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PCR-based approach to detect the Prevalence of *exo S.* Gene in Local Isolates of *P. aeruginosa* isolated from Otitis

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Abstract:This study was designed to detect *exo S.* gene in local isolate of *Pseudomonas aeruginsoa* using PCR technique. It includes 49 isolates of bacteria diagnosed by classical methods, DNA isolated and subjected to amplify using specific primers for bacteria.

The results of PCR amplification for the gene encoding for exotoxin S indicated that out of the forty nine local isolates of *P. aeruginosa* enrolled in this study, thirty nine (39) isolates showed the presence of the genes encoding for exotoxin S corresponding to 79.5% of the isolates (629 bp PCR products) while only ten (10) isolates corresponding to 20.5% of the total forty nine (49) isolates were recorded to be negative. With a result of 72% of the bacterial isolates demonstrating positive reaction for the presence of *exo S.*, it can be concluded that *exo S.* is a prominent virulence factor for *P. aeruginosa* that is expressed along the infection process.

Key words: Keratitis, Otitis, Exotoxin S., polymerase chain reaction (PCR), electrophoresis.

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استخدام تقنية التفاعل البلمري للتحري عن الجين *exo S.* المشفر للذيفان الخارجي في عزلات محليه من بكتريا الزائفة الزنجارية المسببة لالتهاب الاذن

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الخلاصة: صممت هذه الدراسه للكشف عن جين *exo S* المسئول عن انتاج الذيفان الخارجي *S.* في عزلات من بكتريا الزائفه الزنجاريه و تضمنت الدراسه تسعة واربعين عزله من بكتريا الزائفه الزنجاريه المسببه لالتهابات الاذن والتي تم تشخيصها بالطرق البكتريولوجيه القياسيه كما تم استخلاص الدنا البكتيري وتعريضه لتفاعل السلسله المتكرر من اجل الكشف عن وجود الجين *exo S.* اظهرت نتائج الدراسه ان تسعة وثلاثين عينه من اصل التسعة واربعين التي تضمنتها الدراسه قد اظهرت نتيجه موجبة للفحص بتفاعل السلسلة المتكرر للكشف عن وجود الجين المسئول عن التشفير للذيفان الخارجي اس وبنسبة 79.5٪ وقد كان حجم ناتج حزمة الدنا المضاعف 629 زوج قاعدة في حين ان عشرة عزلات فقط من اصل التسعة والاربعين التي تضمنتها الدراسة الحالية اظهرت نتيجة تفاعل سلبي لوجود الجين المشفر للذيفان الخارجي *S.* وبنسبة 20.5 ٪ من كل العينات.

Introduction

Pseudomonas aeruginosa is a ubiquitous, environmentally beneficial bacterium that can adapt to become a highly virulent opportunistic pathogen in compromised individuals. The versatility and pathogenicity of *P. aeruginosa* are multi-factorial and relate to its ability to respond to its environment by the regulated production of a variety of cell-associated and extracellular products. The establishment of infection begins with the adherence of *P. aeruginosa* to host cells through type IV pili or nonpilus adherence mechanisms (1). After colonization, the organism ensures its survival in the host through the secretion of virulence factors, including exotoxin A (2), hemolysins (3), elastases LasA and LasB (4), and pigments (5). In addition to secreted virulence factors, *P. aeruginosa* is able to directly affect eukaryotic cell function through the contact-dependent translocation of effector proteins by the type III secretion system (6).

Four type III cytotoxins contribute to *P. aeruginosa* cytotoxicity, Exo S, Exo T, Exo U, and Exo Y (7). Exo S. is a bifunctional cytotoxin that has a Rho GTPase-activating protein (RhoGAP) activity (residues 96 to 219) and a 14-3-3-dependent ADP-ribosyltransferase activity (residues 234 to 453) (8). Iglewski and coworkers identified exoenzyme S as an ADP-ribosyltransferase that ADP-ribosylated Ras and several related GTPases. ExoS RhoGAP activity was identified for Rho, Rac, and Cdc42 (9, 10).

The type III toxins were initially identified as virulence factors that facilitated the dissemination of *P. aeruginosa* from burn wounds (11). The specific roles of the individual type III toxins in the pathogenesis of pneumonia have been studied using bacterial mutants constructed in different genetic backgrounds and tested in animal models of infection, with some using intact hosts (12) and others using neutropenic animals (13). The TTS system function as a molecular syringe to deliver toxin directly into the cytosol of cells and host immune response (14). While each of the type III toxins has some role in virulence, biological effects vary depending upon the route of bacterial delivery and the nature of the host. The aim of the study was to detect the prevalence of *exo S.* gene in number of local isolates of *P. aeruginosa*.

