proper fingerprints of individual strains (29).

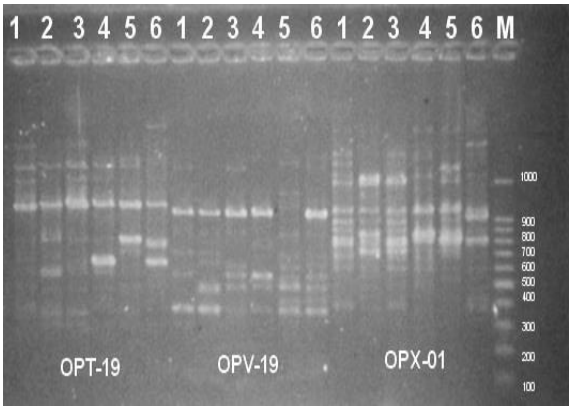

Fig (1): RAPD patterns of *P.aeruginosa* isolates obtained with primer OPT-19, OPV-19, OPX-01. Electrophoresis carry out in a 1.5% agarose gel and run with 3 volt/cm. M: λDNA, Lan 1 to 6 represent six isolates.

RAPD-PCR has been used to differentiate strains of various species, various serotype within species, and various subtypes within a serotype (30). Interestingly, the current study clearly demonstrated the possibility of discrimination, at the molecular level, this agreement with previous studies (31,32), that RAPD-PCR markers help in diversity analysis as well as fingerprinting of *P. aeruginosa* isolated. The results showed that some primers gave main and for all isolates such as primers OPT-19, OPA-11, OPF-16, OPG-13, this indicates that all *P. aeruginosa* isolates have the same primer annealing sites on the template DNA of the tested isolates (figure 1,2,3,4 and 5).

On the other hand, some primers gave unique bands, here two types of unique bands (absent & present band), these unique bands may indicate as a fingerprinting or marker for these isolates. These primers produced a specific DNA band which distinguished one variety from the rest. This may lead to the suggestion of using these primers(OPA-11, OPB-17, OPC-05, OPG-13 and OPI-06) to define clinical isolates of *P.aeruginosa* in future studies. The origin of these unique markers may be attributed to mutation at the priming site of the primers or to insertion/deletion mutation in the distance between the reverse and forward priming sites of the primers. The presence of unique RAPD markers among the various *P.aeruginosa* genotypes confirms the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications including the determination of cultivar purity, efficient use and management of genetic resources collection, particularly in identification of mislabeled accessions (24).

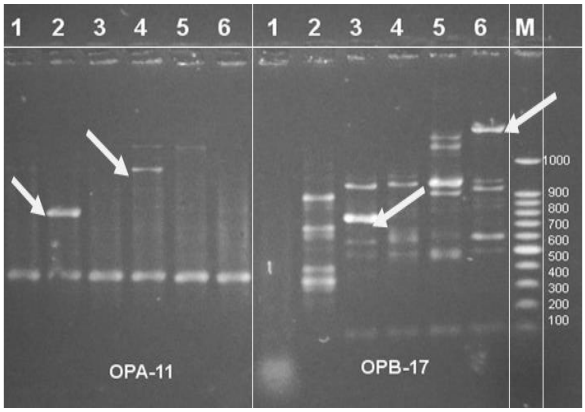

Fig (2): RAPD patterns of P.aeruginosa isolates obtained with primer OPX-01, OPA-11. Electrophoresis carry out in a 1.5% agarose gel and run with 3 volt/cm. M: λDNA, Lan 1 to 6 represent six isolates.

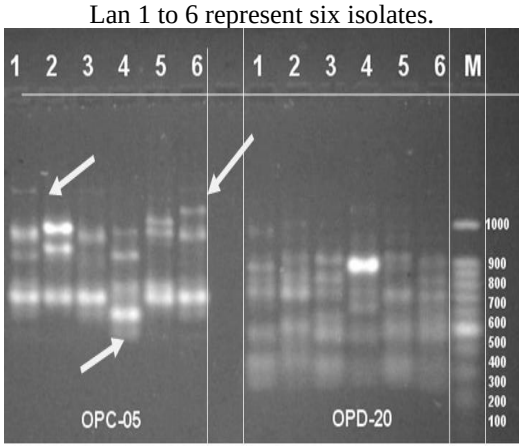

Fig (3): RAPD patterns of P.aeruginosa isolates obtained with primer OPC-05, OPD-20. Electrophoresis carry out in a 1.5% agarose gel and run with 3 volt/cm. M: λDNA, Lan 1 to 6 represent six isolates.

