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Role of Two Different Annealing Temperatures in Genetic Variability Determination of *Pseudomonas aeruginosa*

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ABSTRACT

Seven isolates of *Pseudomonas aeruginosa* were obtained from bacterial strain bank department of Biology/ College of Science/ Mosul University. The DNA was extracted from bacterial culture. Genetic variability was achieved by (RAPD-PCR), and ERIC2 primer was used. Two programs were used in thermal cycling containing two different annealing temperatures, the first was 50^{o} C, its amplification and electrophoresis results showed that all seven isolates had no band except the isolate No. (3) which has revealed a band (approximately 600 bp) when compared with DNA ladder, so this temperature was not suitable for detection of genetic variation for our local bacteria.

The annealing temperature for the second program was 35^{o}C, its amplification and electrophoresis result has illustrated that there are genetic variability among just three isolates which had number 2, 3, and 4, while the other isolates have failed to show any band. So, the 35º C was better than 50º C to determine genetic variation in this study.

Keywords*:* Genetic variation, *P.aeruginosa,* RAPD-PCR, ERIC2 primer.

دور درجتي تلدن في تعيين التباين الوراثي للزوائف الزنجارية

الملخص

استخدمت سبع عزلات من جرثومة *P.aeruginosa* تم الحصول عليها من بنك السلالات البكتيرية/ قسم علوم الحياة/ كلية العلوم/ جامعة الموصل. استخلص الـ DNA من المزارع الجرثومية للعزلات للكشف عن التباين الوراثي لها باستخدام تقنية مؤشرات التضاعف العشوائي المتعدد الأشكال للـ DNA وباستخدام البادئ ERIC2. اعتمد برنامجين يحويان درجتي تلدن مختلفتين، الاول : كانت درجة التلدن المتبعة 50 م وتبين من خلال نتائج التضخيم والترحيل الكهربائي إن كل العزلات السبعة لم تظهر حزم تضاعف ماعدا العزلة رقم 3 التي اظهرت حزمة بوزن جزيئي مقاربا للـ 600 زوج قاعدي عند مقارنتها مع مؤشر الـDNA. استنتج ان درجة التلدن هذه لا يمكن اعتمادها لتحديد التباين الوراثي لجرثومة *P. aeruginosa*. اما البرنامج الثاني فكانت درجة التلدن المتبعة فيه 35 م وقد ظهرت تباينات وراثية بين ثلاث عزلات فقط من العزلات السبع تلك التي تحمل الارقام 2 و 3 و4 حيث اظهرت حزم تضاعف متعددة فيما أخفقت باقي العزلات من إظهار أية حزمة. لذا فان درجة 35 م كانت أفضل من درجة 50 م للكشف عن التباين الوراثي في هذه الدراسة.

الكلمات الدالة: التباين الوراثي، الزوائف الزنجارية، مؤشرات التضاعف العشوائي المتعدد الأشكال للـDNA، البادئ ERIC2

INTRODUCTION

Due to the plasticity of the phenotypic characteristics, molecular techniques (which are inherently more stable than phenotypic characteristics) have gained popularity for strain differentiation and epidemiological studies of many organisms (Deschaght *et al*., 2011).

Molecular typing of microbial pathogens is of pivotal importance in the elucidation of transmission routes, detailed genetic analysis at the species level gives insights into the variability within a bacterial population and generates evidence on genome plasticity and evolution, which in turn leads to bacterial adaptation to various environmental conditions. (Renders *et al*., 1996)

For the last two decades, PCR-based genotyping methods have played an important role in bacterial typing schemes. One of the PCR-based methods, namely random amplification of polymorphic DNA (RAPD)-PCR, also known as arbitrarily primed-polymerase chain reaction (AP-PCR), has been described to be useful on account of its simplicity and utility for analysis of large throughput samples (Gürtlet and Mayall, 2001).

Molecular research has been performed to study diversity among *P. aeruginosa* strains, polymorphism of certain of its genes and also genetic comparison of *P. aeruginosa* isolates from different hosts and environments (Onasanya *et al*., 2010 ; Al-Zahrani *et al*., 2012). The aim of the present study was to determine the genetic variation of seven clinical isolates of *P. aeruginosa* by random amplification of polymorphic DNA technique -using two annealing temperatures 50 ^{o}C and 35^{o}C which may give a significant look into the interrelationship of these bacterial isolates that in turn leads to investigate the risk of patient-to-patient transmission.

MATERIALS AND METHODS

Bacterial strains: Seven *P. aeruginosa* isolates were obtained from Bacterial strain bank/ Biology department/ college of science/ Mosul university) collected from the different clinical samples.