Materials and Methods

1. Sampling

Ear swabs were taken from forty nine patients complaining of symptoms of otitis. Patient's age ranged from 6 months to 85 years, and there were 25 males and 24 females. Samples were collected from Al-Kadhumia Teaching Hospital / Baghdad during the period from January to April 2010. All obtained isolates were identified using biochemical tests according to Forbes *et al.* (14).

2. DNA Extraction

The genomic DNA was extracted from bacterial cells using Wizard genomic DNA purification kits (Promega®, USA) and according to the manufacturer's instructions. Agarose gel (1.5%) electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (15).

3. Detection of gene

To determine whether that the isolates of *P. aeruginosa* that caused otitis were exotoxin S producers; primers were selected to detect the presence of the gene that encodes for this toxin (Table 1). These Primers were purchased from Bioneer ® (South Korea) with melting temperatures and PCR product size of 45 °C; 629 bp.

The preparatory step for PCR included the addition of 5 µl of the template bacterial DNA onto preloaded master mix eppendorff tubes followed by the addition of 2.5 µl (10 picomol\µl) of the specific primers, the final volume was completed to 20 µl by the addition of distilled water; finally the PCR program for the amplification of *extS* gene was run using the conditions mentioned in table 2.

Note: Cycling conditions were adopted as trial and error approach relying upon previous study (16)

Table1: Oligonucleotide primers sequence and molecular weight of PCR products of *tox S.*

Gene	Sequence of forward Primer	Sequence of reverse primer	Product
toxS	5´- CGTTTGGGA CAGATTGAG-3´	5´- GATACTCTG CTGACCTCGC-3´	629bp

Table 2: PCR protocol of *exo S.* gene

Step	Temperature (°C)	Time (minutes)	No. of Cycles
Initial denaturation	94	3	1
First loop:			
Denaturation	94	30 sec.	
Annealing	45	45 sec.	
Extension	72	1	35
Final extension	72	5	

PCR product were detected by agarose gel electrophoresis 1% and photographed under UV light

Results and Discussion

The results revealed that all the forty nine bacterial isolates were identified as *P. aeruginosa* because they appeared as Gram negative rods, capable of growth at 42 ^{o}C, oxidase positive, sweet musty odor was produced, and were confirmed to be oxidative when applying oxidation /fermentation test. These results confirmed that all the forty nine isolates enrolled in the study were viable and produced visible growth when activated and sub-cultured; meanwhile the presence and integrity of their chromosomal DNA were also confirmed using agarose gel electrophoresis (figure 1) .

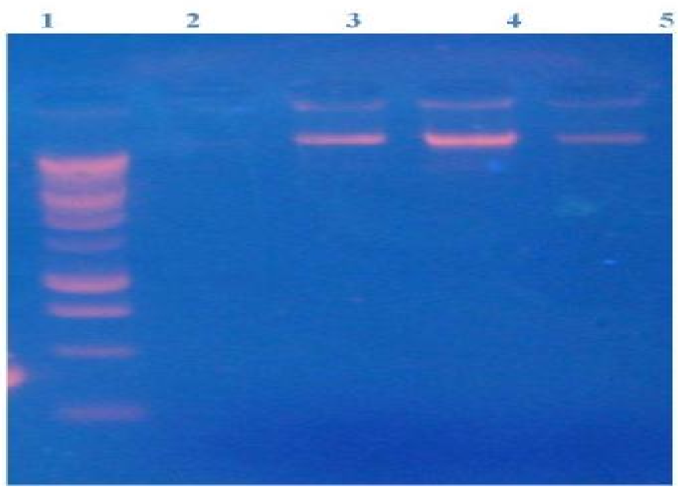

Figure1:Chromosomal DNA bands on 1% agarose gel at 4V/cm^2 for one hour. Lane 1: 100 bp molecular marker, lane 2 : Negative control lane 3, 4, and 5: genomic DNA of bacterial isolates (*P. aeruginosa*)

The results of PCR amplification for the gene encoding for exotoxin S indicated that out of the forty nine local isolates of P. aeruginosa enrolled in this study, thirty nine (39) isolates showed the presence of the genes encoding for exotoxin S corresponding to 79.5% of the isolates (629 bp PCR products) while only ten (10) isolates corresponding to 20.5 % of the total forty nine isolates were recorded to be negative. The presence of the bands reflecting successful PCR amplifications and the absence of these bands indicated positive and negative results, respectively (figure 2).

