# Use Box –PCR to Study Genetic Relatedness of Some *Pseudomonaus aeruginosa* Isolates from Different Clinical and Environmental Sources In Baghdad

**Ebtesam H. Nassir**
College of science / University of al Mustansiriyah

**Summary**

Totally twenty six different isolates of *Pseudomonas aeuruginosa ( P. aeruginosa )* were collected ,sixteen clinical isolates ( 62% of total group ) were isolated from different samples ( Urine , blood , sputum , wound , ear , eye and throat swabs ) from Medical city hospital in Baghdad, and ten environmental isolates ( 38% of total group ) were isolates from different places of soil and water in the same city . All isolates were diagnosed by bacteriological and biochemical tests . The crude DNA was extracted from all clinical and environmental isolates . BOX-PCR was achieved using BOX1AR primer . The BOX-PCR products were analyzed by horizontal agarose gel electrophoresis . The dendrogram for all isolates was draw by using band-based Jaccard and Dice coefficients where the fingerprints were generated by banding patterns using un weighted pair-group method with arithmetic average ( UPGMA ) , in order to study the relatedness among the clinical and environmental isolates . The results were showed that the crude DNA was suitable to BOX-PCR , and presence of the high similarity in banding patterns of clinical and environmental isolates itselves , which was about 45%-99% and 61%-100% in Jaccard and Dice coefficients respectively . The similarity between these two groups was about 40% and 55% in Jaccard and Dice coefficients respectively . Although , the marked relatedness among all clinical and environmental isolates , this study was concluded presence of genetic variation between the clinical and environmental isolates of this opportunistic bacterium , by using BOX-PCR technique and BOX1AR primer .

**Introduction**

*P. aeruginosa* is a ubiquitous environmental Gram- negative bacterium, commonly encountered aerobic microbe [1]. In humans, *P. aeruginosa* can colonize virtually any mucosal surface , invade tissues and blood [1]. Its powerful armamentarium of virulence factors makes it highly pathogenic particularly among immuncompromised patients, causing high morbidity& mortality [2,3,and 4]. It is a well known cause of outbreak in hospitals [2 and 5]. The nutritional versatility enables this bacterium to occupy a variety of

ecological niches [2]. Its primary residence is within the environment, where it can be found in a number of habitats, including soil, water, plants, animal surface, decaying organic matter, environmental recycling within the soil [6] and sewage [3]. Factors that contribute to the virulence of *P. aeruginosa* in the human host are the same as those that contribute to its adaptability in the environment [6].Molecular techniquesoffer a considerable improvement & can complement phenotypic data to obtain a better understanding of bacterial diversity [7].The molecular typing techniques, which have proven useful in typing *P. aeruginosa* in this purpose & for epidemiological purposes include pulsed field gel electrophoresis " PFGE", restriction fragment length polymorphic DNA " RFLP " analysis, random amplified polymorphic DNA " RAPD" analysis, repetitive extra palindromic PCR " rep PCR " analysis & multilocus restriction typing " MLRT " [8].
The term ( REP ) sequences encompasses repetitive & palindromic sequences with between 21 and 65 bases detected in the extragenic space of some bacterial genomes [9]. REP elements are involved in the fine tuning of gene expression, so REP sequences play a role in bacterial DNA physiology[9]. There are families of repetitive DNA sequences are dispersed throughout the genome of diverse bacterial species ; Three families, unrelated at the DNA sequences level are named repetitive extragenic palindromic ( REP ) sequences , enterobacterial repetitive intergenic consensus ( ERIC) sequence and BOX element [10]. The dispersion of the REP , ERIC , and BOX sequences may be indicative of the structure and evolution of the bacterial genome [10 ]. The most common repeat sequences are highly conserved across bacterial species [11 ]. Some of these sequences , such as the repetitive sequences analysis used in forensic medicine , the method developed for assessment of bacterial relatedness ( termed rep PCR )examines the distance between repeat sequences by using primers directed outwardly from these sequences [11 ]. If repetitive sequences are within a distance that can be spanned by *Taq* polymerase extension , products of various molecular size ( depending upon the distance between repeats ) are obtained[11].

The benefits of rep-PCR method have now been widely recognized in the researches of bacterial diversity of clinical isolates as well as strains of industrial , agricultural ,and environmental organisms [7]. BOX-PCR has shown high discriminatory power with reproducibility , stability , fast turnaround times , and cost- effective alternatives for typing bacteria [7]. BOX- elements have since been identified in *P. aeruginosa* as well as *Enterococcus faecalis* [7].
The aim of the present study is to isolate and characterize clinical and environmental isolates of *P. aeruginosa* and study the relationship among these isolates .