Colonies having the typical *P. aeruginosa* morphology were selected and placed in selective and differential media. Biochemical testing was performed to determine the bacterial genus and species, So the purity and identity of each isolate were confirmed by standard microbiological methods.(Forbes *et al*., 2007 ; Todar, 2011).

Preparation of Chromosomal DNA

The DNA was extracted from bacterial culture of *P. aeruginosa* using the method of phenol-chloroform procedure (Ausubel *et al*., 2002). DNAs were stored at -20 ^{o}C until use.

Baterial DNA Amplification:

1- **The Primer:** The RAPD primer ERIC2 (enterobacterial repetitive intergenic consensus) sequence (5' – AAG TAA GTG ACT GGG GTG AGC G – 3') which is considered as universal primer has been used according to (Tazumi *et al*., 2009; Van daele *et al*., 2006; Speijer *et al*., 1999 ; Renders *et al*., 1996). The primer was supplied by BIONEER company as a lyophilized product of picomoles concentration, BIONEER company protocol was adopted for primer resuspension to bring as a final concentration to 5 picomoles/µl with nuclease-free water.

2- **Reaction solution**: Twenty five µl PCR reaction mix contained the ingredients listed in Table (1)

Table 1: preparation of 25 µl of reaction solution

Component	Volume	Final Conc.
GpTag® Green Master Mix, 2X	12.5 µl	1X
Primer	5 µl	5 pmols/ µl
DNA tamplet	2 µl	<250 ng
Nuclease-Free Water to	25 µl	N.A

Twenty Five µl PCR reaction solution was prepared according to Go Taq® Green Master Mix company (Promega, USA). A negative control without template DNA was used in the reaction.

3- **Thermal Cycling conditions (Program):**two programs have been dependent to achieve amplification: The First, according to (Tazumi *et al*., 2009) using thermocycler (Eppendrof AG 22331 Hamburg, Germany, Master Cycler personal) as shown in Table 2.

Table 2: RAPD-PCR amplification condition for first program

No.	Steps	Temperature	Time	No. of cycles
1	Denaturation 1	94° C	5 min.	1
First loop				
2	Denaturation 2	94 ° C	1 min.	40 cycles
3	Annealing	50 ° C	1 min.	
4	Primary Extension	72 ° C	1 min.	
5	Final Extension	72 ° C	10 min.	1

At the end of the first amplification,(10 µl) were removed from each reaction mixture and examined by electrophoresis (100 V, 90 min) (ENDURO^{TM} electrophoresis system, ENDURO power supplies, Labnet international. Inc. model: E 0303, Taiwan.) in gels composed of 2% (w/v) agarose stained with ethidium bromide (5 µg/100 ml) by using TAE buffer (40 mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.3), Stained agarose gel was visualised under UV illumination by (UV transilluminator, model MUV 21-312, Taiwan). A photograph was taken by using digital camera (Canon, Japan). The molecular weights of bands were estimated by using standard molecular markers (100 bp DNA Ladder, BIONEER).

The second amplification was changed just in annealing temperature of 35°C (Table 3) according to (Van daele *et al*., 2006; Van daele *et al*., 2005; Ortiz-Herrera, 2004)

Table 3: RAPD-PCR amplification condition for second program

No.	Steps	Temperature	Time	No. of cycles
1	Denaturation 1	94° C	5 min.	1
First loop				
2	Denaturation 2	94 ° C	1 min.	40 cycles
3	Annealing	35 ° C	1 min.	
4	Primary Extension	72 ° C	1 min.	
5	Final Extension	72 ° C	10 min.	1

Then the procedure was completed as previous steps and the reaction mixture was completed as explained in the first program.

RESULTS AND DISCUSSION

After the extraction of genomic DNA from the seven *P. aeruginosa* isolates, it was amplified by RAPD-PCR using ERIC2 primer, then the products were separated by electrophoresis to study genetic variation in these local bacterial isolates.

The results of the first program did not give successful finding as seen in (Fig. 1); All seven isolates did not appear any band except the isolate No. (3) which has showed a band with molecular weight of approximately 600 bp compared with 100 bp DNA ladder (lane 3 in Fig. 1), so the isolate number (3) was different from the other six isolates by this band only. The absence of bands for

other six isolates might be attributed to lack of complementary sequences to the primer in this temperature among the genomic DNA from *P.aeruginosa* (Devos and Gale, 1992).

