Use Box-PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad

Summary

Totally twenty six different isolates of Pseudomonas aeruginosa (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 drawn by using band-based Jaccard and Dice coefficients where the fingerprints were generated by banding patterns using unweighted 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 itself, 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
Use Box –PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad ...........................................Ebtessam H. Nassir

powerful armamentarium of virulence factors makes it highly pathogenic particularly among immunocompromised 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 techniques offer 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
Use Box–PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad

Ebtessam H. Nassir

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 environmental 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, a positive 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 Coene 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 was stored at 4°C until use as a template for PCR [13].

Amplification of DNA: Rep-PCR typing with a BOX-A1R primer

(5' CTACCGCAAGCGACGCTGACG -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 master mix was included. The BOX-PCR master mix for primer (BOX-A1R), 1mM deoxynucleoside triphosphates, 4.5 Mm MgCl₂, 1X buffer (Sigma, Switzerland) [14]. Amplification was carried out with a programmable thermal cycler (Techne, Cambridge 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 Jaccard and Dice similarity coefficients. The dendrogram was generated with BioNumerics (Applied Maths) using the
Use Box-PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad...........................................Ebtesam H. Nassir

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

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

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.
Use Box–PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad ............................................ Ebtesam H. Nassir

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 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 timeconsuming 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 itselfs), which was about 45% - 99% and
Use BOX-PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad .......................................................... Ebtesam H. Nassir

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 acquired 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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Use Box-PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad .................................................. Ebtesam H. Nassir

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.
Use Box-PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad

Fig 4. Dendrogram (Dice / UPGMA) showing the relatedness of studied P. aeruginosa isolates
Use Box –PCR to Study Genetic Relatedness of Some Pseudomonas aeruginosa Bacterial Isolates from Different Clinic and Environmental Sources In Baghdad ..................................................Ebtesam H. Nassir

References:


استعمال BOX-PCR لدراسة التقارب الوراثي لبعض من بكتريا Pseudomonas aeruginosa مصادر مرضية وبيئية مختلفة في بغداد

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الخلاصة

تم جمع ست و عشرون عزلة بكتيرية مختلفة من بكتريا Pseudomonas aeruginosa، كان منها 16 عزلة مرضية أي 62% من العدد الكلي للعزلات، عزلة من نماذج مختلفة (إلازور، دم، قش، جروح، مسحات الأذن والعين والبلازما) من مستشفى مدينة الطبل في بغداد. وكان منها عشرة عزلات بيئية أي 38% من العدد الكلي للعزلات، عزلة من أماكن مختلفة من الماء والترية في نفس المدينة. شُخصت كل العزلات بأختبارات البلاكروبيولوجية والبيوكيميائية. تم استخلاص DNA الخام من كل العزلات المرضية والبيئية. BOX-PCR أنجز بال:bأستخدام البادي BOX1AR، بعداً تم تحليل نتائج التحضيم Dendrogram بإستخدام الترجم الكهربائي الألفي بهلام الإكراز. تم رسم معايير الأجرام و Jaccard معتمدة - الحزم، إذ أنجزت الطبعة الوراثية لنسق الحزم بإستخدام معالمان UPGMA، لدراسة التقارب الوراثي بين العزلات المرضية والبيئية. بينت النتائج أن BOX-PCR، و وجود تشابه كبير بين نسب الحزم الناتجة عن التصنيف للعزلات المرضية والبيئية نفسها، والتي كانت بحدود 45% - 99% و 61% - 100% على التوالي. أما التشابه بين هذين المجموعتين كان بحدود 40% - 55% حسب المعالمان UPGMA على التوالي. و Jaccard حسب المعالمان على التوالي.

بالنسبة لموازنة العلاقة الملحوظة بين كل من العزلات المرضية والبيئية، استنبذت هذه الدراسة وجود تباين وراثي بين العزلات المرضية والبيئية لهذه البكتريا الالتهابية باستخدام تقنية BOX1AR و بأستخدام البادي PCR.