**Material and Methods :**

**Bacterial isolation and identification :** 16 clinical isolates of *P. aeruginosa* were collected from patients in the Medical city hospital in Baghdad city ,and 10 environm- ental isolates of *P. aeruginosa* were collected from different places in Baghdad city . The table number 1 show the sources and the numbers of the collected isolates . All isolates were identified as *P. aeruginosa* by using Gram-negative staining , apositive oxidase reaction [12 ], and conventional tests using API20NE ( API-System, Bio Merieux , France ) .

**Extraction of DNA isolates** : DNA from each isolate was extracted as described by Coenye *et al.* [13 ] , with a few minor modification . Briefly , one colony was heated at 95° C for 15 min in 20 µl of lysis buffer containing 0.25% (wt/vol ) sodium dodecyl sulfate ( SDS )and NaOH .Following lysis, 180 µl of distilled water was added ,and the DNA solution were stored at 4° C until use as a template for PCR [13].

**Amplification of DNA** : Rep – PCR typing with a BOX - A1R primer ( 5′ CTACGGCAAGGCGACGCTGACG -3′ ) (BOX-PCR fingerprinting)was carried out as described by Hassan *et al.* [14 ]. Briefly , DNA amplification reactions were performed with a 25 µl reaction mixture that consisted of 2.5 µl of DNA sample for each isolate and 22.5 µl of PCR mater mix was included . The BOX-PCR master mix for primer (BOX-A1R) , 1mM deoxynucleoside triphosphates, 4.5 Mm $MgCl_2$ ,1X buffer (Sigma ,siwsser land ) [14 ]. Amplification was carried out with a programmable thermal cycler (Techne , Cambridage Ltd., England ). After initial denaturation for 2 min at 92° C ,35 amplification cycles were completed each consisting of 3 sec at 94 ° C, 30 sec at 92 ° C , 1 min at 50 ° C , and 8 min at 65 ° C .A final extension of 8 min at 65 ° C was applied [13 and 14 ].

**Separation of DNA bands** : PCR products were separated on 25-Cm long 1.5 % agarose gels in 0.5X TBE buffer ( 60 mA for 4 h at room temperature ) .A 100 bp molecular weight ladder ( Promega ) was used on each gel to allow normalization .Following staining with ethidium bromide and visualization by UV illumination [13 and 15 ].

**BOX-PCR fingerprint analysis** : Statistical analysis was used to determine the relatedness of DNA fingerprints and to determine whether the isolates could be successfully assigned to the correct source group. The DNA fingerprints were compared to each other by calculating Jaccared and Dice similarity coefficients . The dendrogram was generated with BioNumerics (Applied Maths ) using the unweighted pair-group method with arithmetic averages (UPGMA ) by using Dice and Jaccard similarity coefficients methods , in order to determine the relatedness of the studied isolates [11 and 13 ].

**Results and Discussion :**

All isolates of *P. aeruginosa* was characterized by using Gram- negative staining , appositive oxidase reaction [12 ], and conventional tests using API20NE ( API-System ,Bio Merieux , France ) . The results of characterization , showed share all the isolates with the same characters , except some little variation in colonies shapes , and the isolates no. 12 & 13 ( which both isolated from eye swabs ) showed significant trait production stain in liquid broth , in contrast to rest almost isolates which produce stain in solid media only .

**Table No. 1 show the sources and the numbers of the collected isolates**

| No. of isolate | The sources | No. of isolate | The sources |
|---|---|---|---|
| 1 | Tigris river | 14 | Ear swab |
| 2 | Al-Jaish canal | 15 | Ear swab |
| 3 | Swamp | 16 | Ear swab |
| 4 | Soil | 17 | Ear swab |
| 5 | Soil | 18 | Wound |
| 6 | Soil | 19 | Wound |
| 7 | Soil | 20 | Wound |
| 8 | Soil | 21 | Sputum |
| 9 | Soil | 22 | Sputum |
| 10 | Soil | 23 | Blood |
| 11 | Urine | 24 | Blood |
| 12 | Eye swab | 25 | Throat swab |
| 13 | Eye swab | 26 | Urine |

BOX-PCR was generated in this study , by using whole cell suspension , which eliminated the need for purification , and this result corresponding with results of Dombek *et al.* [16 ], and Louws *et al.* [10 ].Where the quality of nucleic acid extracted used for the analysis is one of the key factors in molecular typing [11]. We described the use of the BOX-PCR DNA fingerprinting technique to study the relationship among *P. aeruginosa* isolates collected from environmental and clinical ( human ) sources .

