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Research Article

Molecular Detection and Study of some Virulence Factor Genes Among Pseudomonas aeruginosa Isolated from Different Human Clinical Specimens

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A B S T R A C T

Introduction: Pseudomonas aeruginosa is an opportunistic pathogen causing various healthcare-associated acute and chronic infections. The multifactorial virulence of the ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* has been linked to various virulence factors.

Objective: Isolate and identify *Pseudomonas auroginosa* in different types of human clinical specimens, and evaluate the prevalence of four virulence genes (*toxA*, *exoS*, *oprL*, and *oprI*) in these isolates.

Methods: The present study examined ninety-seven *P. aeruginosa* isolates from various types of patient clinical specimens, including wound swabs (n =33), ear discharge samples (n =28), urine samples (n =22) and burn swabs (n =14), during the period from September 2023 to February 2024 in Babylon governorate, Iraq. All colonies of *P. aeruginosa* were sub-cultured onto the bacterial culture, and then confirmed as *P. aeruginosa* by using microbiological tests and the API 20E test. Next, the PCR technique was carried out to screen the four virulence genes (*toxA*, *exoS*, *oprL*, and *oprI*).

Results: P. aeruginosa was the most frequently isolated organism from wounds, followed by the ear, urine, and burns. The urban community shows a higher prevalence (62.9%) than the rural community (37.1%). The *oprI* and *oprL* genes were the most common genes detected among all tested isolates 97 (100%), while the *toxA* gene was detected in 79 (81.4%) isolates, and the *exoS* gene was detected in 68 (70.1%) isolates. Conversely, the results indicated that *oprI*, *oprL*, *toxA*, and *exoS* genes were the most commonly found genes in *P. aeruginosa* isolates from wound swabs, followed by ear discharge samples, urine samples, and burn swabs.

Conclusion: PCR was empirically found to be the most optimal technique to detect and differentiate the virulence factor genes of *Pseudomonas aeruginosa* isolates that are involved in human infective diseases.

Keywords: Molecular Detection, *Pseudomonas aeruginosa*, Virulence Genes

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Introduction

Pseudomonas aeruginosa is a ubiquitous environmental Gram-negative bacillus and an important opportunistic human pathogen. Its key features, including its metabolic capabilities, resistance to many stresses, and more generally, its physiology, make it highly adaptive, both in the environment and in its mammalian host.[1] *P. aeruginosa* has been found in various infectious diseases in patients with compromised host defenses, such as respiratory infections, skin infections, biliary tract infections, urinary tract infections, intestines infections, central venous catheter infections, postoperative infections, endocarditis, osteomyelitis, sepsis, and bacteremia.[2] *P. aeruginosa* is widespread in the environment and is often found in moist environments, including bathrooms, sinks, saunas, whirlpools, and swimming pools. Contact between humans and ambient water takes place frequently, and the biofilm lifestyle of the organism contributes greatly to difficulties in eradicating it from areas of colonization.[3]

The most important determinants of *P.* aeruginosa as a clinical pathogen are its ability to produce a variety of cell-associated and extracellular products that contribute to its pathogenic mechanisms.[4] The most significant contributing element to *P. aeruginosa* pathogenicity is thought to be the existence of several virulence factors. The mechanisms responsible for pathogenicity and how it expresses virulence are well known. There are numerous virulence factors, among which the most well-known is exotoxin A. It is encoded by the *toxA* gene, which is identified in 80% of biofilm-producing isolates, underscoring its significant correlation with pathogenicity and antibiotic resistance.[5] Exoenzyme S, which is encoded by the *exoS* gene, and outer membrane proteins (lipoproteins) are encoded by two outer membrane genes, the *oprL* and *oprI* genes.[6] *P. aeruginosa* exhibiting resistance to several commonly used antibiotics results in the clinical tendency to use antibiotics of last resort as first-line therapy. However, extensive use of these antibiotics has reduced their effectiveness and lead to frequent treatment failures.[3] In recent years, the development and spread of multiple drug-resistant (MDR) bacteria have presented significant challenges to the treatment of bacterial infections.[7]

Materials and Methods

Specimen Collection and Bacterial Identification

It is a cross-sectional study. Ninety-seven *P. aeruginosa* isolates, which are the subject of this investigation, were collected from various types of clinical specimens related to patients (male and female) of varying ages who were admitted to different hospitals in the Babylon governorate, Iraq, during the period from September 2023 to February 2024. These specimens include wound swabs (n =33), ear discharge samples (n =28), urine samples (n =22), and burn swabs (n =14). All demographic information, including patient age, sex, type of infection, residence of patients, and other characteristics, was provided.

