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Research Article

Correlation Between the Exou/Exos Genotype, Biofilm Formation, and Antibiotic Resistance in Pseudomonas Aeruginosa Isolated from Burn Wound Infections

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A B S T R A C T

Introduction: Pseudomonas aeruginosa is widely recognised as the primary etiological agent for serious infections in burn patients worldwide.

Objective: To examine the frequency of the exoU/exoS genotype in clinical isolates of P. aeruginosa obtained from burn wound infections in Iraq and its correlation with antibiotic resistance.

Methodology: 80 P. aeruginosa isolates were obtained from patients diagnosed with burn wound infections in Iraqi hospitals. The isolates were initially identified by biochemical methods and subsequently confirmed by molecular techniques. Conventional polymerase chain reaction (PCR) was used to detect the exoU/exoS genotype. Antibiotic susceptibility was determined by the disc diffusion method, and biofilm production was assessed by the microtiter plates method.

Results: This study found four distinct genotypes in burn wound infections, with substantial differences ($P < 0.001$). Most P. aeruginosa isolates form biofilm (96.25%; 77/80) at different intensities, with significant differences ($p < 0.05$) and a statistically significant correlation with an (exoS+\exoU-) genotype. The (exoS+\exoU+) genotype was more antibiotic-sensitive. While the (exoS-\exoU+) genotype was more antibiotic-resistant than other genotypes. The genotype exoU+\exoS- correlated significantly with fluoroquinolone antibiotic susceptibility, resulting in substantial resistance rates against ciprofloxacin and levofloxacin. While the genotype exoU+\exoS+ showed a statistically significant correlation with susceptibility to cephalosporin antibiotic groups (ceftazidime and cefepime), the sensitivity rate was higher than other genotypes.

Conclusion: These results suggested that genotype influences antibiotic resistance, posing a concern, as horizontal gene transfer of the exoU gene can increase antibiotic resistance in P. aeruginosa.

Keywords: Pseudomonas Aeruginosa, exoU/exoS Genotype, Type III Secretion System, Burn Wound Infections, Biofilm Formation

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium highly prevalent in various environments and recognized as a primary etiological agent of healthcare-associated diseases.[1,2] P. aeruginosa is a significant contributor to mortality and morbidity in individuals with compromised immune systems, including those who have suffered burns.[3] The presence of this bacterium in hospitals can lead to a range of serious diseases in hospitalized individuals, including septicemia, otitis, endocarditis, pneumonia, keratitis, and skin and soft tissue infections.[4] Furthermore, due to innate or acquired resistance to many broad-spectrum antibiotics, P. aeruginosa has become a prevalent pathogen in hospital environments.[5,6] Patients with compromised immune systems, such as those who have recently had burn treatments, are particularly vulnerable to infection with P. aeruginosa.[7] The mortality rate among burn patients who are infected is greater compared to burn patients who are not infected, mainly when the infection is caused by multidrug-resistant bacteria (MDR). The investigation and characterization of bacterial isolates associated with burn wound infections have received significant attention due to the frequent occurrence of MDR P. aeruginosa infections in burn units.[8,9]

Various protein secretion systems can release proteins into the surrounding environment or into the host cell. The type III secretion system, also known as TTSS or T3SS, is the third type of secretion system found in bacterial cells. It appears to be a needle in the structure of pathogenic bacteria[10], and it functions as a sensory transmitter to detect eukaryotic organisms. It releases proteins that facilitate the spread of bacterial infections. Exoenzymes or effector proteins are secreted directly from the bacterial cell into the eukaryotic host cell by this system, where they exert various effects. This helps the pathogen survive, resist, and evade the immune response. P. aeruginosa secretes four types of exoenzymes via T3SS: S, Y, U, and T exoenzymes.[11] Bacteria that possess this system produce exo Y and exo T in 100 %, while others (exo S and exo U) are present in different percentages. However, new isolates appeared to have both genes (exoS and exoU) in addition to the other two genes, exoY and exoT. These isolates are classified as highly virulent, containing all four toxins.[12]

