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Molecular Detection of Type III Secretion System (T3SS) Genes in *Pseudomonas Aeruginosa* Isolated form Babylon Province

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Abstract

Pseudomonas aeruginosa is an opportunistic bacterium that synthesizes multiple virulence factors. This research assessed the comparative prevalence of exoenzymes (exo) A, U, and S genes (II and III).

In the present study, a total of 120 specimens were obtained from different hospitals in Babylon, Iraq and collected from different sources including burn, wound, urine, ear and sputum. The results showed that 42 (35 %) of isolates were *P. aeruginosa* isolates, distributed to 33 burn (78.5%), ear and urine 3 (7 %) , 2 wound (4.7%) and one sputum (2.3%). Class II and III virulence genes were identified using a PCR test on extracted DNA. The Type III Secretion System (T3SS) is crucial for the direct translocation of effector proteins into target cells of the host via a needle-like apparatus.

The PCR findings indicated that 41 (97.6%) *P. aeruginosa* isolates were positive for the ToxA gene, 40 (95%) for the exoS gene, and 39 (92.5%) for the exoU gene. According to the categorization of virulence genes, the count of invasiveness gene-isolates was 40 for the exoS gene (95%), which is somewhat higher than the cytotoxic gene-isolates at 39 for the exoU gene (92%). The findings indicated a statistically significant correlation ($p<0.05$) between virulence genes and T3SS genotypes; however, the prevalence of the ToxA gene was much greater among the cytotoxic genotype isolates. The statistical analysis of the current investigation indicated a strong correlation between the presence of the exotoxin genes exoS, exoU, and ToxA with resistance to various antibiotic classes ($P\leq 0.05$). This study sought to find the pathogenicity genes. The quantity of isolates possessing the invasive gene (exoS) was marginally greater than that of those containing the cytotoxicity gene (exoU).

Keywords: *Type III Secretion System (T3SS); burn wound; exotoxin; Babylon; Iraq; Pseudomonas aeruginosa*

Introduction

The Gram-negative bacteria *Pseudomonas aeruginosa* (*P. aeruginosa*) can survive and adapt to a variety of settings, including soil, water, municipal wastes, and hospitals. Both aerobic and anaerobic environments are conducive to the bacterium's growth. *P. aeruginosa* is an opportunistic pathogen that may infect humans, animals, and plants. Through surgery, burns, wounds, urinary tract infections, keratitis, and otitis externa, it causes many infections in patients with AIDS, cancer, or immunocompromised individuals (1). *P. aeruginosa* may acquire resistance genes on the plasmid or undergo mutational processes that change the expression and/or function of chromosomally encoded pathways in order to achieve antibiotic resistance. The therapeutic choices for treating serious infections may be significantly limited by both approaches to medication resistance (2). Because it makes it more difficult to choose appropriate treatments, the rising incidence of nosocomial infections caused by *P. aeruginosa* strains that are

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extensively drug resistant (XDR) and multidrug resistant (MDR) is linked to markedly higher morbidity and death (3).

Exotoxin A is a critical virulence agent of *P. aeruginosa*, exhibiting significant toxicity to mammalian cells and obstructing protein synthesis. This toxin has necrotizing action at the colonization site and is also implicated in invasion and immunosuppression (4). PA releases many toxins into the extracellular milieu, with a specific subset being directly injected into host cells. This transpires via a macromolecular syringe known as a type III secretion system (TTSS). This mechanism is crucial in the pathophysiology of several animal infection models (5). *P. aeruginosa* synthesizes many toxins, including four type III toxins: Exoenzyme S, ExoU, ExoT, and ExoY. In vitro, the expression of these toxins has been detected in *P. aeruginosa* isolates from diverse infection contexts, especially in acute infections. Exo S is an effector protein associated with the type III secretion system that operates as an ADP-ribosylating enzyme. Exo S levels were markedly elevated in vitro in *P. aeruginosa* isolates from wounds and urinary tract infections compared to tracheal isolates (6).

The ExoS-deficient mutants of *P. aeruginosa* were shown to stay restricted and induce mainly localized infections in burn victims. Elevated levels of ExoS have been documented in *P. aeruginosa* isolates obtained from wound and urinary tract infections, in contrast to tracheal infections, which exhibit greater persistence (7). ExoU has been linked to the pathogenicity of *P. aeruginosa* at the ocular surface. ExoU is a phospholipase that induces mammalian cell death, and its presence is prevalent in strains isolated from ocular illnesses. A link exists between the presence of exoU and increased resistance to fluoroquinolones and aminoglycosides. ExoU is harbored by bacteria on a genomic island that furthermore includes resistance genes for several drugs (8). xoU has phospholipase/lysophospholipase activity and compromises eukaryotic cell membranes after its transfer into the cell via type III secretion systems (9). The objective of this research is to investigate the distribution of virulence genes and T3SS genotypes in *P. aeruginosa* isolates obtained from patients. Classification of *P. aeruginosa* isolates in Iraq based on T3SS genotyping into invasive isolates (containing the ExoS gene) or cytotoxic strains (containing the ExoU gene).

