PCR Ribotyping for Determining the Diversity of Some Clinical Pseudomonas Aeruginosa Isolates

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Abstract

To study the genetic diversity between deferent strains of *P. aeruginosa*, this study was carried out between August and December 2010 by collecting 165 clinical samples from wounds, burn, ear and urinary tract infection taken from general hospitals of Sulaimani, Erbil and Koya governorates. After identifying *P. aeruginos* based upon culture methods using the selective media (cetrimide agar) coupled with biochemical tests, Polymerase chain reaction (PCR) method were used for identifying *P. aeruginosa* using primers targeting 16S rRNA. PCR analysis of 16S-23S internal transcribed spacer primer targeting the interspaced regions between the 16 and 23S rRNA genes (PCR ribotyping) was evaluated for it s effectiveness to differentiate between the isolates, the results show that the clinical isolates of P. aeruginosa belongs to three ribotypes, which were showed in one to three bands in each isolate of *P. aeruginosa*.

Keywords*: Pseudomonas aeruginosa*, genetic diversity, PCR, Ribotyping.

Introduction

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium which has emerged as one of the most problematic nosocomial pathogens .*it is* found commonly in most hospitals in the world, including those of Kurdistan region in Iraq, it is considered an opportunistic pathogen that causes infection in immune depressed subjects [1], the leading cause of wound infections [2], urinary tract, surgical wound and ear infection [3]. A rapid and accurate system for the identification of Pseudomonas is important to isolate patients and prevent further spreading of the diseases. Ribotyping is the analysis of rRNA genes in general, that is used for differentiating between species or strains of bacteria including *P. aeruginosa* [4], it is differ from PCR-ribotyping which uses specific primers that amplifies sequences between the 16S and 23S gene and has been applied for molecular identification of bacteria at the species level [5]. Presently, genetic techniques supported by phenotypic tests provide detailed characteristic of strains isolated from clinical wards[6].16S rDNA sequence has been used as a taxonomic "gold standard" in determining the phylogenies of bacterial species [7]. In order to determine the genetic relationship between these isolates, there are some techniques such as Restriction endonucleases analysis, Multilocus enzyme electrophoresis, Biotyping, Pulse field gel electrophoresis, and Ribotyping. Among them, the PCR-ribotyping is an efficient technique used during last 10 years and based on the amplification of spacer regions or intervening sequences between 16S and 23S rDNA genes. [8]. PCR-ribotyping uses specific primers that amplifies sequences between the 16S and 23S gene and has been applied for molecular identification of bacteria at the species level [9] and to discrimination between bacterial strains [10]. This method detects both the number of tRNA genes and the spacer length within the cluster, using primers complementary to the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene, which reveals length heterogeneity even within the same species, it mean it is used for studying genetic diversity. The phylogenetic classification of prokaryotes with rRNA gene sequences is based on the assumption that the differences in sequences reflect the evolution of the organisms from which PCR ribotyping have extensively been used in the molecular epidemiology of different outbreaks and episodes of infections in which that they have

been extracted [11]. So the aim of the present study was: To assess the genetic diversity between some strains, of *P. aeruginosa* collected from three deferent hospitals using species specific PCR.

Materials and Methods

Sampling: Between August and December 2010, 55 samples were taken from each of three general hospitals of Sulaimani, Erbil and Koya governorates, as a total of 165 clinical samples recruited for the study.The swab samples were taken from patients with burn, wound and ear infections, and urine samples were taken from patients suffering from urinary tract infection (UTI**).**

Phenotypic tests

According to [12], the swabs were enriched in brain-heart infusion broth, plated onto MacConkey agar. A single colony was selected and inoculated on the selective medium (cetrimide agar). Then morphological characteristics *of P aeruginosa* was described after Gram staining, including pigments production after incubation at 37ºC for 1-2 days. The biochemical tests were done according to [13]which include: Growth at 42ºC in trypticase soya agar, Indole production test, Methyl red test, Voges-proskauer (VP)**,** Citrate utilization test, urease activity**,** Oxidase test and the catalase test.