The relevant literature reports a correlation between biofilm formation and the exoU-/exoS+ genotype.[13] Derakhshan et al. reported that 100% of biofilm-producing isolates contain the exoS gene.[14] With regard to antibiotics, the association between antimicrobial resistance (or MDR phenotype) and type III secretion system genes was most investigated for the exoU gene.[15] As previously indicated, this gene is associated with increased resistance to certain antibiotics, and the exoU gene is more commonly found in MDR P. aeruginosa strains.[16] Acquiring knowledge of these genetic types and their associations with antibiotic resistance and biofilm formation will enable us to understand the importance of these genes in disease development.

Material and methods

Specimens' collection, bacterial isolation, and identification. A total of 120 burn swabs have been collected from two hospitals in Baghdad, namely the Teaching Baghdad Hospital and AL-Yarmok Hospital, over the period of January to April 2023. The swabs were cultured on MacConkey agar and then incubated at 37°C for 24 hours. In addition to microscopic examination, a selective cetrimide medium and biochemical tests, including oxidase, catalase, and incubation at different temperatures (4 and 42 °C), were used for the primary identification. The identity was ultimately confirmed by molecular techniques that targeted a fragment of the 16S rRNA gene with specific primers. The identified bacterial isolates were cultivated and stored at 4°C for subsequent examination.

Oligonucleotide primers

The in-silico design of oligonucleotide primers was carried out using Serial Cloner 2-6-1 and Amplifx software. First, the sequences of the target genes were downloaded from the National Center for Biotechnology Information (NCBI) website. The accession number for the 16SrRNA gene was (PP448158.1), and for exoS and exoU, were (X99471.1) and (KX641459.1), respectively. The primers were manufactured by Macrogen Company and provided in a lyophilized form. A stock solution of primer (100 pmol/µl) was prepared by dissolving the lyophilized primer in an appropriate amount of nuclease-free water, according to the manufacturer's instructions. Then, the working solution (10 pmol/µl) was prepared by diluting the stock solution ten times and kept at -20° C in the freezer; a list of the primers is shown in Table 1. Thermo Fisher Scientific's Tm Calculator was used to calculate the annealing temperatures for primers.[17]

Whole bacterial genomic extraction

This study employed a novel technique to efficiently extract whole bacterial genomes, with potential for use in gene exploration. This strategy combines and adapts the boiling method described by Omar et al.18 and the colony PCR technique used by Husam19. In the present experiment, a single bacterial colony was transferred into a 5 ml nutritional broth and incubated for 24 hours at 37°C. Next, cells were collected by centrifugation at 13000 rpm for 15 minutes. The recovered cells were washed twice with 1 ml of distilled water and resuspended in another 1 ml. The cells were then standardized to achieve an optical density (O.D.) value of 1 at a wavelength of 600 nm. Subsequently, 0.3 ml of the standardized bacterial culture was transferred into an Eppendorf tube. The cells were subsequently collected by

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centrifuging for 15 minutes at 13000 rpm. Following this, the cells were resuspended in 0.75 ml of Tris-EDTA buffer (TE) and subsequently heated to 100°C for 10 minutes. The denatured proteins and cellular debris were removed from the genomic DNA using centrifugation for 20 minutes at 13000 rpm. The genomic DNA-containing supernatant was used as a direct PCR template without additional purification, with a supernatant-to-PCR reaction mixture ratio of 1:9.

Amplification of genes by polymerase chain reaction (PCR) technique

The conventional PCR technique was employed to amplify segments of three target genes: the 16SrRNA gene for molecular bacterial identification and the exoS and exoU genes to investigate the prevalence of the exoS/exoU genotype among P. aeruginosa isolates. The reaction mixture and PCR settings for amplifications using Go Taq G2 Green Master Mix are provided in Table 2. All PCR reactions were performed using sterile PCR tubes and an Applied Biosystems thermocycler.