In BOX-PCR DNA fingerprinting , PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain specific DNA fingerprints which can be easily analyzed with pattern recognition computer software .The BOX-PCR technique was chosen , because this technique is simple , accrued , speed and can differentiate between closely related strains of bacteria [10,11,13 and 16 ]. BOX-PCR has been use to classify and differentiate among strains of many bacteria .

The ensure of the stability of the typing assay of BOX-PCR is due to the fact that highly conserved repetitive element of BOX sequences [7].

For all the reason above the BOX primer DNA fingerprints of 26 *P. aeruginosa* isolates were achieved analyzed by using the Jaccard and Dice band-matching algorithm , where banding patterns were compared using the un weighted pair group method with average linkages ( UPGMA ).

The results of PCR products were showed as fig. 1 and fig. 2 . The position of bands on each gel were normalized by using the 100bp molecular weight ladder(Promega) as external reference standard .Normalization with the same external standard allowed us to compare the both gels ( as show in fig . 1 and fig. 2 ) . Four bands that where common to most of the isolates on each gels were also used as internal reference standards .

Individual lanes at fig. 1 generally contained from 7 to 16 PCR product bands , except the lane 23 which contain 4 product bands only . The molecular size of the PCR products ranged from about 100bp to slightly more than 1500 bp .

More prominent bands ( 800 ,700 , 500 ,and 240 )bp , however ,were consistently present in 73% of isolates contain the PCR product bands which have molecular weight about 650 bp .

The BOX-PCR fingerprint profiles among each gel as show in in fig.1 and fig. 2 , contain many bands of equal mobility , but they are distinct , consistent with the concept that selection for a specialized niche affects genome organization and that corresponds to a unique distribution of repetitive sequences in the bacterial genome .

Indeed , for all the genotyping methods under consideration , visual comparison of large number of complex fingerprint patterns , is not only timconsuming but also highly subjective . The use of equipment to digitize patterns and software to perform numerical analysis of these patterns are necessary for this typing study .

Jaccard and Dice are band-based coefficients that consider only the presence or absence of DNA bands, which used to calculate similarities among rep-PCR fingerprints [14 ]. So the fig. 3 and fig . 4 show the resulted dendrogram of studied isolates .

The results of the dendrogram showed, the presence of high similarity in banding patterns of isolates among every group ( among environmental isolates it selves, and among clinical isolates itselves), which was about 45% - 99% and 61% - 100% in Jaccard and Dice coefficients respectively. The similarity between the two group was about 40% and 55% in Jaccard and Dice coefficient respectively.

The isolates no. 23 and no.1 showed low percentage of similarity in the dendrogram which was only 10% and 19% respectively in Jaccard coefficients , and 20 % and 31% respectively in Dice coefficients , this low percentage of

similarity , probably as a result of the variation of isolates source collected in this study . Only two isolates ; no. 11 & no.26 which share in the same source ( urine samples from different patients ) were identical , this may because the fact that *P. aeruginosa* posses a large and diverse genome [1] , or because it is thought that patients acquiered their own unique strains from the environment and only in specific circumstances the patients were found to share the same strain [17 ].

Our results indicated that although , the use of BOX-PCR to find relatedness among different sources of *P. aeruginosa* isolates in Jaccard and Dice /UPGIMA dendrogram , may have been useful for separating isolates in to environmental and clinical groups ,the isolates were highly close related and their was some isolates could not be assigned to the correct source group . This is may be due to certain assumption regarding the parameters of BOX-PCR method itself [11] , or due to the fact that controversially of the main habitat of *P. aeruginosa* [3].

