J. Agric. Sci. Mansoura Univ., 32 (6): 4475 - 4492, 2007

RAPD-PCR TECHNIQUE FOR STUDYING GENETIC RELATIONSHIP AMONG *Pseudomonas aeruginosa* isolates

Youssef, M. A. H; Amina A. H., and M. K. Amin
Genetic Dept., Fac. of Agric., Zagazig Univ.

ABSTRACT

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) analysis was performed to detect the genetic relationships between some *Pseudomonas* human pathogenic isolated from Hospital of Zagazig University. These isolates were fully characterized in Dept. of pharma. Microbiology, Faculty of pharmacy Zagazig University. Six out of 16 arbitrary decamer primers used in this study were informative and detected scoreable polymorphism in banding patterns of RAPD markers between these isolates and the wild type strain of *P. aeruginosa* PAOI and between some bacteriophages isolated from the environment propagating on *Pseudomonas* bacteria with the wild type phage F116 of *P. aeruginosa*. Each of primers used for analysis of individual bacterial isolates and bacteriophages amplified different number of bands. Genetic similarity between bacterial isolates and between bacteriophages, calculated as the total number of band differences. The highest similarity value(0.865) was found between PAO1 and both of ATC1, ATC78 and ATC87.The lowest value (0.486) was found between PAO1 and ATC70.The dendrogram of genetic distances among bacterial isolates based on band polymorphisms generated by RAPD-PCR after using the primers showed that PAO1, ATC1, ATC78, ATC87 and ATC114 are fallen in one cluster, ATC45, ATC58, ATC76 and ATC111 are grouped in the second cluster, while ATC70 and ATC50 are found in the third cluster. All bacterial isolates except for ATC45 and ATC58 were distinguishable by unique RAPD markers. The highest similarity value(0.906) between F116 and bacteriophage isolates was found between F116 and both of PA3 and PA9.The similarity coefficient value between F116 and AMSE2000 was (0.875).The dendrogram of genetic distances among bacteriophages based on band polymorphisms generated by RAPD-PCR after using the primers showed that all bacteriophages except for PA5 and PA7 are fallen in one cluster. All bacteriophages except for AMSE2000, PA9 and Ø111 were distinguishable by unique RAPD markers**.** The data indicated that it could be possible to differentiate DNA polymorphism among bacterial isolates and among bacteriophage isolates with relatively few markers. This study demonstrated the effectiveness of RAPDs for identifying polymorphism and can be useful in fingerprinting**.**

Key words: Pseudomonas, bacteriophage relationship,RAPD-PCR, dendogram.

INTRODUCTION

The genus *Pseudomonas* includes species with functions of ecological, economic and health-related importance. Some species are pathogenic for plants (Stead, 1992), while others are pathogens of animals or human (Palleroni, 1992 and Tyler *et al*., 1995). Some species exhibit plant growth-promoting and pathogen- suppressing functions and may be exploited for use in biological control (Keel *et al*., 1996).

The genus *Pseudomonas* is gram negative, rod. Shaped microorganisms has been subject to repeated taxonomic revisions (Palleroni, 1993). The multitude of important characteristics of *Pseudomonas* species has inspired numerous taxonomic studies, which have led to the development of a better understanding of this genus. The approaches used include selective culturing (Sands *et al*., 1980), PCR amplification of rRNA genes (Tyler *et al*., 1995 and Widmer *et al*.,1998) in conjunction with restriction fragment length polymorphism (RFLP) analysis (Brosch *et al.,* 1996), DNA sequencing (Moore *et al*., 1996), DNA and RNA hybridization (Palleroni, 1973) and lipoprotein genes(De Vos *et al* ., 1993).

The randomly amplified polymorphic DNA (RAPD) assay, which detects nucleotide sequence polymorphisms by means of the polymerase chain reaction (PCR) and a single primer of arbitrary nucleotide sequence, is a useful method for generating molecular markers. RAPD assay was first described by two independent groups, Williams *et al.*, 1990 and Welsh and McClelland, 1990. The success of this method is due to the fact that no prior sequence information about the target is needed and a single short 10-mer oligonucleotide primer can be used in the reaction. The amplification happens at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the DNA sequence variations may work as genetic markers that can be used in genetic similarity and diversity studies. This technique has been used extensively to detect genetic relationships among *Pseudomonas aeruginosa* (Renders *et al.* 1996; De Vos *et al.* 1997; Barnini *et al.* 2004; Curran *et al.* 2004; Cheng *et al.* 2006; Christian *et al.* 2006 and Nazik *et al.* 2007).

The aim of this study was to detect the genetic relationships between some *Pseudomonas* human pathogen. The genetic relationships carried out between these isolates and the wild type strain of *P. aeruginosa* PAOI. The analysis extended also to find a relationship between some bacteriophages isolated from the environment propagating on *Pseudomonas* bacteria with the wild type phage F116 of *P. aeruginosa*.

MATERIALS AND METHODS

This work was carried out in Molecular Genetics Lab. Genetic Dept., Fac. of Agric., Zagazig Univ.

***Pseudomonas aeruginosa* bacterial strain and isolates:**

The standard *P. aeruginosa* bacterial strain PAOI that was used in this study obtained from M. Day, University of Wales, College of Cardiff, UK. The isolates of *P. aeruginosa* were obtained from M. EL- Hussieny, Fac. Pharmacy, Zagazig Univ. (Table 1)

***P. aeruginosa* bacteriophages:**

In this study 12 bacteriophages were used. These phages were: two standared (F116 and AMSE2000), five released spontaneous from lysogenic

bacterial isolates (Ø37, Ø43, Ø50, Ø78 and Ø 111) and five isolated from agriculture drain water (PA1 , PA3, PA5, PA7 and PA9).

Table 1: List of *P. aeruginosa* bacterial isolates.

Strain / isolate	Nutritional requirements	lysogen	Sensitirity to antibiotics			
			Str	Tet	Amp	Chl
PAO1	Prototrophic	-	-	-	-	-
ATC1	Prototrophic	+	-	-	+ up to 1500 µg/ml	+ up to 250 µg/ml
ATC45	Prototrophic	+	-	-	+ up to 1500 µg/ml	+ up to 100 µg/ml
ATC50	Prototrophic	+	+ up to 1500 µg/ml	+ up to 1200 µg/ml	+ up to 1000 µg/ml	+ up to 250 µg/ml
ATC58	Prototrophic	+	+ up to 1500 µg/ml	+ up to 1200 µg/ml	+ up to 1200 µg/ml	+ up to 1500 µg/ml
ATC70	Prototrophic	+	-	+ up to 400 µg/ml	-	+ up to 250 µg/ml
ATC76	Prototrophic	+	-	-	+ up to 500 µg/ml	+ up to 250 µg/ml
ATC78	Prototrophic	+	+ up to 500 µg/ml	+ up to 1200 µg/ml	+ up to 1000 µg/ml	+ up to 250 µg/ml
ATC87	Prototrophic	+	+ up to 500 µg/ml	+ up to 1200 µg/ml	-	+ up to 250 µg/ml
ATC111	Prototrophic	+	+ up to 500 µg/ml	+ up to 500 µg/ml	+ up to 1500 µg/ml	+ up to 1000 µg/ml
ATC114	Prototrophic	+	-	-	-	-

- = Non lysogen or antibiotic sensitive. + = Lysogen or antibiotic resistance.

Bacterial DNA isolation:

The assays were performed with the following: 15 µl from a single colony grown on nutrient agar (the colony was picked and resuspended in 150 µl of distilled water, the suspension was boiled for 5 min, cooled at room temperature, and the supernatant was collected after centrifugation at 10,000 rpm at 4˚C for 10 min).