Materials and Methods

P. aeruginosa Separation

Sample Gathering

Between April and December of 2022, a total of 120 specimens were collected from different hospitals in the province of Babylon. Forty-two *P. aeruginosa* isolates were obtained from patients' burns, wounds, urine, ears, and sputum, among other sources. In order to maintain the sample for further analysis using standard microbiological and biochemical characteristics, specimens were tagged and delivered by transport medium to the laboratory of the Biology Department, College of Science, the University of Babylon.

Culturing and Identification of Bacteria

The samples we obtained were from a variety of medical specimens, including burns, sputum, urine and wounds. Multiple mediums, including Blood agar, MacConkey agar, and the specific medium of PA Cetrimide agar, were used to cultivate each isolate. All cultivated media were incubated for 18 to 24 hours at 37 oC. In order to exhibit the distinctive characteristics exhibited by these bacteria, such as their greenish blue hue and fruity odor, the putative isolates of bacterium that may be PA were cultured on Cetrimide agar. Based on Forbes et al., the additional diagnostic examinations included the identification of microscopic as well as phenotypic features in conjunction alongside biochemical diagnostic procedures [10].

Extraction of genomic DNA

Following a 24-hour incubation period, *Pseudomonas aeruginosa* isolates were submitted to genomic DNA extraction (Favorgen DNA Extraction KIT, Korea). Nano-drop (Analytik Jena, Germany) was used to measure the DNA content and purity for the specimens. Bacteria DNA was isolated from *P. aeruginosa* specimens that were cultivated in culture conditions in accordance with the supplier's methodology (Bacterial). The primer solutions were made in accordance with the manufacturer's instructions (Integrated DNA Technologies firm) via diluting them in a deionized distilled water (ddH2O) to produce a solution of stock with an end concentration of 100 pmol/l. Prior to use, the primer standard solutions were stored at -20 °C. To make a 10 pmol/µl concentration of the work primer dissolved in water, 10 µl of stock solution was added to 90 µl of ddH2O, mixed using a vortex device, and stored at -20 °C until needed. ToxA reverse AAGCCTTCGACCTCTGGAAC, whereas the toxA primer pairs forward 5'-ATGGTGTAGATCGGCGACAT -3'. The exoU gene primer sequences used in this investigation are exoU forward 5': CCAACACATTAGCAG CGAGA -3' and exoU reverse 5': TGGGAGTACATTGAGCAGCA.ExoS gene F primer sequence: CATCCTCAGGCGTACATCCT, R primer sequence: ATCGATGTCAGCGGGATATC [11].

PCR assay

All specimens were examined for the presence of the exoS gene in order to identify the isolates of bacteria known as *P. aeruginosa*.

PCR-based genotyping method

The previously published approach was used to separate genomic DNA from bacterial cells that were taken during overnight culture (Smet et al., 2009). (GeNet Bio, Korea) supplied the extraction kit. Amplification by PCR for each of the toxA, exoU, and exoS genes was carried out in accordance with the standard manufacturer's protocol for the target gene amplification, which included 3 minutes of initial denaturation at 95°C; one cycle of denaturation at 95°C for 30 seconds; 30 seconds of annealing at 53°C; 50 seconds of amplification at 72°C; and five minutes of final amplification at 72°C. Following electrophoresis, 5µl of the product of PCR was run on a 1% agarose gel in trice boric acid EDTA buffer and visualized under an UV light source to examine the results.

Results

From 120 clinical samples collected from both sex with different ages were isolated from different hospitals in Babylon, Iraq, 42 (35 %) were positive for *P. aeruginosa* (Figure 1)

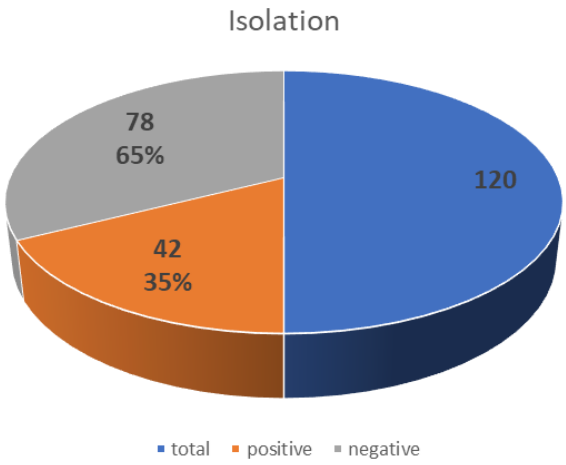

Figure 1: Distribution of *P. aeruginosa* in clinical specimens

33 (78.5%) were isolated from burn, three (7%) were from ear and urine, whereas wound was tow (4.7%) and one (2.3%) for sputum [Figure 2].

