Identification *Pseudomonas aeruginosa* by 16s rRNA gene for Differentiation from Other *Pseudomonas* Species that isolated from Patients and environment

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Abstract:

Pseudomonas aeruginosa is a common and major opportunistic human pathogen, its causes many and dangersinfectious diseases due to death in some timesex: cystic fibrosis , wounds inflammation , burns inflammation , urinary tract infection , other many infections otitis external , Endocarditis , nosocomial infection and also causes other blood infections (Bacteremia). thereforebecomes founding fast and exact identification of *P. aeruginosa*from samples culture very important.However, identification of this species may be problematic due to the marked phenotypic variabilitydemonstrated by samples isolates and the presence of other closely related species. To facilitate species identification, we used 16S ribosomal DNA(rRNA) sequence data toidentify genus- and species-specific 16S rRNA signature sequences,its account a stable part of the geneticcode. Based on these sequences we designed simple, rapid, and accurate PCR assays that allow the differentiation of *P. aeruginosa* from *Pseudomonas* species and other pathogen genus ,also this test considered as the most specific than the other diagnostic tests like API (20) which give 70% while the 16SrRNA test give more than 90 %.

Key words:*Pseudomonas aeruginosa*;16s rRNA;gene;Differentiation

Introduction:

Pseudomonas aeruginosa is an aerobic non-spore forming Gram-negative rod with remarkable adaptable capacity to survive and persist under a broad range of environmental conditions (7)**.**

*Pseudomonas aeruginosa*is a common opportunistic human pathogen acquired in both the hospital and community setting (6)**.***P. aeruginosa* may be found in a variety of aqueous solutions, including disinfectants, soaps and eye drops, as well as in sinks, hot tubs, respiratory equipment and showerheads**.***P. aeruginosa* is rarely part of the microbial flora of healthy individuals, but may colonize the gastrointestinal tract of especially hospitalized patients (3) particularly those who have received previous antibiotic therapy (14)**.**

Commercial test systems and other phenotype-based identification methods may therefore misidentify *P. aeruginosa* (10, 11, 15, 16, 17). Identification is oftenfurther hampered by the presence of other closely related nonfermenting gram-negative bacilli, including other *Pseudomonas*species (4,5) The potential for misidentification of this species from culture presents an obstacle to patient management, particularly with respect to antimicrobial therapy, patient prognosis, and infection control. Genotype-based identification methods circumvent the problem of variable phenotype to provide more accurate species identification.

However, the taxonomic complexity, uncertain phylogeny, and paucity of genomic sequence data of the dozens

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of species within the broad genus *Pseudomonas* present an obstacle to genotypic identification assays.

In the 1980s, a new standard for identifying bacteria began to be developed. In the laboratories and others, it was shown that phylogenetic relationships of bacteria, and, indeed, all life-forms, could be determined by comparing a stable part of the geneticcode (20, 21). Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S (also called the small subunit), and the 23S rRNA and the spaces between these genes. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene (2, 8, 9, 12, 13, 19). The 16S rRNA gene can be compared not only among all bacteria but also with the 16S rRNA gene of archeobacteria and the 18S rRNA gene of eukaryotes(18).

Material and Methods:

by using the MegAlign software package (DNASTAR Inc., Madison, Accumulate eighty-fourstrains were obtained from ministry of environment and the different hospitals (Al-alwia for children, Al-kadhemia, Ebenalbalady, Al-yarmuk) This included *P. aeruginosa*isolates and other *Pseudomonas* species (ex: *P. agarici, P.alcaligenes,P. chlororaphis,P. flavescens,P. fluorescens,P. fulva,P. fuscovaginae, P. mendocina,P. oleovorans,P. pseudoalcaligenes, P. pertucinogena*) all theseisolates cultured on different media (Blood agar base, MacConky agar, Nutrient agar, Nutrient broth, Brain heart infusion broth, Brain heart infusion agar) from differentsamples (soil, water, and patients samples) transport to microorganismlaboratory in (Genetic Engineering & Biotechnology Institute, Baghdad University)and then cultured on selective media (**cetramide agar**) for all *Pseudomonas* species for 24h in 37°C .

DNA extraction: was done using (Reagent Genomic DNA Kit ,Geneaid -Tiawan):Cell Harvesting : transfered the bacterial culture to a 1.5 ml microcentrifuge tubes ,centrifuged for 1 minute at 14-16000x g and then discarded the supernatant .DNA etxtract according to manfcture protocol.