Bacteriophage DNA isolation:

A modification of the procedure of Durmaz and Klaenhammer,2000 was used for bacteriophage DNA isolation and purification. Two hundred milliliters of phage lysate was incubated for 1 h at 37°C. Polyethylene glycol 8000 and NaCl were added to final concentrations of 10% (w/v) and 3% (w/v), respectively. After gentle mixing, the samples were incubated overnight at 4°C. The phages were pelleted by centrifugation at 10,000 rpm at 4˚C for 10 min, and the supernatants were discarded. The phage pellets were resuspended in 100-µl of 50 mM Tris (pH 8.0). Phage suspensions were extracted two times with 100 µl of phenol and then twice with phenol-chloroform-isoamyl alcohol (25:24:1). The nucleic acids were precipitated with 50 µl of 3 M sodium acetate and 1 ml of 70% (vol/vol) ethanol and resuspended in 50 µl of Tris-EDTA buffer.

Primers:

A set of 16 primers was analyzed and based on the accurate amplified bands profiles and the produced polymorphic patterns of DNA fingerprinting selected six different primers were choosen (Table 2).

Table 2: Sequence and operon codes of the random primers used to detection of variation in *Pseudomonas* and bacteriophages.

Primer codes	*Pseudomonas* and / or bacteriophages	Sequence (5^- to 3^-)
OPB-11	*Pseudomonas*	GTA GAC CCG T
OPC-08	*Pseudomonas* and bacteriophages	TGG ACC GGT G
OPC-20	*Pseudomonas* and bacteriophages	ACT TCG CCA C
OPA-11	*Pseudomonas* and bacteriophages	CAA TCG CCG T
OPD-03	*Pseudomonas* and bacteriophages	GTC GCC GTC A
OPD-05	bacteriophages	TGA GCG GAC A

Amplification reaction mixture:

The amplification conditions were based on **Williams *et al*., 1990 with** some modifications. The reaction was prepared using 25µl per tube, containing 2µl DNA of each strain(20 ng), 1 unit of *Taq* DNA polymerase enzyme, 2µl 10X buffer, 2 µl $MgCl_2$ (25 mM), 2µl $dNTP_s$ (2.5 mM of each), 2 µl primer (10 pmol) and 14.8µl H_2O.

DNA amplification cycles:

The temperature cycling program used with a Perkin-Elmer Gene Amp PCR system (model 2400) was as follows: one cycle at 94°C for 5 min followed by 30 cycles consisting of one step of denaturation (94°C) for 1 min, one step of annealing (35°C) for 1 min, followed by one step of synthesis (72°C) for 2 min and a final extension step consisting of 72°C for 7 min and finally 4°C infinitive.

Band analysis:

The reaction products were analyzed by electrophoresis on 1.4% agarose gels, stained with ethidium bromide, and photographed under UV light. The synthetic DNA, ladder 100 bp (Pharmacia) was employed as molecular markers for bands molecular weight. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Profiles were considered different when at least one polymorphic band was identified. Fragments were scored as 1 if present or 0 if absent based on standard marker. Pairwise combinations, genetic similarity and genetic distances were estimated following Lynch (1990 and 1991). The computer package SPSS was used to generate distance matrices to determine isolates relationships using the unweighted pair-group method of analysis (UPGMA) (Sneath and Sokal 1973).

RESULTS AND DISCUSSION

RAPD analysis performed for bacterial isolates:

Single, random oligodeoxyribonucleotide primers were used to generate PCR-amplified fragments, termed RAPD (random amplified polymorphic DNA) markers. The five primers from the initial screening process were OPB-11, OPC-08, OPC-20, OPA-11 and OPD-05 (Table 2) that

exhibit a high level of polymorphism among the isolates and therefore chosen for this study. A fragment was considered polymorphic when absent in at least one strain. These primers detected scoreable polymorphisms in banding patterns among the isolates.

All Polymorphic PCR products were confirmed by repeating the reaction. Each of five primers used for analysis of individual isolate amplified different number of bands. The total number of amplified fragments from all primers for each isolate are summarized in Table 3. Figure 1 (a, b , c, d and e) represents the patterns of the five random primers with the 12 isolates. Each of five different random primers yielded from 5 to 10 DNA fragments whose molecular size ranged from approximately 250 to 2050 bp. The total number of amplified RAPD fragments after using all five primers was 38 bands with an average of 7.6 fragments / primer. The number of polymorphic bands through each primer ranged from 2 to 8 bands per primer with an average of 4.6 polymorphic bands per primer. The total number of polymorphic amplicons produced by the 5 primers was 23, thus, representing a level of polymorphism of 60.5% in all isolates. Primer OPA-11 produced the highest number of fragments among the primers used with an average of 10 fragments while, primer OPC-20 produced the lowest number of fragments with an average of 5 bands. This indicated that the Primer OPA-11 was the highest among the tested five primers in their ability to flank the DNA sequences of 11 isolates tested in this work. Primer OPD-05 produced the highest percentage of polymorphism (89%) in the isolates while, primer OPC-20 produced the lowest percentage of polymorphism (40%). The highest number of amplified RAPD fragments (34 and 33) after using all five primers were detected in ATC70 and ATC50 respectively with an average of 6.8 and 6.6 fragments per primer, while the lowest number (17) with an average of 3.4 fragments per primer was detected in PAO1.

The fragment size of the PCR products generated by primer OPB-11 (Fig. 1a) approximately ranged from 624 to 2050 bp and produced two shared bands in all isolates with sizes of 1083 and 1800 bp. Bands of size 1633, 1883 and 2050 bp were observed in ATC70 and ATC50 but were absent from all isolates. A band of size 888 bp was observed only in PAO1, ATC70 , ATC50 and ATC114. Bands of size 888, 1633, 1883 and 2050 bp were positive unique RAPD markers.

Table 3: Number of amplicons and the number of polymorphic bands produced by each RAPD primer for *Pseudomonas* isolates.

Primers	PAO_1	ATC_1	ATC_{70}	ATC_{50}	ATC_{45}	ATC_{58}	ATC_{76}	ATC_{78}	ATC_{87}	ATC_{114}	ATC_{111}	Total No. of amplicons	Polymorphic amplicons	Polymorphism %
OPB-11	3	2	7	7	2	2	2	2	2	4	2	7	5	71.4
OPC-08	4	4	6	6	4	4	4	4	4	5	6	7	3	42.86
OPC-20	3	4	5	5	5	5	5	4	4	5	5	5	2	40
OPA-11	5	5	9	8	7	7	7	7	7	7	7	10	5	50
OPD-05	2	3	7	7	5	5	8	1	1	1	5	9	8	89
Total	17	18	34	33	23	23	26	18	18	22	25	38	23	60.5
Average	3.4	3.6	6.8	6.6	4.6	4.6	5.2	3.6	3.6	4.4	5	7.6	4.6	

The fragment size of the PCR products generated by primer OPC-08 (Fig. 1b) ranged from approximately 250 to 1222 bp and produced 4 shared bands in all isolates of sizes of 250, 449, 622 and 672 bp. Bands of size 1111 and 1222 bp were observed only in ATC70, ATC50, ATC114 and ATC111. A band of size 941 bp was observed only in ATC114. Bands of size 941,1111 and 1222 bp were positive unique RAPD markers.

The fragment size of the PCR products generated by primer OPC-20 (Fig. 1c) ranged from approximately 280 to 1211 bp and produced three shared bands in all isolates. A band of size 1211 bp was absent only in PAO1 while a band of size 280 bp was absent in PAO1, ATC1, ATC78 and ATC87. Bands of size 280 and 1211 bp were negative unique RAPD markers.