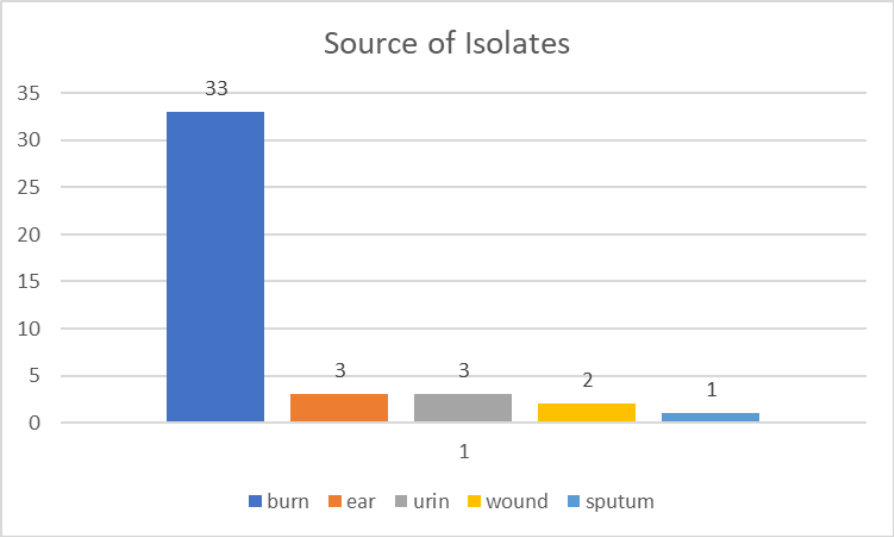

Figure 2: *P. aeruginosa* isolates were mostly gathered from clinical environments.

All isolates were cultured on cetrimide agar. On cetrimide agar, the bacterial colonies of *P. aeruginosa* appeared as greenish-blue because the majority of these colonies produce pyocyanin, a greenish-blue dye (Figure 3).

Figure 3: *P. aeruginosa* colonies on cetrimide agar

Extraction of DNA

Isolation of the genomic DNA of *P. aeruginosa* was conducted using the Favor Prep™ Genomic DNA Mini Kit for DNA extraction. The DNA had an approximate concentration of 65-200 ng/µl with high purity sample of DNA range was between (1.7–1.8) by using Nano drop devise.

Distribution of Type III Secretion System (T3SS) Genes

The exos gene was detected in 40 (95%) isolates of *P. aeruginosa* with an amplification product of 240 kb [Figure 4]. The gene encoding exoU was present in 39 (92.5%) of the clinical isolates of *P. aeruginosa*, with an

amplification product of 94 bp, as shown in [Figure 5]. ToxA gene was detected in 41 (97.6%) *P. aeruginosa* isolates and one (2.38 %) negative strain showed amplification product of about 397 bp [Figures 6].

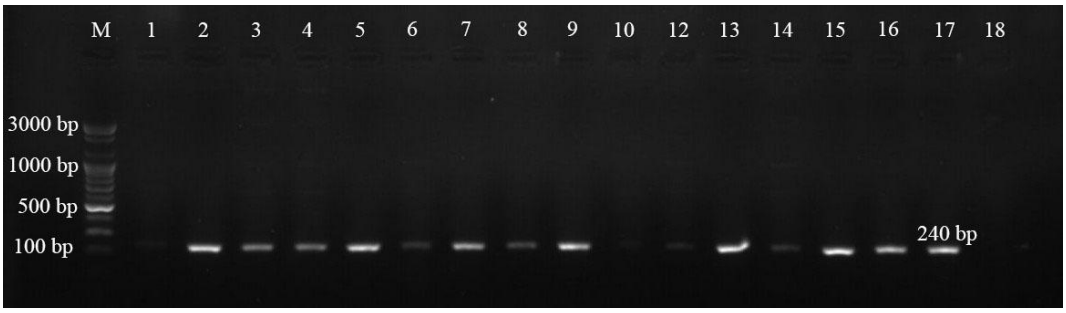

Figure 4: PCR amplification of the 240 bp exoS gene in specimens of *P. aeruginosa* on 1.5% agarose at 70 volts for two hours. Lane M: DNA marker of 100 bp. Positive isolates in lanes 1–18.