DNA Extraction

Genomic DNA was prepared according to [14] with some modification as follow: Ten ml overnight cultures were prepared in broth media from fresh single colony. Cells were harvested in a centrifuge for 5 min at 6000 rpm. After this, they were suspended in 200 µl 1xTE buffer (pH8), then 3 mg/ml lysozyme to the cell Suspensions was added and incubated for 2 hours at 37ºC. After the incubation, 370 µl, 1x TE (pH 8) containing Proteinase K (1mg/ml) was added for 1 hour, and then 30 µl, 10% SDS were added. The samples were then incubated for 1 h at 37°C. then phenol Chloroform extraction was performed twice (if necessary) using one equal volume of phenol/ chloroform/isoamyl alcohol (24/24/1) for 30 minutes and then, samples were centrifuged for 5 min at 6000 rpm, then the aqueous phase was transferred into a clean eppendorf tube and the genomic DNA was precipitated by the addition of cold isopropanol (one equal volume) after addition 10% of the volume by ammonium acetate and then incubates in freezer for 30 minutes. And precipitated DNA was transferred into a fresh eppendorf tube adding 200 µl of (70%) ethanol, and washed. Finally, the pellet was dissolved in TE buffer.

Application of PCR

In order to confirm the isolates as *P. aeruginosa*, PCR assay that based on 16S rDNA sequence with specific primers as described by [7] was carried out in 25 µL reaction volumes (2.5 µl of PCR buffer (500 mM-KCl, 100 mM Tris-HCl [pH 8.4]), 1µl Taq polymerase, 0.8 µl $MgCl_2$ (15 mM) and 2.5 µl dNTPs(dGTP, dTTP, dATP, and dCTP; 2 mM each) and 2 µl of Forward and Reverse prime in concentration of 10Pmol, the volume complete up to 25 µl by sterilized double distilled water , the reaction mixture was overlaid with 18 µl of mineral oil in order to avoid evaporation. The sequence of the primers used are: Pp16S-F GACGGGTGAGTAATG CCTA and Pp16S-R CACTGGTGT TCCT TCC TATA for *P. aeruginosa* which expected to amplifies 956 bp. the amplification program was run as follow: One cycle of 95°C for 2 minutes, Thirty cycles of(92°C for 1 minute, 55 C for 1 minute and One cycle of 72°C for 10 minutes, The amplified product was running by 1.2% agarose gel electrophoresis, then stained using ethidium bromide and visualized by U.V. transilluminator at 366 nm wavelength for detection of the results. PCR-ribotyping of P. aeruginosa isolates was carried out as described by [15] and amplification reaction was performed as the same above but using the pair of primers F: GAAGTCGTAACAAGG and the, R: CAAGGCATCCACCGTand changing the annealing temperature to 50°C.

Results and Discussion

Isolation of *P. aeruginosa*

Out of the 165 isolates that were collected from the three hospitals, of Sulaimani, Erbil and Koya governorate 43 isolates successfully diagnosis as *P. aeruginosa*, representing 26.06% of total isolates, the highest percentage of *P. aeruginosa* was obtained from burn

samples (10.9) and the lowest percentage were obtained from Otitis samples (1.81), this is because *P. aeruginosa* introduced into areas only when devoid of normal defenses or when membranes and skin are disrupted by direct tissue damage.

Phenotypic test

The result showed that *P. aeruginosa* were Gram negative rod shaped an aerobic, non-sporulating, produced greenish-yellow or blue pigment colonies on selective media (cetrimide agar), this is agreed with [1]. *P. aeruginosa* reacted positivly to catalase and oxidase tests, while it was negative for methyl red, Voges Proskauer and indole. It slowly hydrolyzed urea, utilized Simmon's citrate. The biochemical properties of the bacteria recorded in this study are the same as obtained by [16]. In general biochemical methods have, to a certain extent, facilitated the identification of *P. aeruginosa*; however, some of these methods are time consuming and may not be very accurate.

Isolation of the DNA

24 samples were chooses for DNA extraction, The full amount of DNA obtained using this protocol was very efficient method for DNA extraction from *P. aeruginosa*, since good yields of genomic DNA were obtained with average yields between (50-95µg/ ml) using 10ml broth culture media.

PCR Analysis

16SRNA gene based PCR

During PCR analysis, specific amplifications are only observed when PCR-specific primers, are used, which occur only in *P. aeruginosa* and not in other bacteria species**.** The primers used in this study targeted species-specific signature sequences in 16S rDNA variable regions 2 and 8 (V2 and V8), respectively. PCR assays employing each primer pair produced DNA products of the predicted size which was 956bp (Fig.(1)). 16S rRNA gene sequence offered a useful method for the identification of bacteria. It had long been used as a taxonomic method in determining the phylogenies of bacterial species [17]. The specificity of species-specific PCR assays was determined by testing the 24 culture collection of *P. aeruginosa* strains, which were 100%. Beside no amplified product produced using another bacterial species (*Salmonella typhi* and *staphylococcus aureas*), this result is in agreement with [7].Genotypic identification systems are currently preferred since they have proved to be more sensitive and specific in characterizing the different bacterial strains or clones. Within these, the PCR-based methods are most important because they could detect bacteria that were inhibited in their growth by residual concentrations of antimicrobials in the sample, or overgrown by other bacteria present in the sample, or autolyzed during incubation, or difficult to cultivate in vitro and thus under-diagnosed by bacterial culture. However, PCR-based tests are probably not appropriate to determine the number of viable bacteria and should be considered as complementary indicative tests in critical care set tings with culturing.