The fragment size of the PCR products generated by primer OPA-11 (Fig. 1d) ranged from approximately 297 to 1350 bp and produced 5 shared bands in all isolates. Bands of size 100 and 1350 bp were absent only in PAO1 and ATC1. Bands of size 758 and 923 bp were observed only in ATC70 while, a band of size 400 bp was observed only in ATC50. Bands of size 100 and 1350 bp were negative unique RAPD markers while, bands of size 400, 758 and 923 bp were positive unique RAPD markers.

The fragment size of the PCR products generated by primer OPD-05 (Fig. 1e) ranged from approximately 389 to 1240 bp and produced one shared band (781 bp) in all isolates. A band of size 389 bp was observed only in ATC70 , ATC50, ATC76 and ATC111 while a band of size 643 bp was observed only in ATC76 and a band of size 836 bp was observed only in PAO1. Bands of size 389, 643 and 836 bp were positive unique RAPD markers.

Nevertheless, all isolates except for ATC45 and ATC58 were distinguishable by unique RAPD markers. ATC1, ATC78 and ATC87 were identified by negative unique RAPD markers. PAO1 revealed positive and negative unique RAPD markers. ATC70 and ATC50 were characterized by the high number (9 and 8 , respectively) of positive unique RAPD markers.

Cluster analysis of isolates based on nutritional requirements, lysogen and sensitivity to antibiotics:

Nutritional requirements, lysogen and sensitivity to antibiotics (Table 1) were subjected to hierarchical Euclidean cluster analysis to determine the genetic divergence among the isolates. Considering the genetic distance among studied isolates (Table 4), the maximum distance (31.048) was recorded between PAO1 and ATC45 , followed by a distance of (27.622) between ATC45 and ATC111. The minimum Euclidean distance of (1.500) was observed between ATC1 and ATC70. The minimum Euclidean distance between PAO1 and isolates (10.050) was observed between PAO1 and ATC111. Based on the extent of relative dissimilarity among isolates, the 11 isolates were grouped into two clusters (Fig. 2). Cluster I consisted of seven isolates; ATC1, ATC70, ATC114, ATC50, ATC45 and ATC78. Cluster II consisted of five isolates; PAO1, ATC58, ATC76, ATC87 and ATC111.

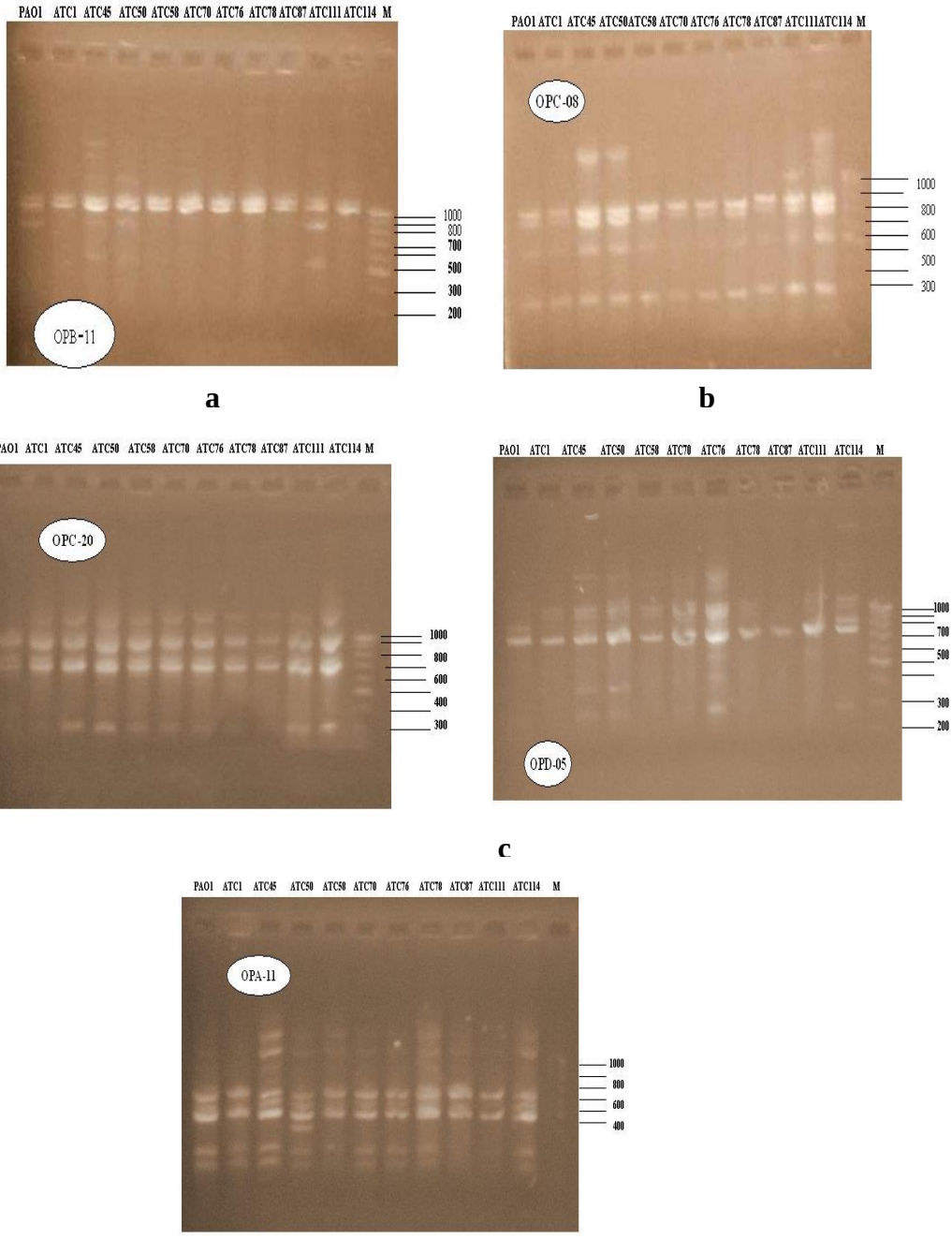


Figure 1 Amplified band profiles generated with primers OPB-11, OPC-08, OPC-20, OPA-11 and OPD-05

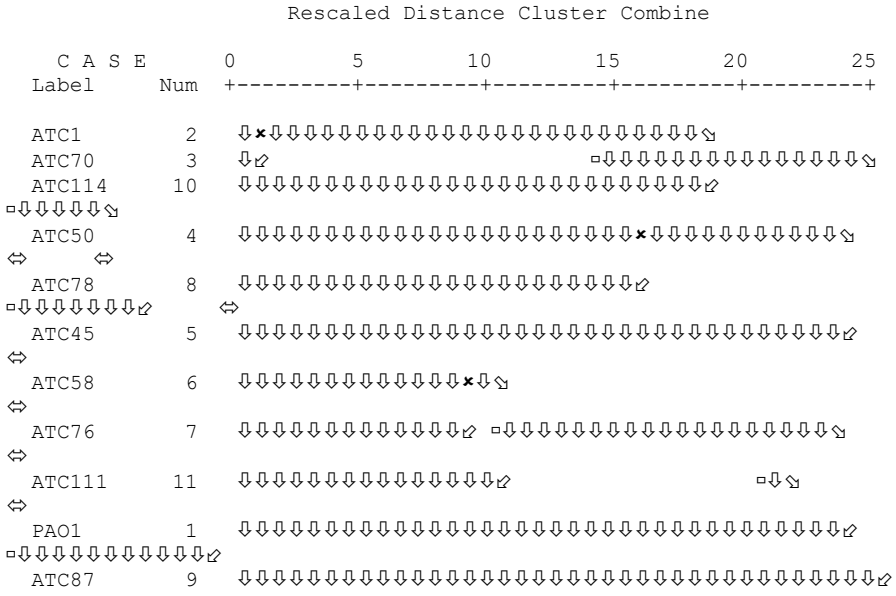


Fig. 2: Linkage dendogram of studied isolates based on nutritional requirements, lysogen and sensitivity to antibiotics

Table 4: Euclidean genetic distances among studied isolates based on nutritional requirements, lysogen and sensitivity to antibiotics.