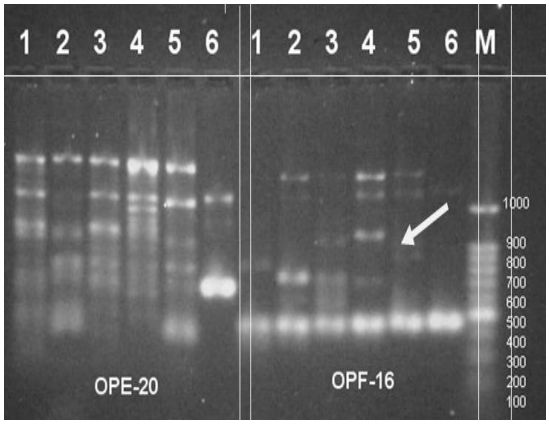

Fig (4): RAPD patterns of P.aeruginosa isolates obtained with primer OPE-20, OPF-16. Electrophoresis carry out in a 1.5% agarose gel and run with 3 volt/cm. M: λDNA, Lan 1 to 6 represent six isolates.

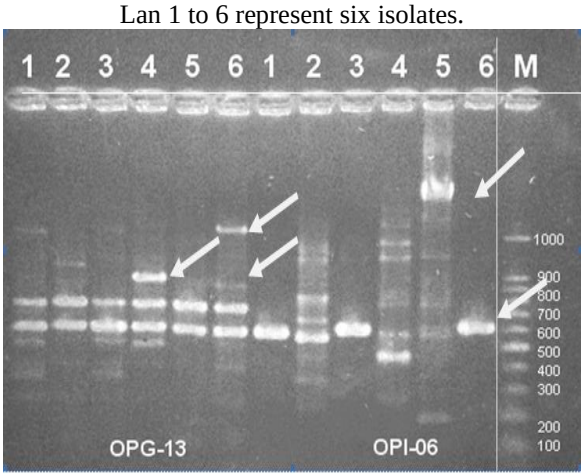

Fig (5): RAPD patterns of P.aeruginosa isolates obtained with primer OPG-13, OPI-06. Electrophoresis carry out in a 1.5% agarose gel and run with 3 volt/cm. M: λDNA, Lan 1 to 6 represent six isolates.

Genetic fingerprinting, phylogenetic diversity and genetic distance of of *P.aeruginosa* isolates was evaluated using RAPD technique. Such information will be useful in its classification, epidemiological survey, ecology and diagnosis. (Table 3) summarize the values of genetic distance for six *P.aeruginosa* isolates from different locations.

The genetic distance values range from 1.0381 to 0.3099. It was clear that the lowest genetic distance (0.3099) was found between isolates number 1 (wound) and 3 (Burn), whereas the highest genetic distance (1.0381) was found between isolates number 2 (ear) and 6 (eye).

Table (3) : The genetic distance values for six *P.aeruginosa* isolates according to RAPD markers:

	1	2	3	4	5	6
1	0					
2	0.7619	0				
3	0.3099	0.6098	0			
4	0.7397	0.6709	0.5875	0		
5	0.6897	0.5941	0.4242	0.5355	0	
6	0.5884	1.0381	0.4880	0.7281	0.7281	0

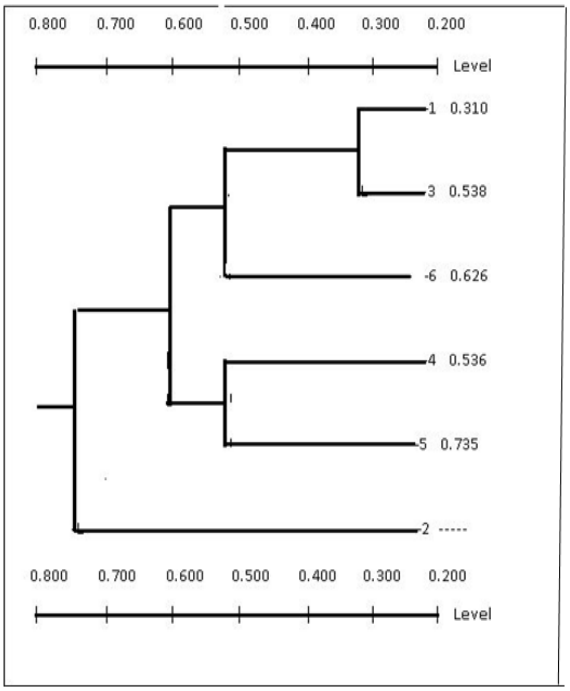

Figure (6): Dendrogram showing Genetic diversity among six *P.aeruginosa* isolates according to RAPD technique

Figure (6) showed the cluster analysis and phylogenetic tree depending on genetic distance in order to reveal the genetic relationship between the six *P. aeruginosa* isolates subjected RAPD-PCR analysis. Analysis this dendrogram, the overall result indicated that all six *P. aeruginosa* isolates were classified into two major group genotypes. The first group was further separated into two subgroups, the first subgroup included isolate number 5 and 4, while the second subgroup included two division, the first isolate number 6 and the second includes isolate number 1 and 3. The second group included only isolate number 2.