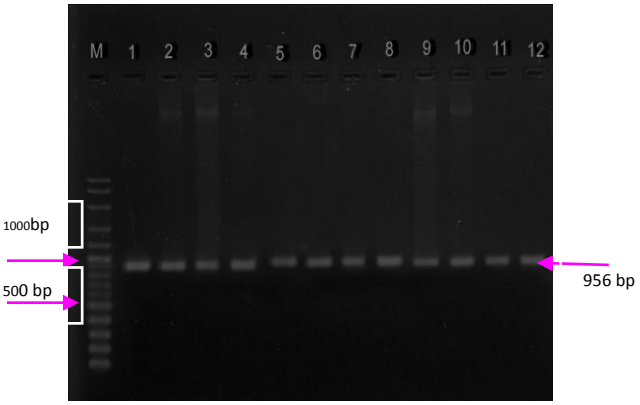

Fig.(1) 956 bp PCR products of 16S rRNA gene which were identified in some isolates of P. aeruginosa used in this study samples in 1.2% agarose gel electrophoresis. M 1kb DNA ladder 1- 12 were various samples of P. aeruginosa isolates.

PCR ribotyping

To determine the diversity of these strain that accurately identified using 16SrRNA gene based PCR, another kind of PCR called: PCR ribotyping was used. All the clinical isolates of *P. aeruginosa* belongs to three ribotypes which were showed in one to three bands in each strain of *P. aeruginosa* Cluster one include eight samples including samples 1, 5, 7, 8, 12, (Fig.(2)) which showed one band while Cluster two include twelve samples including 2, 3, 6, 9, 11, (Fig.(2)) which showed two bands and Cluster three showed

four sample including the samples 4, 10, which showed three bands (Fig.(2)), which varies in their molecular size from 300 bp to 600 bp and all strains shared a common band with 560 bp, these variations reflect the variation in the ribosomal gene structures between these strains, the cluster two was predominate and contained 12 samples which represent 50% of *P. aeruginosa* isolates used in this study. Prokaryotes contain the 16S, 23S, and 5S genes in their rRNA genetic loci. These genes are separated by spacer regions which can vary in length and sequence at the genus and species level. The spacer region between the 16S and 23S rRNA genes can be amplified by PCR and the polymorphisms in the resulting product can be used for identification of bacteria [18]. Nucleotide base sequence information has become available for this region for many bacterial species. [19] Many bacteria have multiple copies (alleles) of the rRNA operons per genome and the spacer region may vary in size within different operons, this relates to the number and type of tRNA genes (tRN*Aglu*, tRN*Aile*, tRN*Aala*) located in some spacer regions, the intergenic spacer regions (ISRs) are subject to lower evolutionary pressure and therefore show wider genetic variation [20]. Collierand coworkers [21] studied 49 strains of *P. aeruginosa* from various origins, concluded that PCR-ribotyping was

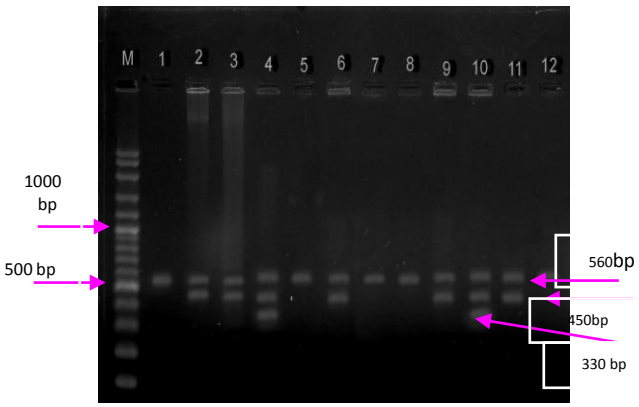

Fig.(2) Showed the result of PCR Ribotyping (300 bp – 600 bp) of some isolates of P. aeruginosa used in this study and all shared 560 bp using 1.2% agarose gel electrophoresis. M 1kb DNA ladder 1-12 were various samples of P. aeruginosa isolates.