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**Fig: 1 BOX-PCR products patterns of environmental studied *P. aeruginosa* isolates generated with primer BOX1AR . The circumstances of electrophoresis ( 25-Cm-long 1.5 % agarose gels in 0.5X TBE buffer ,60 mA for 4 h at room temperature) . Lane M contained 100bp molecular weight ladder .**

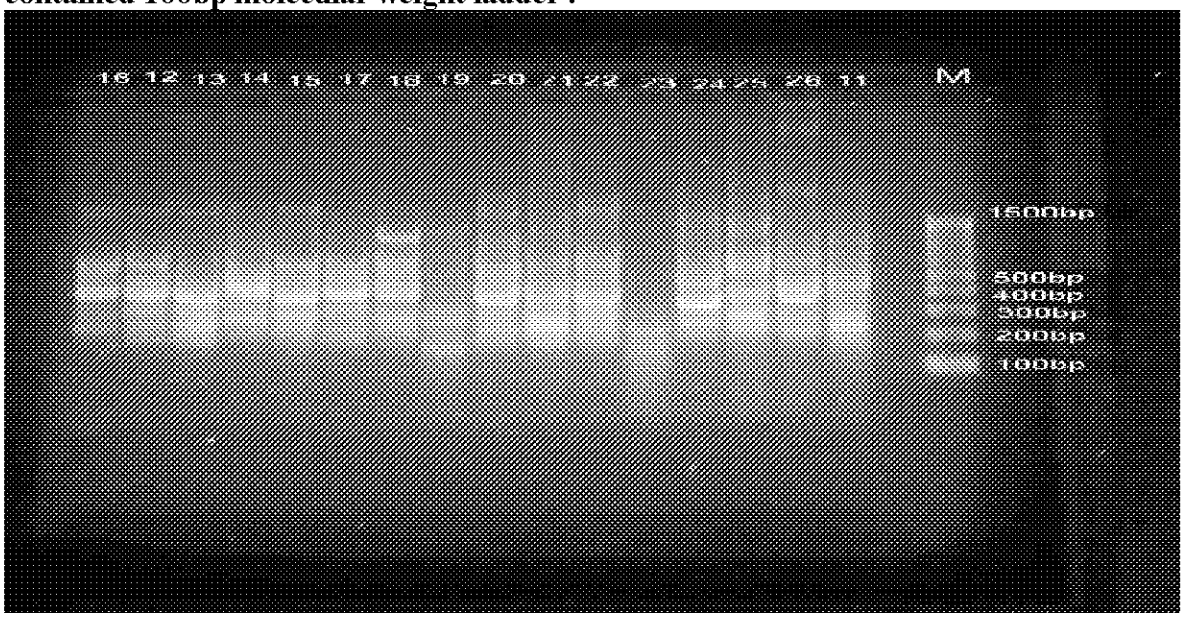


**Fig: 2 BOX-PCR products patterns of clinical studied *P. aeruginosa* isolates generated with primer BOX1AR . The circumstances of electrophoresis ( 25-Cm-long 1.5 % agarose gels in 0.5X TBE buffer ,60 mA for 4 h at room temperature) . Lane M contained 100bp molecular weight ladder .**

