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Detection of *Pseudomonas aeruginosa* in Clinical Samples Using PCR Targeting *ETA* and *gyrB* Genes

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Abstract:

Pseudomonas aeruginosa has variety of virulence factors that contribute to its pathogenicity. Therefore, rapid detection with high accuracy and specificity is very important in the control of this pathogenic bacterium. To evaluate the accuracy and specificity of Polymerase Chain Reaction (PCR) assay, *ETA* and *gyrB* genes were targeted to detect pathogenic strains of *P. aeruginosa*. Seventy swab samples were taken from patients with infected wounds and burns in two hospitals in Erbil and Koya cities in Iraq. The isolates were traditionally identified using phenotypic methods, and DNA was extracted from the positive samples, to apply PCR using the species specific primers targeting *ETA*, the gene encoding for exotoxin A, and *gyrB* gene. The results of this study indicate that 100% of *P. aeruginosa* isolates harbored the *gyrB* gene, whereas 74% of these isolates harbored *ETA* gene. However, the specificity of PCR for detection of *P. aeruginosa* based on the both genes was 100%, since no amplified product obtained using DNA extracted from other bacterial species. Hence by considering the importance of rapid detection of this bacterium due to the presence of problems in biochemical methods, PCR targeting multiple virulence genes is suggested in identification of pathogenic strains of *P. aeruginosa* isolated from some infections which should speed diagnosis of an antimicrobial therapy.

Key words: *Pseudomonas aeruginosa*, identification, PCR, *ETA* gene, *gyrB* Gene.

Introduction:

Pseudomonas aeruginosa is an environmentally ubiquitous Gram-negative bacterium which is a leading nosocomial pathogen causing various infectious syndromes (1). Clinically, this microorganism plays a critical role in the survival rates of affected patient; hence it is important to detect it quickly and accurately. In general, detection of *P. aeruginosa* is done by standard traditional culture such as morphologic and biochemical tests (2). However these tests are lengthy and unreliable and because of the seriousness of the infection, there is a need for a rapid and sensitive technique for early detection of pathogenic *P.aeruginosa* DNA-based techniques, such as polymerase chain reaction (PCR) are shown to have those characters for the identification of pathogenic bacteria in terms of accuracy, specificity and reliability (3). Many of these PCR methods have been applied for identification of *P. aeruginosa* (4, 5).

A variety of virulence factors contribute to the pathogenicity of *P.aeruginosa* and are targeted by PCR for its detection such as *oprL* gene and *exoS* gene (6), *gyrB* gene (2,7), *ecfX* genes (8), Quorum sensing gene (9), gene encoded to phospholipase (*plcH*), rhamnolipid AB (*rhlAB*), alkaline protease (*aprA*) and elastase (*lasB*) (10) beside exotoxin A (*ETA* gene) (11, 12). *ETA* gene is species specific and conserved for *P.aeruginosa* species and is not present in other species of *Psuedomonas* genus. The gene encoding exotoxin A has been sequenced, characterized and is known to contribute to *P. aeruginosa* pathogenesis, since strains of this species of bacteria are deleted for this gene are less damaging than parental strains (13). The main problem of PCR detection methods of *P. aeruginosa* is that they target only one gene which is inadequate for comprehensive and reliable diagnosis (7, 14). Because *P. aeruginosa* strains demonstrate high genotypic diversity (15), and many studies have confirmed the absence of one or more of the virulence genes in some strains (16), this study aimed to detect pathogenic strains of *P. aeruginosa* by targeting *ETA* gene and another gene called *gyrB* that encodes the subunit B protein of DNA gyrase (7, 8).

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Materials and Methods:

Bacterial isolation:

Between June and October 2015, 70 swab samples were taken from infected wounds and burns from patients attending the hospitals in Erbil and Koya cities in the Kurdistan region of Iraq. Brain Heart Infusion Broth was used to enrich the bacteria, and this was cultured on MacConkey agar plates and incubated at 37°C overnight to observe colony morphology. The observed colonies were inoculated on the selective medium (cetrimide agar) and processed further for biochemical tests according to MacFaddin (17) which include: Growth at 42°C in trypticase soya agar, Indole production test, Voges-proskauer (VP), Methyl red test, urease activity, Oxidase test, Citrate utilization test, and catalase test.