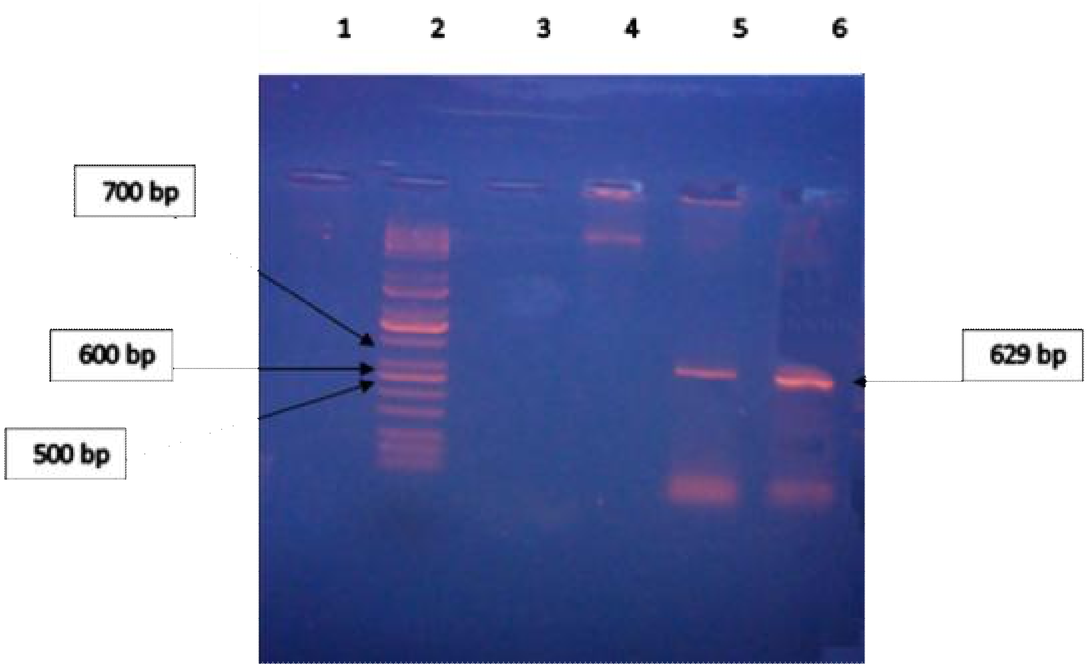

Figure2: Electrophoresis profile of PCR products of exotoxin S found in *P. aeruginosa.* from left to right: lane 2 represents the molecular size marker (1000 bp), lanes5, 6 represent the bands of PCR products belonged to two bacterial isolates. Bands run on 1.5% agarose gel

Consistent with the contact-mediated nature of TTS induction, evidence supports the involvement of TTS in the establishment of the infectious process of many gram-negative bacteria, including *P. aeruginosa* (17,18). Relative to bacterial exotoxins, steps in the internalization and trafficking of the type III cytotoxins are less clear. Current models propose that the TTS system is found in both clinical and environmental *P. aeruginosa* isolates, suggesting an essential role of TTS in *P. aeruginosa* (19). ExoS affects the cell growth, morphology, and adherence of epithelial and fibroblastic cell lines and also exerts antiphagocytic effects on macrophages (20). Although the genes regulating TTS and encoding the needle-structure are conserved among Gram-negative bacteria,

Exo T and Exo S. are both ADP-ribosylating enzymes but whilst ExoT appears to be virtually ubiquitous amongst *P. aeruginosa*, several authors have noted the mutual exclusivity between ExoS and the cytolytic factor Exo U (21,22). General surveys of the proportion of strains carrying each of these TTS effector genes suggest that exo S. is more common. In a result of separate study that mimic the result of the current one, the prevalence of 72% and 28% was reported for exoS and exoU, respectively, amongst a panel of 115 clinical and environmental isolates that did not include any from eye infections (24). Berthelot *et al.*, (23) separated 92 bacteraemia isolates into four groups on the basis of their cytotoxicity against macrophages and analysed secretion of the ExoU and ExoS proteins. Forty-eight (52.2%) of the strains exhibited slower rates of cytotoxicity (type II) and secreted ExoS. Overall gene prevalence levels for *P. aeruginosa* isolates for exoU and exoS were 29 (31.5%) and 65 (70.7 %), respectively, including two isolates that possessed both genes.

In a study of 13 isolates associated with corneal infections in the USA, the authors reported invasive and cytotoxic phenotypes in equal proportions and suggested that since invasive and cytotoxic strains have different effects on corneal cells, they may require different treatment strategies (25).

Conclusion

It can be concluded that Exo S is a prominent virulence factor for *P. aeruginosa* that is expressed along the infection process.

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