slightly more discriminatory than Arbitrary primed PCR (AP-PCR), however, Martin *and* co workers [22] observed a low degree of heterogeneity of rRNA operon using molecular typing of strains of *P. aeruginosa*. So Vinuesa *and coworkers* [23] have found that PCR ripotyping can be improved by digestion of the PCR products with restriction endonuclease, (*Eco*Rl enzyme). However Wolska and Szweda [8] and Stehling and coworkers [4] suggest that maximum discrimination of *P. aeruginosa* can be achieved by a combination of ribotyping with other PCR or traditional Methods.In general epidemiologic studies on *P. aeruginosa* in patients are hindered by problems related to phenotypic variations of bacterial isolates throughout the period of infection, because phenotypic identification systems do not allow for reliable determination of the number of *P. aeruginosa* strains, whether the patient is infected by different strains over a period of time, or whether the isolated strains differ according to the site where the sample was taken, beside it was time consuming so there is need for qualitative and quantitative test that are more rapid than bacterial culture [24].

References

[1] Brooks, E.; Melnick, J. L.; Adelberg, E. A. Medical Microbiology. McGraw–Hill companies. N.Y, U.S.A.Vol.24, PP.189-201. 2007.

[2] Schaechter, M.; Baldauf, S. L.; Baross, J. A.; Baulcombe, D. C.; Haselkorn, R.; Hopwood, D. A. and Ingraham, J. L. Encyclopedia of. Microbiology 3rd ed., AP.USA. . PP.980-985. 2009.

[3] Todar, K. "*Pseudomonas aeruginosa*." Online Text Book for Bacteriology. PP.432-435. 2008.

[4] Stehling, E. G.; Leite, D. S. and Silveira, W. D. Molecular typing and biological characteristics of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in Brazil. *Braz. J. Infect. Dis.*, Vol.14, No.5. PP 23-27. 2010.

[5] Spacov, I. C. G.; da Silva, S. A. M. and Júnior, M. A. and de Morais, M. M. CPolymorphism of the rDNA and tDNA loci in clinical isolates of Pseudomonas aeruginosa: A perspective for molecular

epidemiology surveillance. *Genetics and Molecular Biology.*, Vol.29, No.4, PP 722-729. 2006.

[6] Czekajło-Kołodziej U, Giedrys-Kalemba S, Medrala D. Phenotypic and genotypic characteristics of Pseudomonas aeruginosa strains isolated from hospitals in the north –west region of Poland. Pol J Microbiol.Vol.55, PP.103-112. 2006.

[7] Spilker T, Coenye T, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. J Clin Microbiol . Vol 42, PP.2074-9. 2004.

[8] Wolska K, Szweda P. Comparative evaluation of PCR ribotyping and ERIC PCR for determining the diversity of clinical Pseudomonas aeruginosa isolates. Pol J Microbiol. Vol.57, PP.157-163. 2008.

[9] Agodi, A.; Sciacca, A.; Campanile, F.; Messina, C.; Barchitta, M.; Sciacca, S. and S, Stefani. Molecular epidemiology of *Pseudomonas aeruginosa* from cystic fibrosis in Sicily: genome macrorestriction analysis and rapid PCR-ribotyping. New *M*icrobiol. Vol. 23, PP.319-327. 2000.

[10] Pereira, M. S. V.; Leal, N. C.; Leal, T. C. A.; Sobreira, M. Almeida, A. M. P.; Siqueira-J˙nior, J. P. and Campos-Takaki, G. M. Typing of human and bovine *Staphylococcus aureus* by RAPD-PCR and ribotyping-PCR. *Lett. Appl. Microbiol.*, Vol.35, PP. 32-36. 2002.

[11] Satapathy, A. K.; Pavankumar, T. L.; Bhattacharjya, S.; Sankaranarayanan, R. and Ray, M. K. ATPase activity of RecD is essential for growth of the Antarctic *Pseudomonas syringae* Lz4W at low temperature. *FEBS Journal*., Vol. 275, PP.1835–1851. 2008.

[12] Cheesbrough, M. Medical Laboratory Manual for Tropical Countries. 2nd *ed., ELSB, Cambridge.*, Vol. 11, PP. 508-511. 1991.

[13] MacFaddin, J. F. Biochemical Tests for Identification of Medical Bacteria. 2nd ed., Waverly Press, Inc., Baltimor, USA., PP. 64-67. 2000.

[14] Cardinal, M. J.; Meghrous, J.; Lacroix, C. and Simard, R. E. "Isolation of *Lactococcis lactis* strain producing inhibitory activity against *Listeria*", Food *Biotechnology*, Vol.11, No.2, PP.129-146. 1997.