In the diagnostic laboratories, all colonies of *P.aeruginosa* were sub-cultured onto MacConkey agar, Nutrient agar, and Cetrimide agar, then incubated for 18-24 hour at 37°C. All ninety seven isolates were confirmed as *P. aeruginosa* by using microbiological tests such as colony features and Gram stain, and subsequently specimens have undergone biochemical tests, and those results were then further confirmed with an API 20E system identification kits.[8]

Molecular Methods

DNA Extraction

The isolates of each confirmed *P. aeruginosa* were subjected to nucleic acid extraction according to the manufacturing instructions of Presto Mini-DNA Bacteria Kit, which is provided by (Geneaid, Thailand).

Detection of Virulene Genes

The PCR technique was performed to amplify the four virulence genes (*toxA, exoS, oprL, and oprI*) in the *P. aeruginosa* isolates by using specific primers with the appropriate amplicon size as previously performed by Khattab et al.,[9] The primer sequence is displayed in Table 1.

Multiplex PCR Assays

The PCR assays were performed to amplify virulence genes, including (*toxA, exoS, oprI, and oprL*) and were prepared by using a 20µl master mix (Bioneer, South Korea). The PCR mixture contains a 3µl DNA template, and 1µl each forward and reverse primers. The final volume was achieved to 20 µL using free nucleases deionized water "following the manufacturer's instructions with a few modifications." The mixture of PCR was added to AccuPower PCR-PreMix, which consisted of dNTPs, Taq DNA polymerase, and a 10x buffer reaction. After three minutes of vortex vibration, all of the PCR tubes are put into the thermocycler device. The requirements for the thermal cycling process are stated in Table 2.

Once PCR had been completed, 1% agarose gels in TBE solution were used for electrophoresizing the samples. Then stained the gels with ethidium bromide, observed them under a UV transilluminator, and recorded an image.

Ethical Approval: The study was carried out following the Helsinki Declaration. Before enrolling in the study, the researchers obtained verbal consent from patients. Also, study approval was gained from the research ethics committee. The registration number A34020 bears the date of September 12, 2023.

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Table 1.The Sequence of Specific Primers and Product Size

Genes	Sequence (5´to 3´)	Product Size (bp)
toxA	F-GGTAACCAGCTCAGCCACAT	352
	R-TGATGTCCAGGTCATGCTTC	
exoS	F-CTTGAAGGGACTCGACAAGG	504
	R-TTCAGGTCCGCGTAGTGAAT	
oprL	F-ATG GAA ATG CTG AAA TTC GGC	500
	R-CTTCTTCAGCTCGACGCGACG	
oprI	F-ATGAACAACGTTCTGAAATTCTCTGCT	250
	R-CTTGCGGCTGGCTTTTTCCAG	

Table 2.PCR Thermal Cycling Conditions for All Genes

Gene	PCR Steps and No. of Cycles				
	Initial Denaturation Cycle	Denaturation (35) Cycles	Annealing (35) Cycles	Extention (35) Cycles	Final Extention cycle
toxA	95°C/5 min	94°C/30 sec	58°C/30 sec	72°C/1 min	72°C/5 min
oprL					
oprI					
exoS	95°C/5 min	94°C/30 sec	60°C/30 sec	72°C/1 min	72°C/5 min

Results

Ninety seven pure bacterial isolates were obtained from various clinical specimens distributed as wound swabs (33/97) (34%), ear discharge samples (28/97) (28.9%), urine samples (22/97) (22.7%) and burn swab (14/97) (14.4%), Table 3.

The results revealed that, (61/97) of the bacterial isolates were obtained from patients in urban areas, whereas (36/97) of bacterial isolates were obtained from patients in rural areas. Thus, the higher prevalence of positive *P. aeruginosa* in urban areas than in rural areas (62.9% and 37.1%, respectively). Figure (1).

All (97) isolates of *P. aeruginosa* were submitted to the multiplex PCR technique using specified primers to determine the following virulence genes (*toxA*, *exoS*, *oprL*, and *oprI*) which are based on the sizes product amplicon of the PCR. Figure 2-3.

The results of PCR indicated that the *oprI* and *oprL* genes are the most common genes observed in all studied isolates 97 (100%), followed by *toxA* gene 79 (81.4%), while *exoS* was the least commonly detected gene 68 (70.1%). Figure 4.