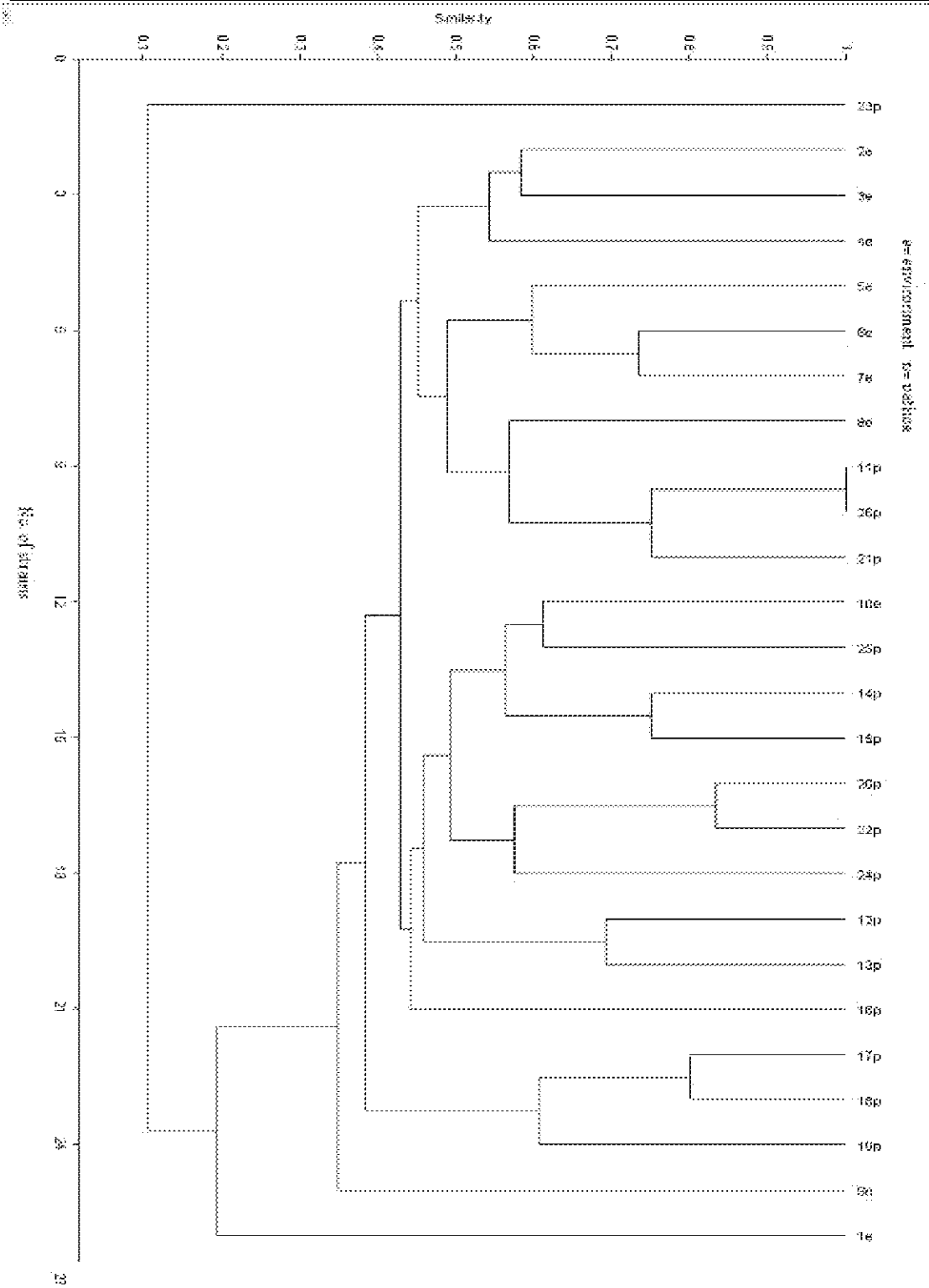


**Fig :3 . Dendrogram ( Jaccard / UPGMA ) showing the relatedness of studied *P. aeruginosa* isolates**

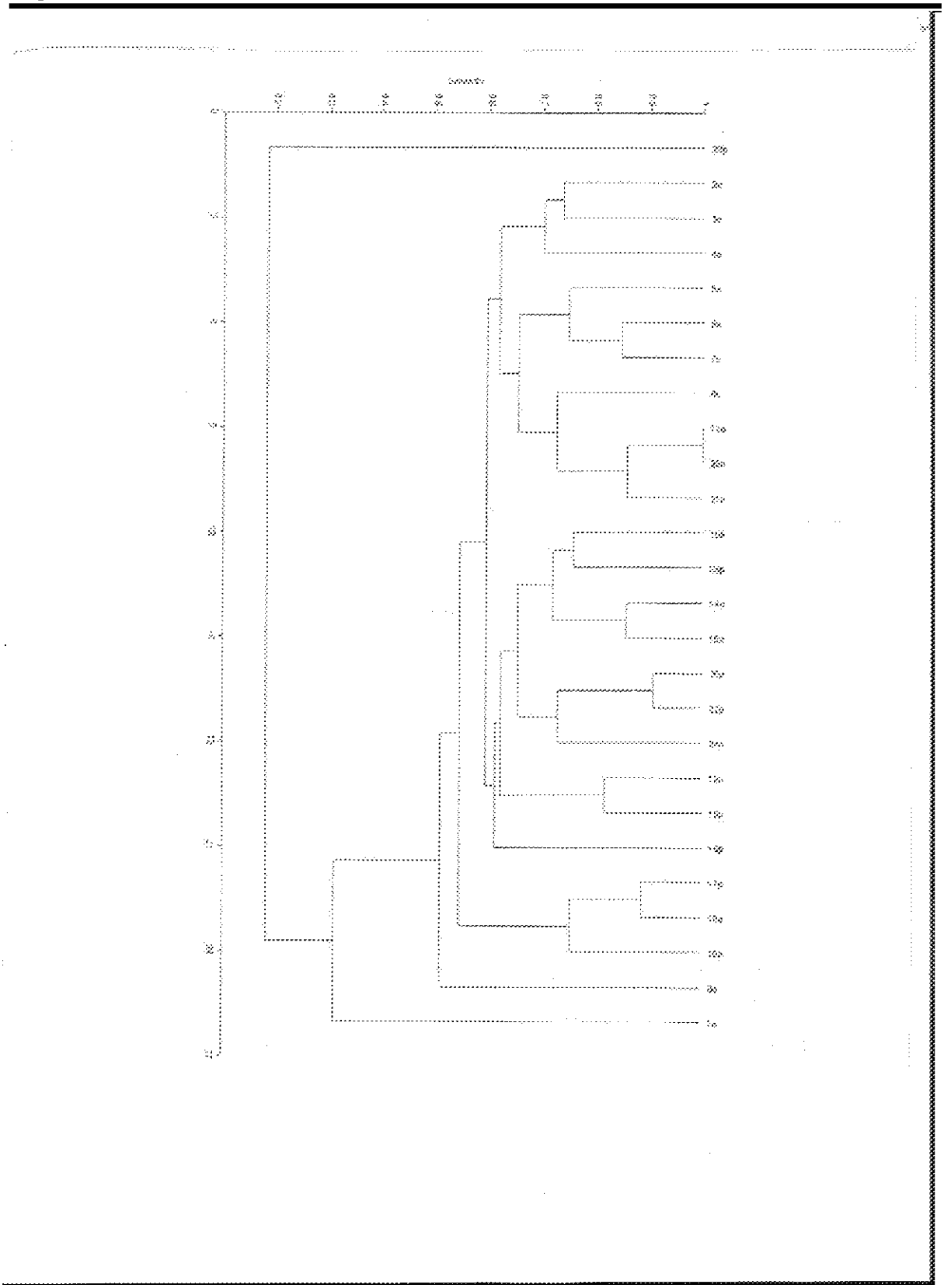

**Fig :4 . Dendrogram (Dice / UPGMA ) showing the relatedness of studied *P. aeruginosa* isolates**

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## "استعمال BOX-PCR لدراسة التقارب الوراثي لبعض من بكتريا *Pseudomonaus aeruginosa* المعزولة من مصادرمرضية و بيئية مختلفة في بغداد "

**ابتسام حمود ناصر**

**قسم علوم الحياة / كلية العلوم/ الجامعة المستنصرية**

**الخلاصة**

تم جمع ست و عشرون عزلة بكتيرية مختلفة من بكتريا *Pseudomonas aeuruginosa*، كان منها 16 عزلة مرضية أي 62 % من العدد الكلي للعزلات ، معزولة من نماذج مختلفة ( ادرار و دم و قشع و جروح و مسحات الاذن و العين و البلعوم ) من مستشفى مدينة الطب في بغداد . و كان منها عشرة عزلات بيئية أي 38% من العدد الكلي للعزلات ، معزولة من اماكن مختلفة من الماء و التربة في نفس المدينة . شخصت كل