Primer design:

Relevant 16S rDNA Primersequences available in paper based on GenBankdatabase were aligned Wis.). These included 136 sequences from 42 validly described by primers PA-SS-F(GGGGGATCTTCGGACCTCA) and PA-SS-R (TCCTTAGAGTGCCCACCCG)(18). This 16S rDNAPrimer of *Pseudomonas aeruginosa*was similared and obtained from the NCBI genomic data base (National Center of Biotechnology Information).

PCR.The PCR amplificationwas performed in a total volumes of 25µl containing 3µl DNA, 20µl Accu power premix (Bioneer,South Korea). 1µl from each primer (10 pmol/1µl) and up to 25µlwith nucleases free water.

After an initial denaturation for 2 min at 95°C, 25 cycles were completed, each consisting of 20 s at 94°C, 20 s at the appropriate annealing temperature (58°C) and 40 s at 72°C. A final extension of 1 min at 72°C wasapplied. With this program, the total time for amplification of target DNA wasapproximately 45 min. using a thermal Cycler (Gene Amp, PCR system 9700, Applied Biosystem).

Results:

Eighty four isolates were cultured on cetramide agar. Only60 Pseudomonas grow after 24h in 37°C while other 24 isolate not grow.From 60 isolate only 48 diagnosis as

*P.aeruginosa*based on biological and API 20 test (fig 3). DNA were extracted from28 Pseudomonas strains and7 nonpseudomonal species this include :2*Escherichia coli*, 1*Klebsiella pneumonia*,2*Neisseria gonorrhoeae* and 2 *Lactobacillus acidophilus*for comparison purpose (fig.1).

PCR reaction was preformed for diagnosisof*P. aeruginosa* using 16S rDNA. The results showed that one band 956bp (product size) as a result onAgarose gel compare with DNA marker (100 bp).

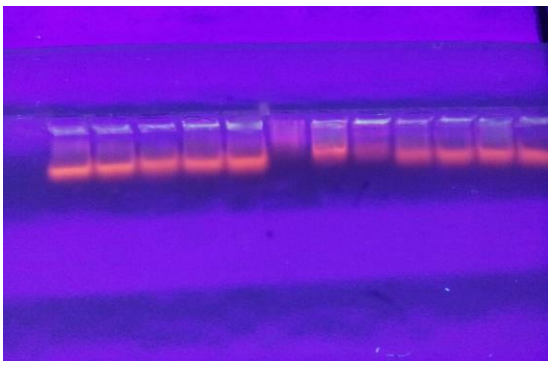

Fig (1): Extracted DNA electrophoresis on 1% agarose (70 vol/ 90 min) to check purity and integrity

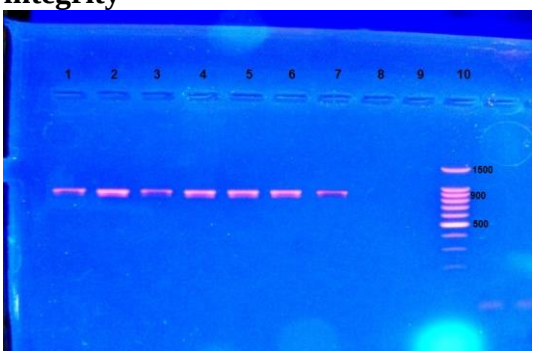

Fig. (2): PCR product electrophoresis on agarose gel 1.5%(1 h /70 vol)

Lane 10 : 100 bp DNA marker;Lane 8 and 9 : Negative control; Lane 1-7: PCR product of *P.aeruginosa* (956 bp)

Fig. (3) show API 20 assay of *P. aeruginosa*

Discussion:

The 16SrRNA gene is used for phylogenetic studies [(22)] as it is highly conserved between different species of bacteria and archaea.[(23)] Carl Woese pioneered this use of 16S rRNA[24.] In addition to these, mitochondrial and chloroplastic rRNA are also amplified.The most common primer pair was devised by Weisburg *et al.*[(22)] and are currently referred to 27F and 1492R, however, for some applications shorter amplicons may be necessary for example for 454 sequencing with Titanium chemistry (500-ish reads are ideal) the primer pair 27F-534R covering V1 to V3 (25).

In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification(26,27). As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid and cheap alternative to phenotypic methods of bacterial identification. (26) Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species, or even genera (29,30) It has also been used to describe new species that have never been successfully cultured (31,32).