This put to rest the fact that there is definitely a high rate of genetic recombination or mutations leading to a high level of genetic diversity among the *P.aeruginosa* isolates population(32). High level of genetic diversity in *P.aeruginosa* isolates population had also been reported as the basis of *P.aeruginosa* multi-drug resistance and high tenacity in environmental adaptability (34,35,36). In agreement with other studies, there was substantial diversity among the *P.aeruginosa* isolates. The large number of genotype suggest that most *P.aeruginosa* isolates were derived from the patients themselves, as shown previously (37, 38, 39).

The relationship between the isolates site of infection in the body with their genetic diversity shows

that isolates 1 and 3 were predominant in wound and burn infection cases and have high genetic similarity. Isolates 4 and 5 were predominant in were urinary tract infection, while the isolate number 2 (ear) have high genetic distance compared with the other isolates. This reveals the fact that genetic differences in P. aeruginosa strains could affect the site at which the organism is predisposed to be virulent, suggesting possible relationship between genetic diversity of *P. aeruginosa* isolates and the type of environment they preferentially survive (34, 36, 37). *P. aeruginosa* isolates in the first subgroup were predominant in surviving in the most adverse of the three environments in the blood stream. This suggests that these isolates could be the more virulent group. This would not be unconnected with their genetic makeup which possibly would have enhanced the expression of virulence factors necessary to survive despite the blood immune responses (40, 41, 42). On the other hand, the second group has a significantly larger proportion of urinary tract infection *P. aeruginosa* isolates. The urinary tract is also a special environment with high osmotic pressure, pH fluctuations and movement of fluid. Bacteria known as pathogens and flora of this terrain have been known to possess good ATPase membrane pumps and good adhesive properties, and *P. aeruginosa* is generally known to be rich in such properties (43,44,45). The genetic diversity captioned in the second subgroup must involve a good number of genes with expressions that ensure survival in that terrain (46).

In conclusion this study has demonstrate that there is high DNA polymorphism in the six *P.aeruginosa* isolates from patients that hospitalized in Tikrit Teaching Hospital, which is apparently due to high rate of genetic recombination. The high genetic diversity of *P.aeruginosa* strains also affects the site at which they preferably cause colonization and infection. Therefore, we can say that RAPD method could be an additional rapid typing method for studding the molecular characterization and the epidemiology of *P.aeruginosa* bacteria.

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التوصيف الجزيئي لعزلات *Pseudomonas aeruginosa* معزولة من مرضى سريرين باستخدام تقنية RAPD-PCR

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الخلاصة:

هدفت هذه الدراسة إلى إجراء التوصيف الجزيئي وتقييم درجة التباين والعلاقة الوراثية لست عزلات من بكتريا Pseudomonas aeruginosa باستخدام تقنيةRAPD-PCR المعتمدة على التضخيم العشوائي المتعدد الأشكال لسلسة ألدنا. جمعت 86 عينة من المرضى الوافدين إلى مستشفى تكريت التعليمي في مدينة تكريت. أخذت هذه العينات من مصادر مختلفة واعتمادا" على مصدر الإصابة أخذت العزلات من الجروح، الأذن، الحروق، البول، البلغم والعيون. عزلت وشخصت بكتريا P. aeruginosa باستخدام الأوساط الاغنائية والانتخابية بالإضافة إلى الاختبارات الكيموحيوية. تم عزل ألدنا المجيني بنجاح من عزلات P. aeruginosa الستة المعزولة من مصادر مختلفة بتركيز ونقاوة مناسبة لاستعماله في تفاعلات تقنية RAPD-PCR. أنجزت تقنية RAPD-PCRباستخدام 15 بادئ عشوائي مختلف وأظهرت النتائج أن 11 بادئا" أعطت نتائج ناجحة من التجارب المتكررة. كنتيجة، العدد الكلي للحزم المتضاعفة كان270 والعدد الكلي للحزم المتباينة كان 234 حزمة. أعلى عدد من الحزم المتباينة أنتج من قبل البادئ OPX-01 (39 حزمة)، تراوحت كفاءة البادئات مابين 3.7 للبادئ OPA-11 إلى 14.44 للبادئ OPX-01، أما قوة التباين فتراوحت مابين 1.7% للبادئ OPA-11 إلى 16.66% للبادئ OPX-01. بالإضافة إلى، تحديد البعد الوراثي والتباين العرقي لعزلاتP. aeruginosa باستخدام برنامج UPGMA الحاسوبي اعتمادا" على أنماط التضاعف المستحصلة من هذه الدراسة باستخدام تقنية التضاعف العشوائي متعدد الأشكال لسلسة ألدنا (RAPD-PCR).تقترح هذه النتائج حدوث تغيرات وطفرات وراثية محتملة ومتكررة في تسلسل ألدنا لبكتريا P. aeruginosa من المصادر والمناطق المختلفة. أثبتت هذه الدراسة بوضوح وجود اختلافات وراثية على مستوى ألدنا بين عزلات P. aeruginosa الستة المعزولة من المصادر المختلفة. لذلك يمكن القول أن تقنية RAPD-PCR تقنية فعالة لدراسة التوصيف الجزيئي ووبائية بكتريا P. aeruginosa.