العزلات بألاختبارات البكتريولوجية و البايوكيميائية . تم استخلاص DNA الخام من كل من العزلات المرضية و البيئية . أنجز BOX-PCR بأستخدام البادئ BOX1AR ، بعدها تم تحليل ناتج التضخيم BOX-PCR بأستخدام الترحيل الكهربائي الافقي بهلام الاكاروز . تم رسم Dendrogram لكل العزلات بأستخدام معاملان Jaccard و Dice معتمدة – الحزم ، اذ أنجزت الطبعة الوراثية لنسق الحزم باستعمال UPGMA ، لدراسة التقارب الوراثي بين العزلات المرضية و البيئية . بينت النتائج ان DNA الخام مناسب لأجراء BOX-PCR ، و وجود تشابه كبير بين نسق الحزم الناتجه عن التضخيم للعزلات المرضية و البيئية نفسها ، و التي كانت بحدود 45 % - 99% و 61% - 100% حسب المعاملان Jaccard و Dice على التوالي . اما التشابه بين هاتين المجموعتين كان بحدود 40% و 55% حسب المعاملان Jaccard و Dice على التوالي . بالرغم من العلاقة الملحوظة بين كل من العزلات المرضية و البيئية ، استنتجت هذه الدراسة وجود تباين وراثي بين العزلات المرضية و البيئية لهذة البكتريا الأنتهازية بأستخدام تقنية BOX-PCR و بأستخدام البادئ BOX1AR .