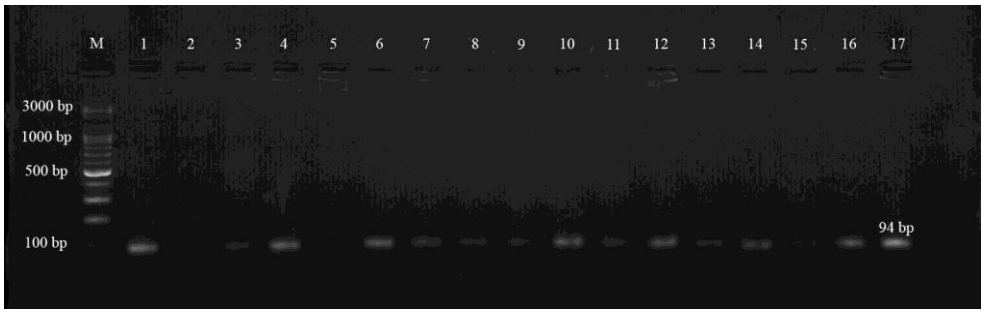

Figure 5: PCR amplification of exoU gene (94bp) in *P. aeruginosa* isolates. 2% agarose gel at 70 volt for 2 hrs. Lane M: 100 bp DNA marker. Lane (1-17) Positive isolates. Lane (2) isolates Negative.

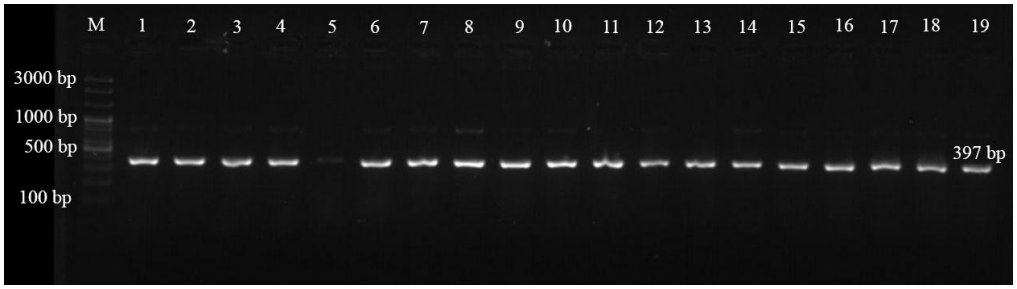

Figure 6: PCR amplification of the ToxA gene (397 bp) in *P. aeruginosa* isolates was conducted on 1.5% agarose at 70 volts for 2 hours. Lane M: 100-base pair DNA marker. Lane 1-19: Positive isolates.

The results of present study reported that most of *P. aeruginosa* isolates produce the T3SS exotoxins; ToxA 41(97.60%), exoS 40 (95%), and exoU 39 (92.50%) (Figure 7).

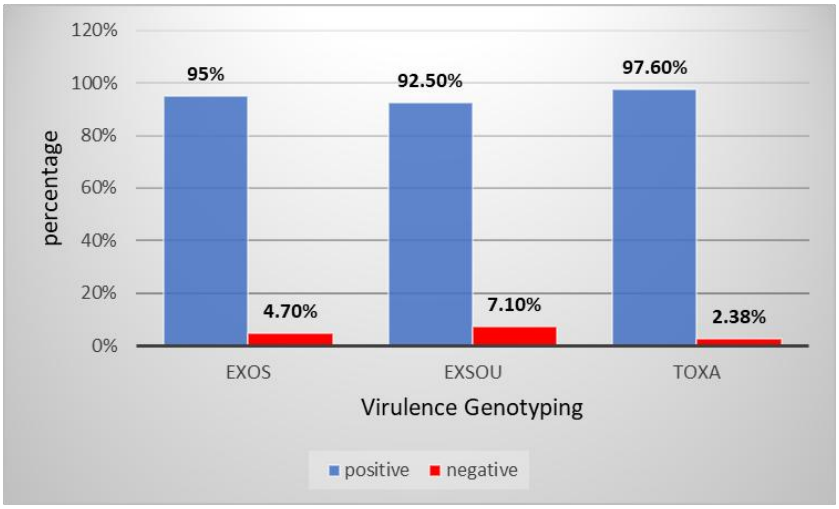

Figure 7: Distribution of T3SS exotoxins of *P. aeruginosa* isolates

Discussion

This investigation was a hospital-based cross-sectional research designed to detect and isolate *P. aeruginosa* from various clinical specimens and to ascertain the presence of type III Secretion System (T3SS) genes (exoA ,exoU, and exoS) in isolated clinical isolates of *P. aeruginosa*. This research involved 120 patients from various units across many hospitals in Babylon.