DNA Extraction:

Genomic DNA was prepared according to Oliveira *et al.* (18) as follows: From a single colony 10 ml cultures were prepared in broth media for 12 hours, then centrifuged for 5 min at 6000 rpm to pellet the cells, The pellets were resuspend in TE buffer (PH 8) and 30 mg/ml lysozyme and incubated for 2 hours at 37°C. After that, TE buffer (pH 8) containing Proteinase K (1mg/ml) was added for 1 hour for denaturation of cell protein, and 10 µl of 20% Sodium Dodocyl Sulfate (SDS) was added and incubated for 1 h at 37°C. One equal volume of phenol /chloroform/isoamyl alcohol (24/24/1) was added and put in to a shaker for 30 minutes, and was centrifuged for 5 min at 6000 rpm. The supernatant was transferred into a clean micro tube and then ammonium acetate was added by 10% of the volume with one equal volume of cold isopropanol to precipitate the genomic DNA. The precipitated DNA was transferred into another micro tube and washed with 200 µl of (70%) ethanol. Finally, the washed DNA was dissolved in TE buffer and stored at -20°C until use.

Application of Polymerase Chain Reaction:

Two sets of primers were used in the application of PCR. The first one was forward primer 5' GACAAC GCCCTCAGCATCACCAGC3' and the second was reverse primer 5'CGCTGGCCCATTTCGCTCCAGCGCT 3', which targeted exotoxin A (ETA) gene, which amplified a 222 bp snippet. The second set of primers was a forward primer 5'AAGTACGAAGGCGGTCTGAA3' and reverse primer 5'GTTGTTGGTGAAGCAGAGCA3' which was targeting *gyrB* gene which amplifies a 367bp sequence, with both genes were specific for *P. aeruginosa*. The reagents required for PCR reaction include 25µl reaction mixture, with 2U of Taq

DNA polymerase, 2.5 µl of 10 x PCR buffer (10 mM of Tris-HCl (pH8.5), 30 mM of KCl, 1.5 mM of MgCl₂, and 0.4mM of each dNTPs with 50 ng template DNA beside 10pmol of each primer (0.5µM). The amplification program was run as follow: One cycle at 95°C for 2 min, 30 cycles of 92°C for 60 s, 59°C for ETA gene and 55°C for *gyrB* gene as annealing temperature and 72°C for 1 min, and finally One cycle at 72°C for 8 min. The amplified product was run on 1.2% agarose gel electrophoresis for 90 min at 75 Volts, stained with ethidium bromide and visualized by a U.V. transilluminator.

Results and Discussion:

Identification of *P. aeruginosa* using phenotypic methods.

Out of 70 samples collected from patients with infected wounds and burns, 24 (34%) of the isolates were successfully diagnosed as *P. aeruginosa*, using phenotypic methods. This reflects that *P. aeruginosa* is widely exists in hospital environments such as air and distribution systems (19). Moreover, *P. aeruginosa* has acquired many antibiotic resistance genes and therefore dominant compared to other kinds of bacteria. As this bacteria is introduced into areas when membranes and skin are disrupted by direct tissue damage, this makes *P. aeruginosa* prevalent in wound and burn trauma. The results of phenotypic tests include formation of large, pale, translucent and mucoid colonies on MacConkey agar plates, and formation of a greenish blue color on nutrient agar (due to production of pyocyanin and fluorescein pigments). The identification of the isolates was confirmed by API 20E system and biochemical assays results (20, 21). Despite the successful use of traditional methods for identification of *P. aeruginosa*, it is often lengthy and still needs validation. Because *Pseudomonas* species are phenotypically very unstable and therefore, detection of this bacteria at the molecular level is very important especially for its pathogenic strains.