Conversely, as Table 4 illustrates, the results revealed that the greatest frequency of (*oprI*, *oprL*, *toxA*, and *exoS*) genes in *P. aeruginosa* strains was found in wound swabs, which were then followed by ear discharge samples, urine samples, and burn swabs.

Table 3.Frequency of *Pseudomonas aeruginosa* Isolates Depending on Specimen Type

Specimen Type	Positive Specimen	
	No.	%
Wound swabs	33	34
Ear discharge samples	28	28.9
Urine samples	22	22.7
Burn swab	14	14.4
Total	97	100

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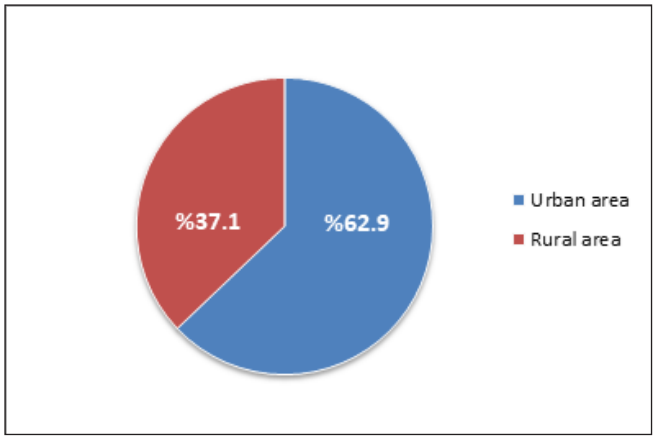

Figure 1. Distribution of *P. aeruginosa* Isolates Depending on Patients Residences

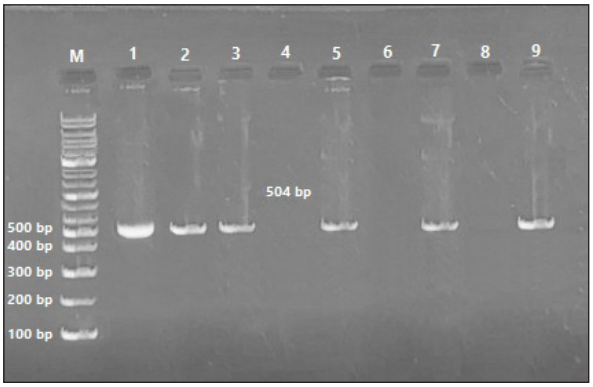

Figure 3.Agarose Gel Electrophoresis of PCR Technique to Determine exoS Gene in *P. aeruginosa* Isolated from Various Clinical Specimens. Lane M: Marker with (100-3000 bp) Ladder. Lanes 1-3,5, 7, and 9: Positive for *exoS* Gene, Product Size: 504 bp.

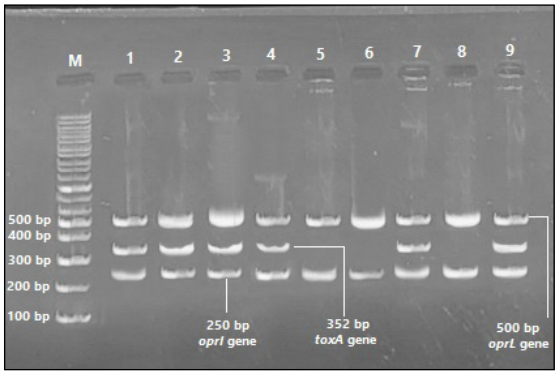

Figure 2. Agarose Gel Electrophoresis of PCR Technique to Determine (*toxA, oprI, and oprL*) Genes in *P. aeruginosa* Isolated from Various Clinical Specimens. Lane M: Marker with (100-3000 bp) Ladder. Lanes 1-9: Positive for *oprI* Gene, Product Size: 250 bp, While Lanes 1-4, 7, 9: Positive for *toxA* gene, Product Size: 352 bp. Lanes 1-9 positive for *oprL* gene, Product Size: 500 bp.