This study gathered 42 isolates of *P. aeruginosa* from 120 patients with wounds, burns, ear infections, urinary issues, and sputum samples. A significant proportion of *P. aeruginosa* isolates was incinerated. The research results indicate that 41 (97.60%) of the isolates had the ToxA virulence gene, 40 (95%) possessed exoS, and 39 (92.50%) possessed exoU.

The virulence gene ToxA was more prevalent than exoU as well as exoS in the isolates, corroborating a prior report which indicated that the exo genes in the *P. aeruginosa* isolates in this research. The results showed the exoU gene was present in 24.70% (n = 83/336) of the isolates, while the exoS gene was present in 69.94% (n = 235/336) of the isolates. Conversely, another investigation (13) indicated that the toxA gene was present in 83.3%, a finding corroborated by El-Gohary. A research conducted by (14) reported that 83 (19.6%) *P. aeruginosa* isolates were obtained from diverse clinical sources. Four (6.3%) exoU+ strains and one (1.6%) exoU+exoS+ multidrug-resistant strain were identified, including a total of 11 serotypes. The prevalence of toxA was 93.8%.

The type three secretion system (T3SS), or injectisome, is a prominent virulence component of these bacteria and a significant contribution to acute infections. This investigation evaluated 22 clinical isolates of *P. aeruginosa* for the secretion of protein effectors via the T3SS (15). Recent researches have shown that among the identified genes, exoA (81.5%) was the most abundant, followed by T3SS, exoS (63.9%), and exoU (46.3%), which were less frequent (16). The current investigation revealed a higher prevalence of ExoS toxin compared to ExoU toxin. This aligns with the results of other investigations (17,18). This conclusion aligns with the results of the present investigation and contradicts another study that reported exoU genotypes were more prevalent than exoS genotypes in clinical *P. aeruginosa* isolates (19). Numerous studies indicate that ExoU is more deleterious than ExoS toxin, with ExoU associated with cytotoxicity, while ExoS is recognized for its role in modulating bacterial invasion. Furthermore, 100% of the isolates had exoT, 88.46% included exoY, and 57.69% and 38.46% harbored exoU and exoS, respectively.

The ToxA gene was found in 120 (97.6%) of the isolates studied. However, the presence rate is higher than in a recent investigation carried out in Erbil, Iraq (90.56%) (21), and convenient with another study conducted in Egypt virulence genes toxA (86%), exoS (82%) and exoU (19%) of the 100 isolates (22). Also consistent with another study of (26) of the isolates tested positive for *P. aeruginosa* from AlTurkey Hospital and private clinics in Thi-Qar province that showed presence of 84.62% of ToxA gene (23). In addition, higher prevalences of toxA gene were reported by

(24) and 95.7% by (25). ToxA was present in 77% of wound isolates as well as 51% of burn isolates, but the exoS gene was found in 69% of wound isolates and 49% of burn isolates. According to (26), toxA exhibited a prevalence of 88.3%, whereas (27) reported a prevalence of 87.33%.

This mechanism delivers exotoxins or proteins into the cytoplasm of the eukaryotic cell via a translocation needle-like machinery and porin-forming proteins. The toxins include exoenzyme S (exoS), which is encoded by the ExoS gene; exoS facilitates immune evasion by altering the cytoskeletal architecture. ExoU, released by the type III secretion system, is a cytotoxin that induces fast host cell death within 1 to 2 hours. This death is marked by the loss of cell membrane integrity, indicative of typical necrosis. ExoU exhibits phospholipase A2 activity and impedes phagocyte absorption. ExoU is believed to possess cytotoxicity that is 100 times greater than that of ExoS (28).

Exotoxin A (ExoA) is a toxin released from the cell via the type II secretion system. ExoA is crucial to the pathogenicity of *P. aeruginosa*. Like diphtheria toxin, ExoA has been shown to inhibit the host response during infection. Furthermore, it has been asserted that ExoA substantially influences local tissue injury and invasion [29].

Conclusion

P. aeruginosa is a formidable bacterium characterized by significant virulence characteristics and substantial antibiotic resistance, which may lead to refractory infections in burn victims within the healthcare community. The PCR test for T3SS genes indicated that the predominant genes were ToxA, ExoS, and ExoU.

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