	ATC_1	ATC_{70}	ATC_{50}	ATC_{45}	ATC_{58}	ATC_{76}	ATC_{78}	ATC_{87}	ATC_{114}	ATC_{111}
PAO_1	21.383	21.260	26.481	31.048	15.756	16.039	22.389	14.186	24.515	10.050
ATC_1		1.500	19.849	23.114	15.524	10.000	13.928	22.226	10.308	16.008
ATC_{70}			19.906	23.958	15.596	10.112	14.009	22.277	11.446	15.843
ATC_{50}				12.659	19.723	19.849	10.000	17.321	15.174	22.366
ATC_{45}					24.274	23.964	16.132	22.366	13.528	27.622
ATC_{58}						6.403	13.748	13.748	17.529	6.874
ATC_{76}							13.928	17.146	14.361	7.500
ATC_{78}								14.142	11.413	17.328
ATC_{87}									20.742	14.151
ATC_{114}										20.000

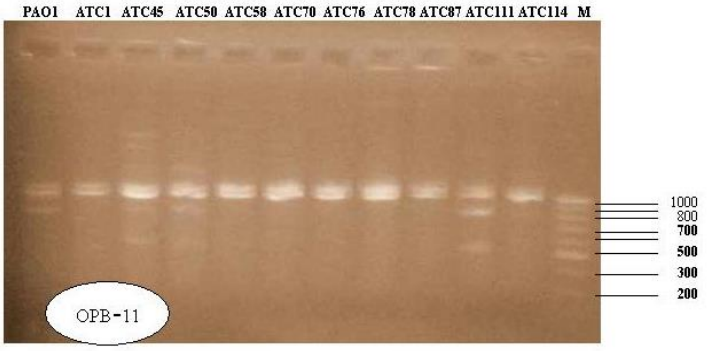
Phylogenetic relationship among isolates based on amplified RAPD fragments (bands):

The similarity coefficient values among isolates based on band polymorphisms generated by RAPD-PCR after using the primers are presented in Table 5. The highest similarity value (1.000) was found between ATC45 and ATC58 and between ATC78 and ATC87**.** The highest similarity value between PAO1 and isolates (0.865) was found between PAO1 and both of ATC1, ATC78 and ATC87.The lowest value (0.486) was found between PAO1 and ATC70.

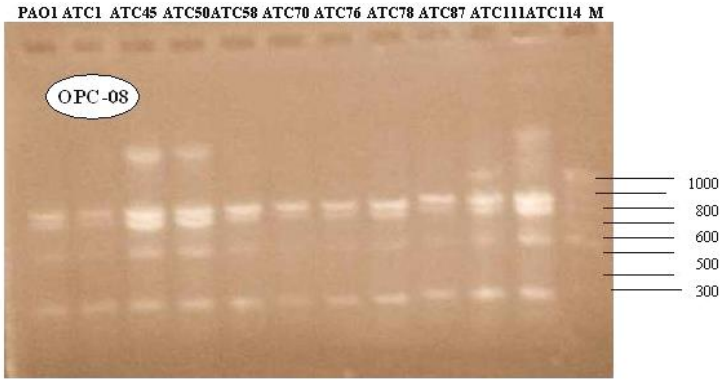
Table 5: The similarity coefficient among bacterial isolates based on combined analysis of amplified RAPD fragments after using all primers.

	ATC_1	ATC_{70}	ATC_{50}	ATC_{45}	ATC_{58}	ATC_{76}	ATC_{78}	ATC_{87}	ATC_{114}	ATC_{111}
PAO_1	.865	.486	.514	.730	.730	.649	.865	.865	.811	.649
ATC_1		.568	.595	.865	.865	.784	.892	.892	.784	.784
ATC_{70}			.919	.703	.703	.730	.568	.568	.676	.784
ATC_{50}				.730	.730	.757	.595	.595	.703	.811
ATC_{45}					1.000	.919	.865	.865	.811	.919
ATC_{58}						.919	.865	.865	.811	.919
ATC_{76}							.784	.784	.730	.892
ATC_{78}								1.000	.892	.784
ATC_{87}									.892	.784
ATC_{114}										.784

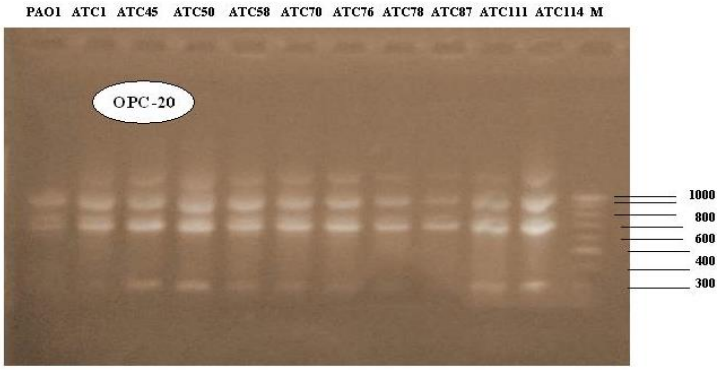
The dendogram of genetic distances among isolates based on band polymorphisms generated by RAPD-PCR after using the primers is shown in (Fig. 3). It revealed that PAO1, ATC1, ATC78, ATC87 and ATC114 are fallen in one cluster; ATC45, ATC58, ATC76 and ATC111 are grouped in the second cluster, while ATC70 and ATC50 are found in the third cluster.Compiring data from Figs.2 and 3 depeding on phenotypic relationship and RAPD-PCR analysis showed that the wild type strain PAO1 and ATC87 were closed to each other.However,strain ATC70 and ATC50 were also close to each other. These finding has been proved when assessing similarity coefficient which showed that similarity coefficient between PAO1 and ATC87 was 0.865 wheres between ATC70 and ATC50 reached up to 0.919.