PCR Analysis

Recently, it has become difficult to cure infections caused by *P. aeruginosa* due to its acquisition many new antibiotic resistance genes that allow it to survive and distribute easily and convert to chronic colonization (22). Therefore early diagnosis and control of this pathogen have become vital for positive patient outcomes. Many researchers attempted to develop DNA –based techniques especially PCR for detection of *P. aeruginosa* due to its ease and accuracy. However, a definitive methodology still lacking. Two pairs of primers specific to *P. aeruginosa* genes were used

in this study. The benefit of using more than one targeting gene for identification of one organism is to provide more confirmation and confidence in the identity of the organism, and to reduce the potential of false negative results caused by sequence variation in the primers (23). The first pair was targeting exotoxin A (*ETA*) gene as many studies targeted the same gene for identification of *P. aeruginosa* (24, 25). Amplified products of the predicted size of 222bp were obtained using the DNA extracted from 74% of *P. aeruginosa* isolates (Fig1). These results were in accordance with that recorded by Naiem (26) who detect this gene in 66% of the strains that were collected from human clinical infection samples from Al-Diwanyia (Iraq) hospital. This ratio increased to 73% in other studies conducted on samples collected in Baghdad (27). The ratio increased to 75% in a study conducted on samples collected from Kirkuk Hospital (28). Exotoxin A is produced by the majority of pathogenic *P. aeruginosa* isolates and can inhibit protein biosynthesis of the host at the level of stopping polypeptide chain elongation factor 2 (29). The absence of *ETA* gene from some strains is due to the mutation of that gene. By applying PCR targeting of another virulence gene (*gyrB* gene), the results showed amplified fragment with 367bp in 100% of the isolates (Fig 2), the same results obtained with other studies (25, 30), indicating the presence of this gene in the conserved region of the genome of this bacteria. The difference in percentage of virulence genes is due to several reasons including nature of place, and type of prevalence strains (31). Since the specificity of PCR primers is very important criterion needed for detection of any bacteria, it was investigated for detection of *P. aeruginosa* based on the two genes used in this study, It was 100% accuracy, this result is agreed with that reported by other studies (32). Since no amplified product obtained using the DNA that extracted from other various bacterial species including *Salmonella typhimurium*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Escherichia coli*. The present study is first of its kind in Erbil/Iraq to detect the presence and distribution of two virulence genes *toxA* and *gyrB* gene across the genome of *P. aeruginosa* isolates by PCR. Moreover, this technique was a confident assay and specific for identification of this bacterium in short time with low cost.

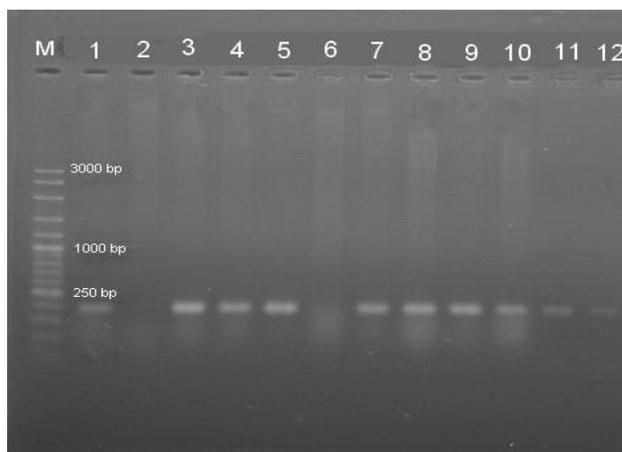


Figure 1. PCR amplification of *ETA* gene of *P. aeruginosa*, M: DNA ladder, lanes 1, 2, 3,...12 represent amplified product (222bp) of *P. aeruginosa* isolates.