[15] Agarwal G, Kapil A, Kabra SK, Chandra R, Das B, Diwedi SN Phenotypic and genotypic variants of *Pseudomonas aeruginosa* isolated from children with cystic fibrosis in India. Indian J Med Res, Vol.116, PP. 73-81. 2002.

[16] Abro, S. H.; Wagan, R.; Tunio, M. T.; Kamboh, A. A. and Munir, M. Biochemical Activities of Bacterial Species Isolated from the Frozen Semen of Cattle. *Journal of Agriculture and Social Sciences.*, Vol.5, PP. 109–113. 2009.

[17] Drancourt, M.; Bollet, C.; Carlioz, A.; Martelin, R.; Gayral, J. P. and Raoult, D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clini. Microbiol.*, Vol. 38, PP. 3623-3630. 2000.

[18] Kostman, J. R.; Alden, M. B.; Mair, M.; Edlind, T. D.; LiPuma, J. J. and Stull T. L. An universal approach to bacterial molecular epidemiology by polymerase chain reaction ribotyping. *J. Infect. Dis*., Vol. 171, PP. 204-208. 1995.

[19] Tannock, G. W. Studies of the intestinal microflora: a prerequisite for the development of probiotics. *International Dairy Journal*. Vol. 8 PP.527-533. 1998.

[20] GarcÌa-MartÌnez, J.; Acinas, S. G.; AntÛn, A. I. and RodrÌguez-Valera, F. Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. *J. Microbiol. Meth*., Vol. 36, PP. 55-64. 1999.

[21] Collier, J. G.; Fraser, G. A.; Marmion, P. B.; Simmons, A. Practical Medical Microbiology.., Churchill Livingstone, New York. Vol.4, PP. 413-418. 1996.

[22] Martin, C.; Ichou, M. A.; Massicot, P.; Goudeau, A. and Quentin, R, Genetic diversity of *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis revealed by restriction fragment length polymorphism of the rRNA gene region. *J. Clin. Microbial.* Vol. 33, PP.1461-1466. 1995.

[23] Vinuesa, P.; Rademaker, J. L. W.; de Bruijn, F. J. and Werner, D. Genotypic characterization of Bradyrhizobium strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA DNA sequencing. *Appl. Environ. Microbiol.*, Vol.64, PP. 2096–2104. 1998.

[24] Pirnay, J. P.; Vos, D. D.; Duinslaeger, L.; Reper, P.; Vandenvelde, C.; Cornelis, P. and Vanderkelen, A. Quantitation of *Pseudomonas aeruginosa* in wound biopsy samples: from bacterial culture to rapid 'real-time' polymerase chain reaction. *Crit Care*, Vol.4, PP.255-261. 2000.

الخلاصة

لدراسة التنوع الوراثي لعزلات سريرية من بكتريا الـ *P. aeruginosa* اجريت هذه الدراسة وذلك بجمع ١٦٥ نموذج شملت: الجروح والحروق والتهابات الأذن والمجاري البولية والتي تم أخذها من المستشفيات العامة في ثلاث مناطق من الإقليم وهي كويه، سليمانية و اربيل وذلك للفترة بين شهر آب الى شهر كانون الاول من عام ٢٠١٠. فبعد تشخيص بكتريا الـ *P. aeruginosa* اعتمادا على الطرق التقليدية المعتمدة على استخدام الأوساط الزرعية، إذ تم استخدام الوسط الانتخابي (cetrimide agar) مقترنا مع الاختبارات الكيماوية. تم تطبيق التفاعل التسلسلي لانزيم بلمرة الدنا (PCR) في تشخيص عزلات الـ *P. aeruginosa* على مستوى الـ DNA والمعتمدة على الجينات الخاصة بالرايبوسومات وهي الـ 16S rRNA. لإيجاد العلاقة الوراثية بين تلك العزلات تم استخدام نوع أخر من هذه التفاعلات وهي الـ PCR ribotyping) والتي تستخدم فيها بادئات تستهدف جينات الرايبوسامات في المنطقة المحصورة بين 16S-23S وذلك لكفائتها في التمييز بين العزلات البكتيرية، إذ تم التوصل إلى وجود اختلاف بين تلك العزلات نتيجة وجود اختلاف في عدد وحجم الحزم المتضاعفة والتي تراوحت بين ١–٣ حزم مما يدل على انتماء تلك السلالات الى ثلاثة انواع من الانماط الرايبوسومية (ribotypes) ممايدل على وجود اختلافات وراثية بين تلك العزلات وما يعقبه من اخلاف في التعامل مع كل عزلة.