PAO1 ATC1 ATC45 ATC50 ATC58 ATC70 ATC76 ATC78 ATC87 ATC111 ATC114 M
OPB-11
1000
800
700
500
300
200

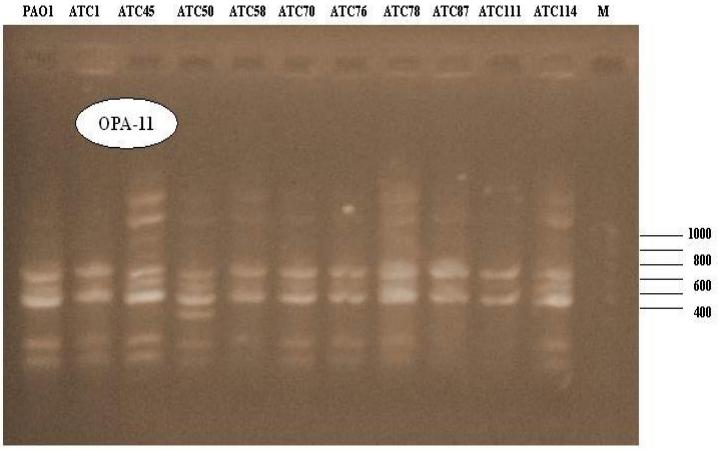
a


PAO1 ATC1 ATC45 ATC50ATC58 ATC70 ATC76 ATC78 ATC87 ATC111ATC114 M
OPC-08
1000
800
600
500
300

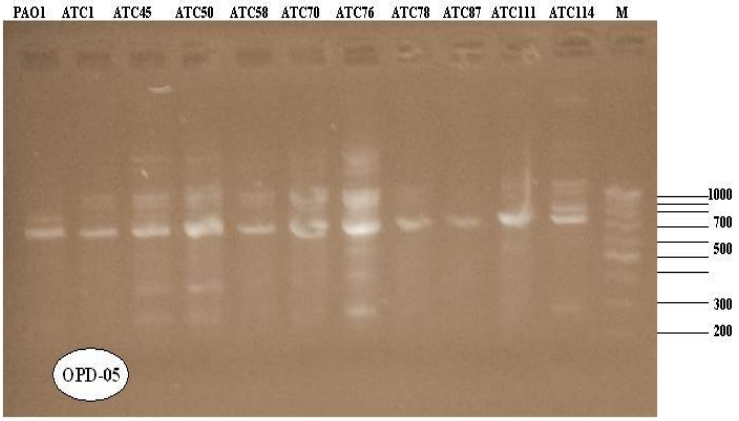
b


PAO1 ATC1 ATC45 ATC50 ATC58 ATC70 ATC76 ATC78 ATC87 ATC111 ATC114 M
OPC-20
1000
800
600
400
300

c



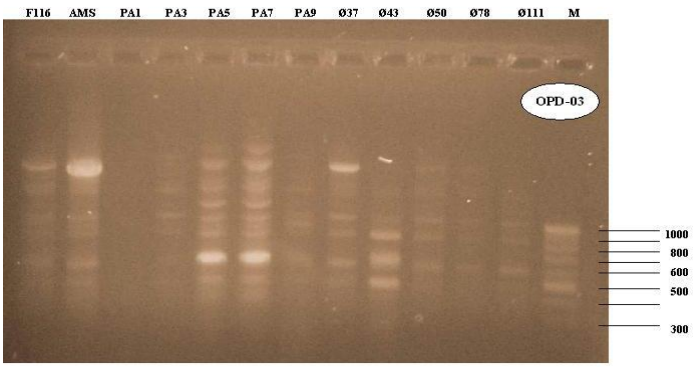
d



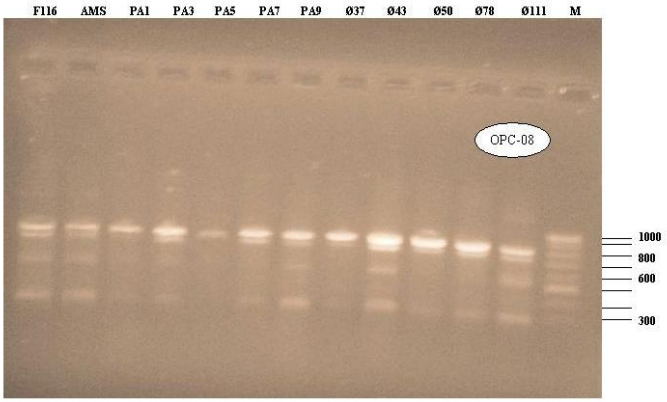
e

Figure 3: Amplified band profiles generated with primers OPB-11, OPC-08, OPC-20, OPA-11 and OPD-05

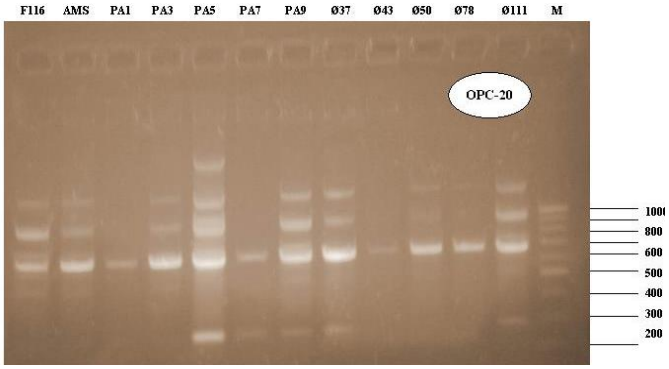
3


F116
AMS
PA1
PA3
PA5
PA7
PA9
Ø37
Ø43
Ø50
Ø78
Ø111
M
OPD-03
1000
800
600
500
300

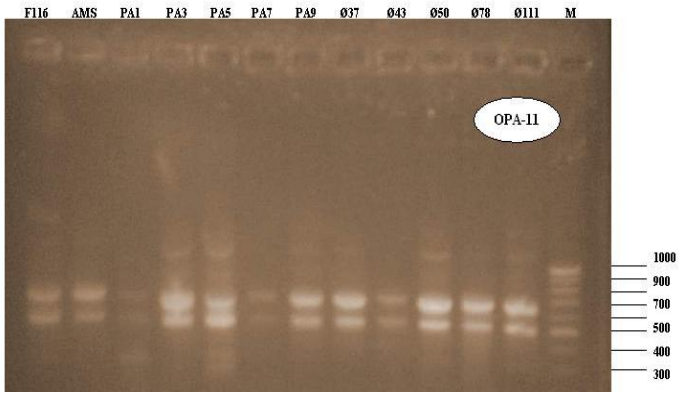
a


F116
AMS
PA1
PA3
PA5
PA7
PA9
Ø37
Ø43
Ø50
Ø78
Ø111
M
OPC-08
1000
800
600
300

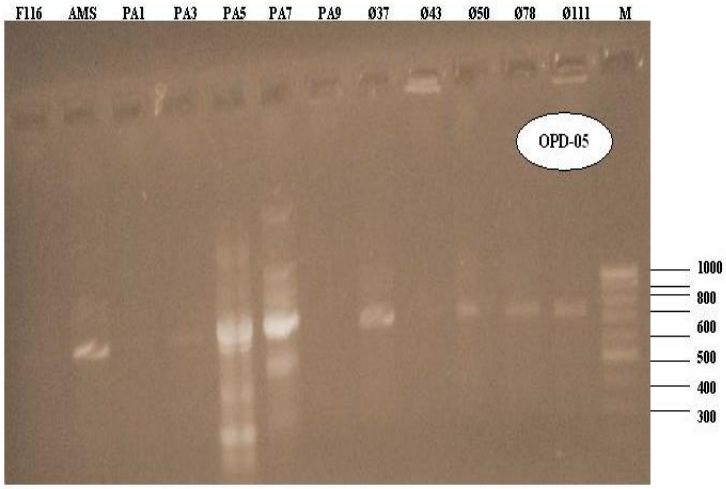
b


F116
AMS
PA1
PA3
PA5
PA7
PA9
Ø37
Ø43
Ø50
Ø78
Ø111
M
OPC-20
800
600
500
400
300
200

c



d



e

Figure 4: Amplified band profiles generated with primers OPD-03, OPC-08, OPC-20, OPA-11 and OPD-05

Analysis of genetic relationships among *Pseudomonas aeruginosa* based on RAPD-PCR technique was done by Aires de Sousa *et al.* 1996; Mahenthiralingam *et al.* 1996; Hernandez *et al.* 1997; Mereghetti *et al* 1998; Feil and Enright 2004; Ortiz-Herrera *et al* 2004; Ruiz *et al* 2004; Sazakli *et al* 2005,;Lodeng *et al* 2006; Sevillano *et al* 2006; Trautmann *et al* 2006; Zietz *et al* 2006 and Shoumali *et al* 2007.