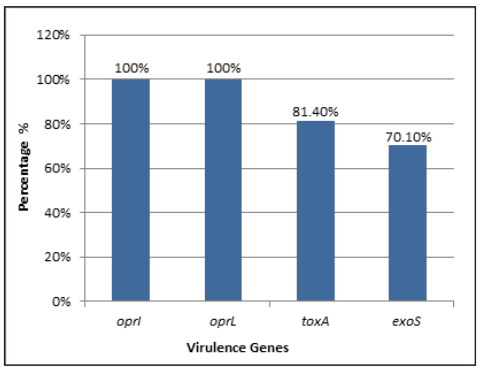

Figure 4. Prevalence The Existence of Virulence Genes in *P. aeruginosa* isolates from Various Clinical Specimens

Table 4. Examination of Virulence Gene Distribution *P. aeruginosa* Isolates from Various Clinical Specimens

Virulence Genes	Wound Swabs (n=33) %	Ear Discharge Samples (n=28) %	Urine Samples (n=22) %	Burn Swabs (n=14) %	Total (n=97)%
oprI	33 (100%)	28 (100%)	22 (100%)	14 (100%)	97 (100%)
oprL	33 (100%)	28 (100%)	22 (100%)	14 (100%)	97 (100%)
toxA	30 (90.9%)	23 (82.1%)	15 (68.2%)	11 (78.6%)	79 (81.4%)
exoS	27 (81.8%)	20 (71.4%)	12 (54.5%)	9 (64.3%)	68 (70.1%)

Discussion

Pseudomonas aeruginosa is an opportunistic pathogen that may infect practically all organs of the body. Its pathogenicity can be increased by several virulence factors. The spread of resistant and extremely virulent pathogens is likewise a major issue worldwide.[10]

The present study showed that (97) pure bacterial isolates were recovered from various clinical specimens, with the highest percentage of *P. aeruginosa* in the wound swabs (34%), followed by ear discharge samples (28.9%), then urine samples (22.7%), and burn swabs (14.4%). These results corresponded to the results recorded in Wasit province by Al-Saeedi and Raheema,[11] who observed that

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wounds are the common sites for *P. aeruginosa*, followed by the ear, urine, and burn. While the other local previous study in Baghdad city, carried out by Ahmad,[12] showed that the greatest percentage of *P. aeruginosa* was isolated from wound (32%), UTI (27%), ear (17%), and burn (24%). These findings revealed that the variations in the results between other studies are most likely attributable to the "geographical place of residence, the time of sample collection (seasons), and the use of various kits for the same procedures", all factors that may impact results across numerous Iraqi cities.

Additionally, there is a higher prevalence of positive *P. aeruginosa* in urban areas than in rural areas based on residence (62.9% and 37.1%, respectively). These factors likely account for the variation in distribution: increasing antibiotic resistance, as people can easily buy any drug over the counter, and a lack of hygiene, particularly in hospitals, contribute to an increase in opportunistic agents such as *P. aeruginosa*, as well as the general adaptability of bacteria to various environments.

The results of the current study have shown that the *oprI* and *oprL* genes were the most common genes present in all *P. aeruginosa* isolated from various clinical specimens at a rate of (100%). This result coincides with the results done by Al-Mayyahi[13] in Baghdad city, where he found that (100%) of isolates were positive for the *oprL* and *oprI* genes. Another local study recorded in Babylon province by Almuttairi and Abdulla[14] indicated that (100%) of isolates were positive for the oprL, but (98%) were positive for oprI gene. Also, the results revealed that PCR detection for the *toxA* gene showed a positive percentage of 79 (81.4%), which is in agreement with the findings of a study in Baghdad city by Ahmad,[12] who noted a rate of *toxA* of (80%). Additionally, the result of PCR showed that *exoS* was the least commonly detected gene 68 (70.1%), as demonstrated in Figure (4). In relevant studies performed in Iran by Azimi et al.,[15] the rate of the *exoS* gene detected was found to be lower than that found in the current study, at a rate of (26.3%). Moreover, the results indicated that *oprI*, *oprL*, *toxA*, and *exoS* genes were the most commonly found genes in *P. aeruginosa* isolates from wound swabs, followed by ear discharge samples, urine samples, and burn swabs.

Global differences in the distribution of virulence factors could be related to the fact that some *P. aeruginosa* strains have the potential to adapt to infection regions and take advantage of the local circumstances of those sites.[16] Another potential reason for these variable strains is that the isolates from the same patient may have different physiological and clinical circumstances.[17]

Conclusion

The study concluded that *Pseudomonas aeruginosa* was the most frequently found organism in wounds, followed by the ear, urine, and burns, respectively. The PCR technique has demonstrated its high sensitivity in detecting and identifying virulence factor genes from *P. aeruginosa* isolates responsible for human illness, revealing that the *oprI* and *oprL* genes were the two most frequently found genes in all 97 (100%) of the isolates, followed by the *toxA* and *exoS* genes, which exhibited varying frequencies.

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