16S rDNA sequence has long been used as a taxonomic "gold standard" in determining the phylogenies of bacterial species (35). Selective amplification of Pseudomonas 16S rDNA by PCR followed by restriction fragment length polymorphism analysis or denaturing gradient gel electrophoresis has been used to detect and differentiate Pseudomonas species from clinical and environmental samples (12, 27, 30, 34). Karpati and Jonasson (16) used a conserved 16S rDNA primer with a Pseudomonas genus-specific primer in a PCR assay to detect Pseudomonas DNA in CF sputum; this assay was not designed to differentiate P. aeruginosa from other Pseudomonas species .

In this study, we took advantage of a recent reassessment of the phylogenetic affiliation of the pseudomonads (1) to reexamine the rapidly expanding 16S rDNA sequence data available in public databases. Based on an alignment of 16S rDNA sequences. We identified Pseudomonas genus-specific and P. aeruginosa-specific signature sequences. PCR assays were used to test 84 isolates . We included in this test isolates representing 24 nonpseudomonal species that collected from different sources to check our primers specificity.

Clarke and colleagues (3) described a PCR assay that amplifies a fragment of the groE heat shock protein gene from several Pseudomonas species. Restriction fragment length polymorphism analysis of the amplified DNA was reported to differentiate P. aeruginosa from P. stutzeri, P. fluorescens, and P. putida. Qin and colleagues (28) used real-time PCR amplification of multiple targets, including exotoxin A, algD, oprL, and gyrB, together with biochemical tests and 16S rDNA sequencing to identify phenotypically atypical P. aeruginosa isolates recovered from CF specimens .

Our examination of recent (60) isolates further showed the utility 16s rDNA PCR assays. 48 were *pseudomonas aeruginosa* while other were only pseudomonas spp. This is explain the highly specifity of primers and accuracy of PCR. Api 20 test consists of a plastic strip of 20 individual, miniaturized tests tubes (cupules) each containing a different reagent used to determine the metabolic capabilities, and, ultimately, the genus and species of enteric bacteria in the family Enterobacteraceae. Each cupule is inoculated with a saline suspension of a pure bacterial culture, rehydrating the dried reagent in each tube. Some of the tubes are to be completely filled (tests CIT, VP and GEL), whereas others are topped off with mineral oil so that that anaerobic reactions (reactions that occur in the absence of oxygen) can be carried out (tests ADH, LDC, ODC, H2S, URE).So, the test depend on chemical reaction and may be this reaction done by gene transfer from another bacteria by horizontal gene transfer.

In summary, we have designed 16S rDNA-based PCR assays that provide rapid, simple, and reliable identification of P. aeruginosa and its differentiation from other phylogenetically closely related Pseudomonas species. Both assays have 100% sensitivity and specificity for their intended targets. We have also demonstrated the utility of these PCR assays in accurately identifying P. aeruginosa among isolates not correctly identified by phenotypic analyses. Comparing with API 20 , PCR assay was more accuracy and faster .

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تشخيص بكتريا *Pseudomonas aeruginosa* بـجين 16s rRNAلتفريقها عن بقية انواع جنسها المعزولة من المرضى والبيئة

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الخلاصة:

تعد بكتريا الـ*Pseudomonas aeruginosa* الاكثر شيوعا وخطورة من بين الممرضات الانتهازية التي تصيب الانسان ،اذ تسبب العديد من الامراض المعدية الخطرة التي تؤدي الى الموت في بعض الاحيان مثال ذلك التكيس الليفي ، التهاب الجروح، التهاب الحروق ، التهاب المجاري البولية ، والعديد من الاصابات الاخرى . لذلك اصبح من المهم جدا ايجاد طريقة سريعة ودقيقة لتشخيص بكتريا الـ *Pseudomonas aeruginosa* من العينات المرضية المزروعة على الاوساط الزرعية وذلك لان تشخيص هذا النوع يواجه مشكلة التغاير في النمط المظهري الملحوظ في العينات المعزولة التي تحتوي على العديد من الانواع المتقاربة جدا فيما بينها وابسط طريقة للتفريق بين هذه الانواع هي استخدام الـ 16SrRNA الذي يعطي تشخيصا للجنس والنوع اذ يعد الشفرة الوراثية الثابتة للنوع وبذلك . هدفت هذه الدراسه الى ايجاد تشخيص سريع وبسيط ودقيق بتقنية PCR يهيأ لنا تشخيص بكتريا الـ*Pseudomonas aeruginosa*وتفريقها عن باقي انواع جنسها واجناس مرضيه اخرى وكذلك يعتبر هذا الاختبار الأدق من بين الاختبارات التشخيصية الاخرى مثل الـ(20) API حيث يعطي نسبة 70% في حين يعطي التشخيص بالـ16SrDNA اكثر من 90%.