RAPD analysis performed for bacteriophages with selected primers;

The five primers from the initial screening process were OPD-03, OPC-08, OPC-20, OPA-11 and OPD-05 (Table 2) that exhibit a high level of polymorphism among the bacteriophages and therefore chosen for this study. A fragment was considered polymorphic when absent in at least one bacteriophage. These primers detected scoreable polymorphisms in banding patterns among the bacteriophages.

All Polymorphic PCR products were confirmed by repeating the reaction. Each of five primers used for analysis of individual bacteriophage amplified different number of bands. The total number of amplified fragments from all primers for each bacteriophage are summarized in Table 6. Figure 4 (a, b , c, d and e) represents the patterns of the five random primers with the 11 bacteriophages. Each of five different random primers yielded from 4 to 11 DNA fragments whose molecular size ranged from approximately 212 to 1509 bp. The total number of amplified RAPD fragments after using all five primers was 33 bands with an average of 6.6 fragments / primer. The number of polymorphic bands through each primer ranged from 3 to 10 bands per primer with an average of 5.6 polymorphic bands per primer. The total number of polymorphic amplicons produced by the 5 primers was 28, thus, representing a level of polymorphism of 85% in all bacteriophages. Primer OPD-03 produced the highest number of fragments among the primers used with an average of 11 fragments while, primer OPC-08 produced the lowest number of fragments with an average of 4 bands. This indicated that the Primer OPD-03 was the highest among the tested five primers in their ability to flank the DNA sequences of 12 bacteriophages tested in this work. Primer OPC-08 produced the highest percentage of polymorphism (100%) in the bacteriophages while, primer OPA-11 produced the lowest percentage of polymorphism (60%). The highest number of amplified RAPD fragments (30) after using all five primers was detected in PA5 with an average of 6 fragments per primer, while the lowest number (11) with an average of 2.2 fragments per primer was detected in PA1.

The fragment size of the PCR products generated by primer OPD-03 (Fig. 4a) ranged from approximately 552 to 1509 bp and produced one shared band (984 bp**)** in all bacteriophages. Bands of size 552, 1174 and 1509 bp were observed in PA5 and PA7 but were absent from all bacteriophages. A band of size 771 bp was absent only in PA3. Bands of size 552, 1174 and 1509 bp were positive unique RAPD markers while, a band of size 771 bp was negative unique RAPD marker.

The fragment size of the PCR products generated by primer OPC-08 (Fig. 4b) ranged from approximately 295 to 830 bp and did not produce shared bands in all bacteriophages. Bands of size 295 and 763 bp were absent only in PA5, a band of size 830 bp was absent only in Ø50 while, a

band of size 558 bp was absent in Ø37 and Ø78. Bands of size 295, 558, 763 and 830 bp were negative unique RAPD markers.

The fragment size of the PCR products generated by primer OPC-20 (Fig. 4c) ranged from approximately 230 to 1411 bp and produced one shared band (536 bp) in all bacteriophages. A band of size 1411 bp was observed only in PA5 while, bands of size 784 and 1089 bp were absent in PA1, PA7 and Ø43. A band of size 1411 bp was positive unique RAPD marker while, bands of size 784 and 1089 bp were negative unique RAPD markers.

The fragment size of the PCR products generated by primer OPA-11 (Fig. 4d) ranged from approximately 307 to 1150 bp and produced two shared bands (553 and 666) in all bacteriophages. A band of size 307 bp was observed only in PA5 while, a band of size 386 bp was observed in F116, PA1 and PA5. A band of size 1150 bp was absent in PA1, PA7 and Ø43. Bands of size 307 and 386 bp were positive unique RAPD markers while, a band of size 1150 bp was negative unique RAPD marker.

Table 6 : Number of amplicons and the number of polymorphic bands produced by each RAPD primer for ever of bacteriophage isolates.

Primers	F116	AMSE 2000	PA1	PA3	PA5	PA7	PA9	Ø37	Ø43	Ø50	Ø78	Ø111	Total No. of amplicons	Polymorphic amplicons	Polymorphism %
OPD-03	8	8	2	6	11	11	7	8	7	6	6	6	11	10	91
OPC-08	4	4	4	4	2	4	4	3	4	3	3	4	4	4	100
OPC-20	3	3	1	3	5	4	4	4	1	3	3	4	5	4	80
OPA-11	4	3	3	3	5	2	3	3	2	3	3	3	5	3	60
OPD-05	1	4	1	1	7	5	1	3	1	3	3	1	8	7	87.5
Total	20	22	11	17	30	24	19	21	15	18	18	18	33	28	85
Average	4	4.4	2.2	3.4	6	4.8	3.8	4.2	3	3.6	3.6	3.6	6.6	5.6	

The fragment size of the PCR products generated by primer OPD-05 (Fig. 4e) ranged from approximately 212 to 1418 bp and produced one shared band (627 bp) in all bacteriophages. Bands of size 212, 331 and 551 bp were observed only in PA5 while, a band of size 1418 bp was observed in PA5 and PA7. Bands of size 212, 331, 551 and 1418 bp were positive unique RAPD markers.

Nevertheless, all bacteriophages except for AMSE2000, PA9 and Ø111 were distinguishable by unique RAPD markers. PA3, Ø37, Ø43, Ø50 and Ø78 were identified by negative unique RAPD markers while, F116 revealed positive unique RAPD markers. PA5 and PA7 were identified by positive and negative unique RAPD markers and PA5 was characterized by the high number (10) of positive unique RAPD markers.

Phylogenetic relationship among bacteriophages based on amplified RAPD fragments (bands):

The similarity coefficient values among bacteriophages based on band polymorphisms generated by RAPD-PCR after using the all five primers are presented in Table 7. The highest similarity value (1.000) was found between Ø50 and Ø78 while**,** the lowest value (0. 313) was found between PA1 and PA5. The highest similarity value between F116 and all bacteriophages (0.906) was found between F116 and both of PA3 and PA9.The similarity coefficient value between F116 and AMSE2000 was (0.875).

The dendogram of genetic distances among bacteriophages based on band polymorphisms generated by RAPD-PCR after using the primers is shown in (Figure 5). It was not surprising that all bacteriophages except for PA5 and PA7 are fallen in one cluster.

Analysis of genetic relationships among bacteriophages based on RAPD-PCR technique was done by Johansson *et al.* 1995, Bruttin *et al.* 1997; Holt and Cote 1998; Tilsala-Timisjarvi and Alatossava 1998; Labrie and Moineau 2000; Moschetti *et al.* 2000; Barrangou *et al.* 2002; Yoon *et al.* 2002; Comeau *et al.* 2004; XU *et al.* 2005; and Daniela *et al*. 2006.

Table 7: The similarity coefficient among bacteriophage isolates based on combined analysis of amplified RAPD fragments after using all primers.

	AMSE	PA1	PA3	PA5	PA7	PA9	Ø37	Ø43	Ø50	Ø78	Ø111
F116	.875	.719	.906	.594	.625	.906	.813	.844	.813	.813	.875
AMSE		.594	.844	.656	.750	.844	.875	.781	.875	.875	.813
PA1			.688	.313	.531	.688	.594	.813	.656	.656	.719
PA3				.500	.594	.938	.781	.813	.844	.844	.906
PA5					.656	.563	.594	.438	.531	.531	.531
PA7						.656	.688	.719	.625	.625	.625
PA9							.844	.813	.844	.844	.969
Ø37								.781	.938	.938	.875
Ø43									.781	.781	.844
Ø50										1.000	.875
Ø78											.875

Fig. 5: Linkage dendogram for bacteriophage isolates based on combined analysis of amplified RAPD fragments after using all primers.