The present study concluded that this temperature is not the optimum for determination the genetic variation of our bacterial isolates.

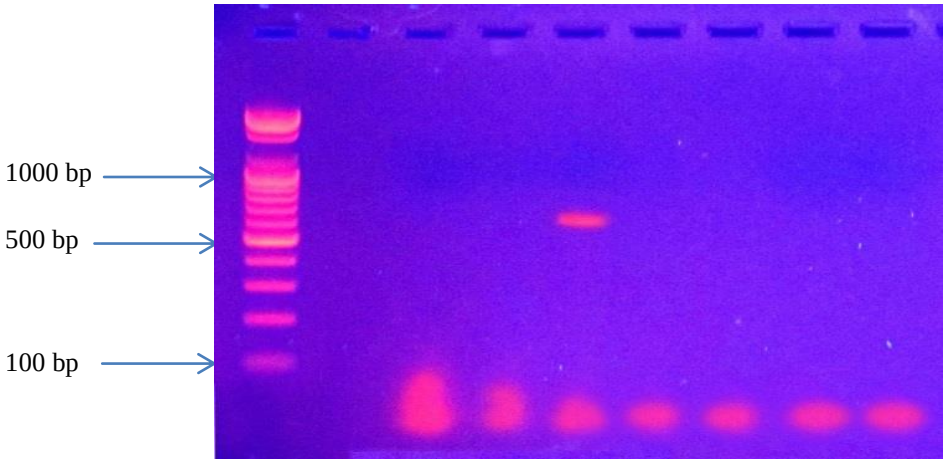

Fig. 1: Agarose gel electrophoresis of RAPD PCR amplification for *P.aeruginosa* isolates. Lanes: M, mw marker; -ve, negative control; 1-7 *P.aeruginosa* DNA (annealing temp. in PCR program was 50°C)

Although it has been reported by various groups that RAPD typing is reproducible, historically there have been several reports of problems associated with its reproducibility. Grif *et al*. (1998) reported that RAPD suffered from the lack of standardization using different methods in different laboratories and this may affect the quality of results. RAPD-PCR can also suffer from the same factors that affect ordinary PCR, including magnesium concentration, primer and reagent 'batch-to-batch' variation, annaeling temperature. and quality of the thermal cycler. Power (1996) suggested that for RAPD to be a definitive typing technique, the reproducible generation and interpretation of RAPD fingerprints needs to be developed, to achieve this criterion, the use of an automated system for DNA preparation combined with the use of manufactured RAPD master mixes, as well as use of the same thermal cycler and standard (Jamasbi and Proudfoot, 2008).

According to a previous result, annealing temperature was decreased to 35^{o} C according to the researchers(Van daele *et al*., 2006; Van daele *et al*., 2005).

In attempt to detect on distinct genetic variations among the isolates, 35^{o} C was shown to be better than 50^{o} C in demonstrating the genetic variations among the isolates.

Variations among studied isolates and RAPD results analysis are achieved by depending on presence or absence of amplified bands, also by the difference of molecular weights for these bands which differ by different sites number complemented to primer sequence on genomic DNA, and also differ by the distance between site and another (Mayer *et al*., 1997).

Our PCR products data indicated high diversity of *P. aeruginosa*, by using the primer ERIC2, this study showed the presence of genetic variation in just three isolates among *P.aeruginosa* as can be seen in (Fig. 2). Results analysis for polymorphism is usually scored in three different forms in experiments: presence or absence of amplified DNA bands, differences in molecular weight of amplified DNA bands and differences in the intensity of amplified bands (Bardakci, 2001).

The isolate No.4 revealed a largest numbers of amplified bands (6 bands) represented by <100, 300, 365, 410, 800 and 1000 bp. while the isolate No. 2 had four amplified bands represented by <100, 300, 400 and 600 bp, but three bands (100, 400, 650 bp) for the isolates No.3. (Table 4). This indicates that the primer has found specific areas that bind it along with the DNA template, consequently amplification of the fragments was achieved, bands have appeared, and finally polymorphism has demonstrated.

The other isolates of *P. aeruginosa* (which had number 1, 5, 6, and 7) had no revealed bands.

This technique utilizes a variable short length arbitrary primer, and is advantageous, as it does not require any previous knowledge of the target DNA sequence data. The primer is amplified arbitrarily at low stringency, where the oligonucleotide binds at complementary and partially mismatched sites and generates bands which differ in length and nucleotide composition. (Tazumi *et al.,* 2009).