Table 1.Primers sequences, annealing temperatures, and amplicon sizes

Primer name	5- Sequence -3	Annealing temperatures (˚C)	Amplicon size bp
For 16s rRNA gene (bacterial identification)			
16S Pseud-F	AGGCCTAACACATGCAAGTCGA	55	1400
16S Pseud-R	GGTTAGACTAGCTACTTCTGGAGC		
For exo U gene detection			
ExoU-F	CCGTTGTGGTGCCGTTGAAG	57	800
ExoU-R	TCATGTGAACTCCTTATTCCGCC		
For exo S gene detection			
ExoS-F	GCGGACCTGAATCGCGCTCT	55	500
ExoS-R	CGTACATCCTGTTCCTGGACC		

Table 2.Composition of PCR Mixture and PCR conditions

<table>
<tr><th colspan="4">Materials</th><th colspan="2">Volume in µl</th></tr>
<tr><td colspan="4">Go Taq G2 Green Master Mix (2X)</td><td colspan="2">50</td></tr>
<tr><td colspan="4">Forward Primer (10 µM/ µl)</td><td colspan="2">1.5</td></tr>
<tr><td colspan="4">Reverse Primer (10 µM/ µl)</td><td colspan="2">1.5</td></tr>
<tr><td colspan="4">Ultra-pure water</td><td colspan="2">47</td></tr>
<tr><td colspan="4">Total reaction mixture</td><td colspan="2">100</td></tr>
<tr><td colspan="6">Aliquot 9.5 µl into 10 PCR tubes and add 1 µl of DNA-containing supernatant</td></tr>
<tr><td colspan="6">PCR conditions</td></tr>
<tr><td rowspan="2">Initial denaturation</td><td colspan="3">30 reaction cycles</td><td rowspan="2">Final Extension</td><td rowspan="2">Hold</td></tr>
<tr><td>Denaturation</td><td>Annealing</td><td>Extension</td></tr>
<tr><td>5 min (95˚C)</td><td>30 s (95˚C)</td><td>30 s (X˚C) *</td><td>50 s (72˚C)</td><td>5 min (72˚C)</td><td>5 min (4˚C)</td></tr>
<tr><td colspan="6">* X= Tm used according to the primer pairs listed in Table 1</td></tr>
</table>

Agarose gel electrophoresis

The PCR results were analysed using agarose gel electrophoresis, using 2% (w/v) agarose gel. The agarose gels were prepared by dissolving 2 g of agarose powder (Promega, USA) in 100 ml of 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.8) using a microwave. After cooling to approximately 50-40°C, the gel was then enhanced with 4 µl of ethidium bromide (10 mg/mL, Promega, USA). Subsequently, the gel was poured into a gel tray and left to solidify at room temperature (20-25°C). A volume of 5 µl of each sample was subjected to analysis with a volume of 3 µl of a DNA ladder (1 kb DNA, Promega, USA). The electrophoresis procedure was conducted at a voltage of 75 volts for 60 minutes. The DNA bands were detected using ultraviolet (UV) light in a gel imaging device (Fisher Scientific, UK).

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Assessment of biofilm formation by bacterial isolates

The biofilm development experiment used a microtiter plate approach, as outlined by Djordjevic et al.20 In summary, isolates were cultured in brain heart infusion (BHI) broth at 37°C for 24 hours. Subsequently, it underwent calibration using a McFarland standard of 0.5. Three wells of the sterile polystyrene microplate with a U-shaped bottom and 96 wells filled with 180 µl of BHI and 20 µl of calibrated bacterial culture. The plate was sealed and incubated for 24 hours at 37°C. Negative controls were conducted using BHI broth wells. Following incubation, the sample was subjected to three gentle rinses using distilled water. Subsequently, it was dried at 65°C for one hour. Next, methanol was applied to fix the biofilm for 30 minutes, followed by washing with distilled water. For staining the biofilm, the plates were incubated for 10 minutes at room temperature with 200 µL of a 1% crystal violet solution. Subsequently, the plates were washed with distilled water and dehydrated for 30 minutes at 37°C. 200 µl of glacial acetic acid was added to dissolve the cells adhering to the surface, and the optical density (OD) at 580 nm was measured for each well using a Microplate ELISA reader (Diagnostic Automation, Inc., USA). The cut-off optical density (ODC) is defined as three standard deviations (SD) above the average OD of the negative control. The ODC is calculated as follows: ODC = average OD of negative control + (3 x standard deviation of negative control). The isolates were classified into four categories according to their ODC value: OD ≤ ODC indicates a non-producer of biofilm. ODC<OD≤2* ODC signifies a weak biofilm producer. 2*ODC<OD≤4*ODC represents a moderate biofilm producer, while 4*ODC<OD indicates a strong biofilm producer.