REFERENCES

Aires de Sousa, M.; I. S. Sanches; A. van Belkum; W. van Leeuwen; H. Verbrugh and H. de Lencastre (1996). Characterization of methicillin-resistant *Staphylococcus aureus* isolates from Portuguese hospitals by multiple genotyping methods. Microb. Drug Resist., 2: 331-341.

Barnini, S.; C. Dodi and M. Campa (2004). Enhanced resolution of random amplified polymorphic DNA genotyping of *Pseudomonas aeruginosa*.Lett. Appl. Microiol., 39: 274-277.

Barrangou, R.; S. S. Yoon; F. Breidt; H. P. Fleming and T. R. Klaenhammer (2002). Characterization of six *Leuconostoc fallax* bacteriophages Isolated from an industrial sauerkraut fermentation. Appl. Environ Microbiol., 68 : 5452–5458.

Brosch, R.; M. Lefevre and P. Grimont (1996). Taxonomic diversity of *Pseudomonads* revealed by computer- interpretation. Syst. Appl. Microbiol., 19: 541-555.

Bruttin, A., F. Desiere, N. d'Amico, J. P. Guerin, J. Sidoti, B. Huni, S. Lucchini, and H. Brussow. (1997). Molecular ecology of *Streptococcus thermophilus, Streptococcus thermophilus* bacteriophage infections in a cheese factory. Appl. Environ. Microbiol., 63:3144-3150..

Cheng, X.; W. Q. Yang; L. Shi; X. L. Yin; Y. Xie; W. Zeng; X. H. Li; J. Y. Su; Y. L. Kou and W. X. Jia (2006). Quantification for biofilm formation of *pseudomonas aeruginosa* isolates and their genotypic analysis. Sichuan Da Xue Xue Bao Yi Xue Ban., 37: 666-669.

Christian G. G.; B. Libisch; C. Colinon; E. Scoulica; L. Pagani; M. Füzi; G. Kronvall and G. M. Rossolini (2006). Establishing clonal relationships between VIM-1-Like Metallo-ß-Lactamase-producing *Pseudomonas aeruginosa* strains from four european countries by Multilocus Sequence Typing. Journal of Clinical Microbiology, 44: 4309-4315.

Comeau, A. M.; S. Short and C. A. Suttle (2004). The use of degenerate-primed random amplification of polymorphic DNA (DP-RAPD) for strain-typing and inferring the genetic similarity among closely related viruses. J. Virol Methods, 15 : 95-100.

Curran, B.; D. Jonas; H. Grundmann; T. Pitt and C. G. Dowson (2004). Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. J. Clin. Microbiol., 42: 5644-5649.

Daniela, M. G.; J. A. Reinheimer; A. G. Binetti; G. Giraffa; D. Carminati and A. Quiberoni (2006). Characterization of spontaneous phage-resistant derivatives of *Lactobacillus delbrueckii* commercial strains. International Journal of Food Microbiology, 111 :126-133.

De Vos, D. ; A. Lim; P. Devos and P. Cornelis (1993). Detection of the outer membrane lipoprotein I and its gene in fluorescent and non-fluorescent *pseudomonads*. J. Gen. Microbiol., 139: 2215-2223.

De Vos, D.; A. Lim; J. P. Pirnay; L. Duinslaeger; H. Revets; A. Vanderkelen; R. Hamers and P. Cornelis (1997). Analysis of epidemic *Pseudomonas aeruginosa* isolates by isoelectric focusing of pyoverdine and RAPD-PCR: modern tools for an integrated anti-nosocomial infection strategy in burn wound centres. Burns., 23 :379-386.

Feil, E. J. and M. C. Enright(2004). Analyses of clonality and the evolution of bacterial pathogens. Curr. Opin. Microbiol., 7: 308-313.

Hernández. J.; M. A. Ferrus; M. Hernández and R. J. Owen (1997). Arbitrary primed PCR fingerprinting and serotyping of clinical *Pseudomonas aeruginosa* strains. FEMS Immunol. Med. Microbiol.,17: 37-47.

Holt, S. M., and G. L. Cote (1998). Differentiation of dextran-producing *Leuconostoc*Leuconostoc strains by a modified randomly amplified polymorphic DNA protocol. Appl. Environ. Microbiol., 64:3096-3098.

Johansson, M. L., M. Quednau, G. Molin, and S. Ahrne (1995). Randomly amplified polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum and Lactobacillus plantarum* strains. Lett. Appl. Microbiol., 21:155-159.

Keel, C.; D. Weller; A. Natsch, and L. Thomashow, (1996). Conservation of the 2,4 diacetylphlo- roglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Appl. Environ. Microbiol., 62 : 552- 563

Labrie, S., and S. Moineau (2000). Multiplex PCR for detection and identification of lactococcal bacteriophages. Appl. Environ. Microbiol., 66:987-994..

Lodeng, A. G.; C. Ahlen; H. Lysvand; L. H. Mandal and O. J. Iversen (2006). Development and evaluation of a new PCR assay for detection of *Pseudomonas aeruginosa* D genotype. Clin Microbiol Infect.,12 : 761-768.

Lynch M. (1990). The similarly index and DNA fingerprinting. Molecular Biology and Evolution, 7: 478-484.

Lynch M. (1991). Analysis of population genetic structure by DNA fingerprinting. In: DNA Fingerprinting Approaches and Applications (ed. by T. Burke, G. Dolf, A.J. Je‹reys & R.Wolf), pp.113-126. Springer Verlag, Basel.

Mahenthiralingam, E.; M. E. Campbell; J. Foster; J. S. Lam and D. P. Speert (1996). Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J. Clin. Microbiol.,34: 1129-1135.

Mereghetti, L.; N. Marquet-van der Mee; J. Loulergue; J. C. Rolland and A. Audurier(1998). *Pseudomonas aeruginosa* from cystic fibrosis patients: study using whole cell RAPD and antibiotic susceptibility. Pathol. Biol. (Paris).46 :319-324.

Moore, E.; M. Mau and K. Timmis (1996). The determination and comparison of the rRNA gene sequen as of species of the genus *pseudomonas*. Syst. Appl. Microbiol., 19: 478- 482.

Moschetti, G., G. Blaiotta, F. Villani, and S. Coppola (2000). Specific detection of *Leuconostoc mesenteroides* subsp. *mesenteroides* with DNA primers identified by randomly amplified polymorphic DNA analysis. Appl. Environ. Microbiol., 66:422-424.

Nazik, H.; B. Ongen; Z. Erturan and M. Salcioglu (2007). Genotype and antibiotic susceptibility patterns of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* Isolated from cystic fibrosis patients. Jpn. J. Infect Dis., 60 : 82-86.

Ortiz-Herrera, M.; A. Gerónimo-Gallegos; F. Cuevas-Schacht; L. Pérez-Fernández and R. Coria-Jiménez (2004). RAPD-PCR characterization of *Pseudomonas aeruginosa* strains obtained from cystic fibrosis patients. Salud. Publica. Mex., 46:149-157.

Palleroni, N. (1992). Human and animal pathogenic *pseudomonas*. P 3086-3103 in A. Balows, Truper, M. and schleifer, H. (ed). The prokaryotes Springer Verlag, New York, N.Y.

Palleroni, N. (1993). *Pseudomonas* classification Antonie Leeuwenhoek, 64: 231-251.

Palleroni, N.; J. Kunisawa and M. Doundoroff (1973). Nucleic and homologies in the genus *Pseudomonas*. Int. J. Sys Bact., 23: 333-339.