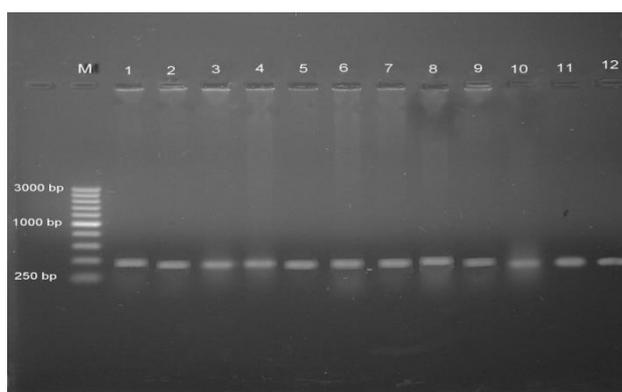


Figure 2. PCR amplification of *gyrB* gene of *P. aeruginosa*, M: DNA ladder, lanes 1, 2, 3,...12 represent amplified product (367bp) of *P. aeruginosa* isolates.

Conclusion:

The results indicate that 100% of *P. aeruginosa* isolates from burn and wound infections harbored *gyrB* gene, whereas 74% of these isolate had *ETA* gene. Considering the importance of rapid and early detection of pathogenic strains of any bacteria due to the time and resources required for biochemical methods, PCR targeting multiple virulence genes (*ETA* and *gyrB*) is suggested for the identification of pathogenic strains of *P. aeruginosa*. This assay can be used for screening of some infections for effective antimicrobial therapy.

Conflicts of Interest: None.

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الكشف عن السلالات المرضية لبكتريا *Pseudomonas aeruginosa* باستهداف جيني الضراوة *ETA* و *gyrB*

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الخلاصة :

تمتلك بكتريا *Pseudomonas aeruginosa* مجموعة متنوعة من عوامل الضراوة التي قد تساهم في قدرتها المرضية، لذلك فإن الكشف السريع بدقة عالية يلعب دورا حاسما في السيطرة على هذه البكتيريا المسببة للأمراض. ان الهدف من هذه الدراسة هو تقييم دقة تقانة تفاعل البلمرة المتسلسل (PCR) ، باستهداف جيني *ETA* و *gyrB* ، وذلك للكشف عن السلالات المرضية لبكتريا *P. aeruginosa*. وولتحقيق ذلك ، تم أخذ 70 مسحة من المرضى المصابين بالجروح الملتهبة، والحروق من مستشفيات في مدينتي أربيل وكويا في العراق . شخّصت العزلات باستخدام الطريقة التقليدية المعتمدة على الصفات المظهرية والصفات الكيموحيية، بعدها استخدمت الطرق الجزيئية في التشخيص بعد استخلاص الحمض النووي من العينات الموجبة للصفات السابقة، وذلك لتطبيق تفاعل البلمرة المتسلسل (PCR) باستخدام البادئات النوعية المحددة للنوع والتي تستهدف الجين *ETA* والجين الـ *gyrB* . اظهرت نتائج هذه الدراسة إلى وجود حزمة تمثل القطعة المتضاعفة من الجين *gyrB* في جميع العينات المدروسة في حين تم الكشف عن وجود الجين *ETA* في 74% من العينات عند تطبيق تفاعلات PCR. إلا أن تخصص الـ PCR وباستهداف كلا الجينين لتشخيص بكتريا *P. aeruginosa* كانت 100% وذلك بسبب عدم الحصول على اي قطعة متضاعفة باستخدام DNA المستخرج من أنواع بكتيرية أخرى ، لذلك و لاهميه الكشف السريع عن هذه البكتيريا بسبب وجود مشاكل في الطرق التقليدية البطيئة تعد تقنية PCR التي تكشف عن اكثر من جين ضروريه جدا في تشخيص السلالات المرضية من *P. aeruginosa* والتي تساهم بدورها في تحديد المضاد البكتيري الفعال لتلك البكتريا.

الكلمات المفتاحية : كشف، شخيص، *Pseudomonas aeruginosa* ، *gyrB* ، *ETA* ، PCR