Antibiotic susceptibility patterns test

The antibiotic susceptibility patterns were assessed using the disk diffusion method on Mueller-Hinton agar plates, following the recommendations provided by CLSI 2023 and the protocol reported by Velican et al.21 The antimicrobial discs used in the study were Amikacin (AK, 30 µg), Gentamycin (GN, 10 µg), Imipenem (IMP, 10 µg), Meropenem (MEM, 10 µg), Ceftazidime (CAZ, 30 µg), Cefepime (FEP, 30 µg), Ciprofloxacin (CIP, 5 µg), Levofloxacin (LEV, 5 µg), Piperacillin-tazobactam (TPZ, 100\10 µg) and Ticarcillin-clavulanate (TCC,75\10 µg). P. aeruginosa isolates were categorized as resistant, sensitive, or intermediate by comparing the inhibition zone diameter to the CLSI, 2023 recommendations, after incubating at 37˚ C for 24 hours.

Statistical analysis

All features were presented as frequencies and percentages, and the Pearson chi-square test was used to assess significant differences in percentages. P≤0.05 was considered significant. The data were analyzed using SPSS v. 22.0 and Excel 2013 statistical software.

Results and Discussion

Primary bacterial isolation and identification

A total of 80 P. aeruginosa isolates (66.6%) were isolated from 120 patients diagnosed with burn wound infections in Iraqi hospitals, compared to 40/120 (33.32%) for other microbial growth, with significant differences (p<0.05). The primary identification of P. aeruginosa isolates was based on culturing characteristics on selective and differential medium, microscopic examination, and a few biochemical tests, as illustrated in Table 3.

Table 3.Results of cultural characteristics and biochemical tests of P.aeruginosa isolates

Tests	Result
Growth on MacConkey Agar	Lactose non-fermenter, pale colony
Growth on Cetrimide agar	A present fluorescent green colour with growth
Gram stain	Gram negative, Bacilli
Oxidase test	Positive
Catalase test	Positive
Grow at 42°C	Positive
Grow at 4°C	Negative

Primary identification is considered an essential step in isolating bacteria from infection sites. This identification helps avoid other uninteresting bacteria that may be involved in infections, thereby saving time and materials, especially since many bacterial species can cause infections in burn wounds.[22,23] However, the identification of the 80 bacterial isolates was confirmed using a molecular technique by targeting a segment of the 16S rRNA gene (1400 pb) that shares 100% identity with P. aeruginosa using specifically designed primers. Four sets of results are illustrated in Figure 1, each representing a different group of isolates (1 to 20, 21 to 40, 41 to 60, and 61 to 80). The consistency of the banding patterns across all samples, as shown in Figure 1, indicates the existence and successful amplification of the target 16S rRNA gene, confirming the identity of the isolates as P. aeruginosa.[24] This demonstrates the reliability and precision of this molecular technique in bacterial identification. This technique achieved high accuracy in confirming the identity of bacterial isolates.[25,26]