Renders, N.; U. Romling; H. Verbrugh and A. van Belkum (1996). Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments. J. Clin. Microbiol., 34: 3190-3195.

Ruiz, L.; M. A. Domínguez; N. Ruiz and M. Viñas (2004). Relationship between clinical and environmental isolates of *Pseudomonas aeruginosa* in a hospital setting.Arch.Med.Res ., 35 :251-257.

Sands, D.; C. Schroth and D. Hildebrand (1980). *Pseudomonas* American phytopathological Society. St. Paul. Minn.

Sazakli, E.; M. Leotsinidis; A. Vantarakis and M. Papapetropoulou (2005). Comparative typing of *Pseudomonas* species isolated from the aquatic environment in Greece by SDS-PAGE and RAPD analysis. J. Appl. Microbiol., 99 :1191-1203.

Sevillano, E.; C. Valderrey; M. J. Canduela; A. Umaran; F. Calvo and L. Gallego (2006). Resistance to antibiotics in clinical isolates of *Pseudomonas aeruginosa*. Pathol. Biol. (Paris).54 :493-497.

Shoumali, L.; H. Masoud; H. Khlaif; H. Migdadi and S. Masoud (2007).Serologic and molecular characterization of *Pseudomonas aeruginosa* Jordanian clinical isolates compared with the strains of International Antigenic Typing Scheme. Diagn. Microbiol. Infect Dis. [Epub ahead of print].

Sneath P. H. A. and R. R. Sokal (1973). Numerical Taxonomy.W.H. Freeman, San Francisco, USA.

Stead, D. (1992). Grouping of plant- pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. Int. J. Syst. Bacteriol., 42: 281-295.

Tilsala-Timisjarvi, A., and T. Alatossava (1998). Strain-specific identification of probiotic *Lactobacillus rhamnosus* with randomly amplified polymorphic DNA-derived PCR primers. Appl. Environ. Microbiol., 64:4816-4819.

Trautmann, M.; C. Bauer; C. Schumann; P. Hahn; M. Höher; M. Haller; and P. M. Lepper(2006). Shared RAPD pattern of *Pseudomonas aeruginosa* from patients and tap water in a medical intensive care unit. Int. J. Hyg. Environ Health, 209 :325-331.

Tyler, S.; C. Strathdee and W. Johnson (1995). Oligonucleotide primers designed to differentiate pathogenic *pseudomonads* on the basis of the sequencing of genes coding for 16s rRNA. Clin. Diagn. Lab. Immun., 2: 448-453.

Welsh J. and M. McClelland (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res., 18: 7213-7218.

Widmer, F.; R. Seidler and G. Di- Giovanw (1998). A high selective PCR protocol for detecting 16S rRNA genes of the genus *pseudomonas*. Appl. Environ. Microbiol., 64: 2545- 2553.

Williams J. G. K.; A. R. Kubelik; K. J. Livak; J. A. Rafalski and S. V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

XU, Y.; D. R. Peng; H. Y. Xiong; X. N. Zhang; M. Q. Su; Y. Q. Sun and X. K. Hao (2005). Study on the nucleic acid of *E. coli* bacteriophage with broad host range and its sterilization effect to sewage samples from the environment. Zhonghua Liu Xing Bing Xue Za Zhi. , 26 :356-360.

Yoon S. S.; R. Barrangou-Poueys; F. Breidt; T. R. Klaenhammer and H. P. Fleming (2002). Isolation and characterization of bacteriophages from Fermenting Sauerkraut. Applied and Environmental Microbiology, 68:973-976.

Zietz, B. P.; H. Dunkelberg; J. Ebert and M. Narbe (2006). Isolation and characterization of *Legionella* spp. and *Pseudomonas* spp. from greenhouse misting systems. J. Appl. Microbiol.,100 :1239-1250.

تكنيك الـ RAPD-PCR لدراسة العلاقات الوراثية بين عزلات بكتريا *Pseudomonas aeruginosa*

محمد أبو بكر حسن يوسف – أمينة أحمد حسن- ممدوح كامل أمين
قسم الوراثة – كلية الزراعة – جامعة الزقازيق

أجريت تحليلات ال RAPD-PCR على ال DNA الجينومى المستخلص من ١١ عزلة من بكتريا ال *Pseudomonas aeruginosa* وكذلك من ١٢ بكتريوفاج بهدف تحديد العلاقات الوراثية بين بعض عزلات بكتريا *Pseudomonas aeruginosa* الممرضة للإنسان المعزولة من مرضي بمستشفى

جامعة الزقازيق و التى تم توصيفها بالكامل في قسم ا لميكروبيولوجى بكلية الصيدلة جامعة الزقازيق. وقد تم استخدام ٦ بواديء من ١٦ باديء ، و قد أعطت البوادئ الستة المستخدمة في هذه الدراسة تعدد صور في طرز حزم واسمات الرابيد بين هذه العزلات والسلالة البرية لبكتريا ال *Pseudomonas aeruginosa* وهىPAO1 وكذلك بين بعض بكتريوفاجات بكتريا السيدوموناس المعزولة من مياه الصرف الزراعي و من عزلات بكتريا السيدوموناس الليسوجينية المستخدمة في هذه الدراسة وبين الطراز البرى لفاج بكتريا ال *Pseudomonas aeruginosa* وهو F116 وقد أعطى كل بادئ عدد مختلف من الحزم. وقد تم تعيين التماثل الوراثي بين عزلات البكتريا وكذلك بين البكتريوفاجات على أساس العدد الكلى للفروق في حزم الرابيد. وكانت أكبر قيمة للتماثل الوراثى٠,٨٥٦ بين العزلات البكتيرية ATC1 و ATC78 وATC87 والطراز البرى PAOI ، وكانت أقل قيمة ٠,٤٨٦ بين الطراز البرى و العزلة البكتيرية ATC70 ، وقد أوضح دندوجرام المسافات الوراثية بين العزلات البكتيرية على أساس تعدد صور حزم واسمات الرابيد بعد استخدام البوادئ بأن العزلات البكتيرية PAO1 و ATC1 و ATC78 و ATC87 و ATC114 تقع فى مجموعة واحدة بينما ATC45 و ATC58 و ATC76 وATC111 تقع في مجموعة ثانية وكل من ATC70 و ATC50 تقع في مجموعة ثالثة. وقد كانت لكل عزلة بكتيرية تم تحليلها في هذه الدراسة تركيب وراثى مميز على أساس بصمات الرابيد فيما عدا العزلتين ATC45 و ATC58 .أما في البكتريوفاج فكانت أكبر قيمة للتماثل الوراثى٠,٩٠٦ بين الطراز البرى F116 و PA3 و PA9 وكانت قيمة التماثل الوراثى بين F116 و AMSE2000 هى٠,٨٧٥ ، وقد أوضح دندوجرام المسافات الوراثية بين البكتيريوفاجات على أساس تعدد صور حزم واسمات الرابيد بعد استخدام البوادئ بأن كل البكتيريوفاجات فيما عدا العزلة PA7 تقع في مجموعة واحدة. وقد كانت لكل البكتيريوفاجات التى تم تحليلها في هذه الدراسة تركيب وراثي مميز على أساس بصمات الرابيد فيما عدا AMSE200 و PA9 و Ø111.

توضح النتائج أنه من الممكن التمييز بين العزلات البكتيرية وكذلك بين عزلات البكتريوفاج باستخدام عدد قليل من الواسمات. وتوضح هذه الدراسة فاعلية تقنية الرابيد في التعرف على تعدد الصور الوراثية ويمكن أيضا أن تكون مفيدة في عمل البصمة الوراثية.