As the primer efficiency depends on the total number of bands amplified by the primer and this could include a number of common ones (representing conserved) sequences among various species, efficiency would not be very informative in identifying species. in generating patterns of banding that are unique to the species and second in exhibiting or lacking unique band (s) that distinguish an individual from the rest of the population (Dhahi *et al*., 2011)

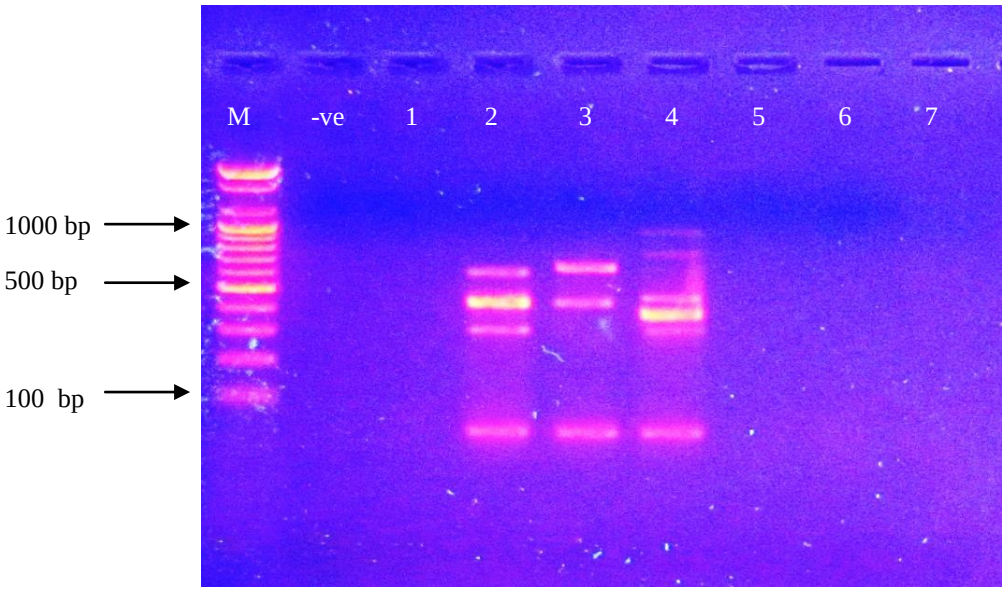

Fig. 2: Agarose gel electrophoresis of RAPD PCR amplification for *P.aeruginosa* isolates. Lanes: M, mw marker; -ve, negative control; 1-7 *P.aeruginosa* DNA (annealing temp. in PCR program was 35°C)

Table 4: Molecular weights(bp) of the amplified fragments of isolates 2, 3, and 4. Lanes 2, 3 and 4 respectively.

Type of band	Molecular weights of bands (bp)	Isolates		
		2	3	4
Polymorphic	1000	0	0	1
=	800	0	0	1
=	650	0	1	0
=	600	1	0	0
=	410	0	0	1
=	400	1	1	0
=	365	0	0	1
=	300	1	0	1
monomorphic	<100	1	1	1
Total	9 bands	4	3	6

0 :abscence of band
1 : presence of band

This technique uses random primers under low specificity conditions; it is less costly and faster and easier to perform than analogous systems, either phenotypic or genotypic.(Loutit and Tompkins, 1991)

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 (Hurtado and Rodriguwz, 1999). Other sources of polymorphisms may include 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 (Williams *et al*., 1990).

Multidrug resistance (MDR) is a common feature of *P. aeruginosa* strains. Rapid identification of the organism is necessary to expedite the onset of treatment and to reduce the duration and dosage of antibiotic therapy, which in turn could minimize the development of antibiotic resistance. (Jamasbi and Poudfoot, 2008)

A significant decline in susceptibility of *P. aeruginosa* to B-lactams, aminoglycosides, and quinolones has been reported, Nosocomial outbreaks MDR in different hospitals have appeared that the frequency and rate of resistance to individual antibiotics are different in different genotype (Gales *et al*., 2001).

The differences in antibiotic susceptibility of *P. aeruginosa* in different genetic variations could be attributed to the differences in patient population, the duration of hospitalization, cross-infection, and the dose and types of antibiotics. Transmission of resistant fragments within smaller hospital units may be responsible for this phenomenon, and the determination of antimicrobial profiles is another typing method used frequently as a supplemental epidemiological tool for strain differentiation of *P. aeruginosa*. It should be noted, however, that antibiotic susceptibility profiles are less stable than O-antigenic markers, given the resistance factors that can occur under pressure of antibiotic therapy. (Jamasbi and Poudfoot, 2008).

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