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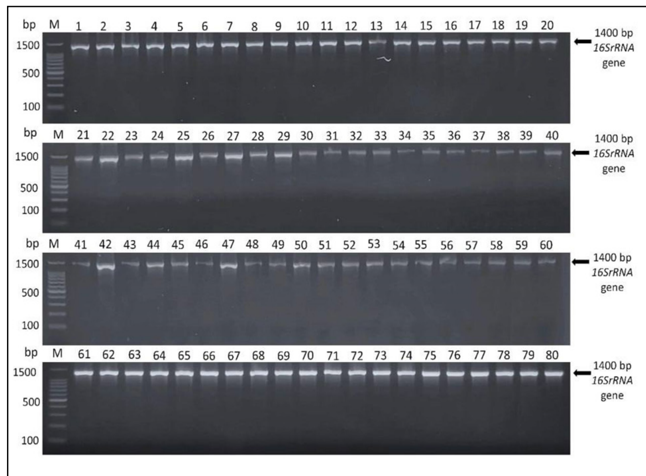

Figure 1.Agarose gel electrophoresis (2%) of PCR amplicon showing a segment of 16S rRNA gene (1400 bp) amplification for P. aeruginosa identification. Numbers 1 to 80 correspond to P. aeruginosa isolates. M corresponds to the DNA marker (100 bp).

Molecular detection of exoU/exoS genotype amongst P. aeruginosa

Two genes were targeted to determine the exoU/exoS genotype: exoU and exoS. The results showed a variable distribution of the exoU and exoS genes among the bacterial isolates under study with significant differences (P< 0.001), as shown in Figure 2. This distribution categorized the P. aeruginosa isolates into four genotypes. The first genotype (exoU-/exoS+), which was the highest one, was found in 75% (60/80) of the bacterial isolates. In contrast, the second genotype (exoU-/exoS-) was relatively rare, found in only 5% (4/80) of the bacterial isolates, indicating a lack of both key virulence factors. The third and most important one was the (exoU+/exoS+) genotype, indicating the presence of both virulence factors, which was observed in 13.75% (11/80) of the isolates. Lastly, the fourth genotype (exoU+/exoS-) was detected in 6.25% (5/80) of isolates. The results are in line with another local study that found that exoS and exoU genes were present in 90.47% and 60.31% of P. aeruginosa isolates from different clinical sources, respectively.[27]

Biofilm formation and correlation with exoU/exoS genotype

The results showed that 96.25% (77/80) of the isolates formed biofilm. In comparison, 3.75% of isolates (3/80) were non-biofilm formers with significant differences (p<0.05) under laboratory conditions. The positive biofilm-forming isolates (77) revealed different levels of biofilm formation. Strong biofilm producers (38.96%), followed by moderate- biofilm producers (35.06%) and weak biofilm producers (25.97%), as shown in Table 4. In general, most isolates are biofilm producers, with the highest prevalence among strong-intensity biofilm producers, followed by moderate and weak-intensity biofilm producers. Several researchers have reported similar results. In the previous investigation, all P. aeruginosa isolates obtained from infected wounds and burns were found to be capable of producing biofilms (100%). Among these isolates, 50% were classified as strong biofilm producers, 37% as moderate biofilm producers, and 13% as weak biofilm producers. Ghasemian et al. reported that all P. aeruginosa isolates isolated from burn wounds were biofilm producers, of which 42.5%, 35%, and 22.5% were strong, moderate, and weak biofilm producers, respectively 28. Similarly, Tiba and Huda reported that 100% of P. aeruginosa isolates were biofilm producers. The prevalence of isolates forming biofilm was 52%, 32%, and 16% for strong, moderate, and weak biofilm, respectively.[29]

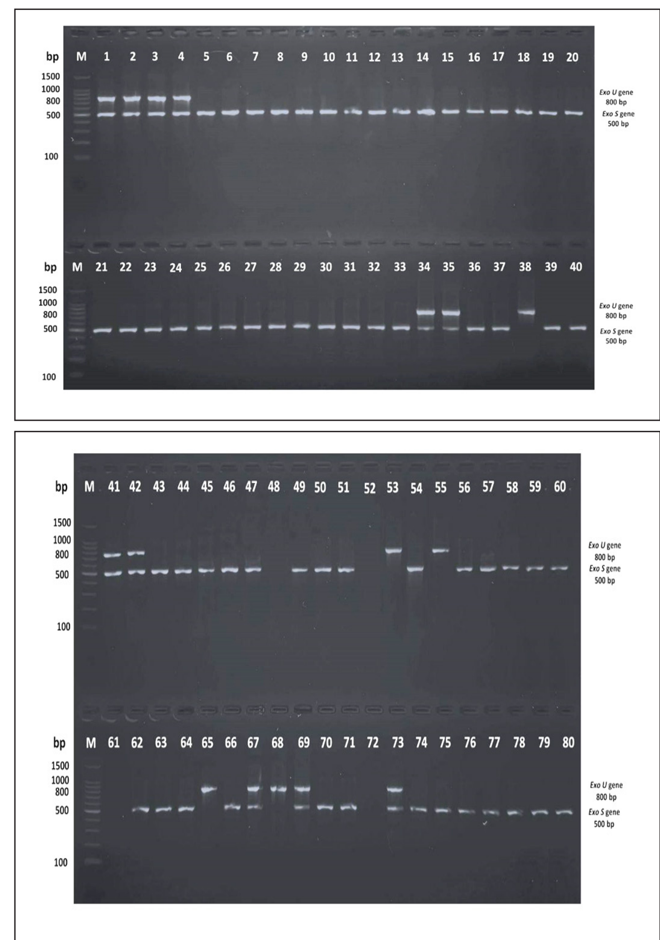

Figure 2.Agarose gel electrophoresis (2%) of PCR amplicon showing amplification of a segment of exoU gene (8000 bp) and exoS gene (500bp) for P. aeruginosa. Numbers 1 to 80 correspond to P. aeruginosa isolates. M corresponds to the DNA marker (100 bp). Each line in the gel represented a combination of separated PCR products for ExoU and ExoS

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Table 4.Frequency and percentage of biofilm types in P. aeruginosa isolates

Biofilm formation	Number of isolates	Percentage	P value
Non-producers	3	3.75%	P<0.05**
Producers	77	96.25%	
Total	80	100%	
Type of biofilm in biofilm producer isolates			
Strong producers	30	38.96%	p<0.05
Moderate producers	27	35.06%	
Weak producers	20	25.97%	
Total	77	100%	

The ability to form a biofilm is controlled by a variety of components and biofilm-related genes, which play a role in the initial attachment of P. aeruginosa to surfaces.[30] These components include flagella, type IV pili, Cup fimbriae, extracellular DNA (eDNA), Psl polysaccharides, and alginate.[31] Biofilm formation would also be due to the release of compounds such as iron siderophores and biosurfactants into the surrounding environment.[32] However, variation in the quality and quantity of autoinducers produced by each isolate, as well as genetic factors and other components that affect biofilm formation, could explain isolates' ability to generate different biofilm intensities.[33,34]

Statistical analysis of the distribution of the genotype amongst the biofilm producers revealed a significant correlation (P<0.05) between biofilm formation and the exoU-/exoS+ genotype; 75.32% (58/77) of biofilm producer isolates were exoU-/exoS+, followed by exoU+/exoS+, which accounted for 12.98% (10/77) of biofilm producer isolates, with no significant difference. In contrast, the genotypes that lack the exoS gene represented a lower percentage among biofilm-producing isolates: 5.1% (4/77) and 6.49% (5/77) for exoU-/exoS- and exoU+/exoS genotypes, respectively, as shown in Table 4. Results revealed no correlation between biofilm types and other genotypes.

Edward and Cottage showed 75% of isolates that have the exoS gene produce biofilm35, and these results were lower than our study, which showed (88.31%; exoU-/exoS+ (75%) + exoU+/exoS+(12.98%)) of isolates that produce biofilm have exoS. Another study revealed similar results. The exoU-/exoS+ genotype was the most common in biofilm-producer isolates (79.8%)13. Derakhshan et al. reported that 100% of biofilm-producing isolates carried the exoS gene14.

The high prevalence of the exoS gene in biofilm producers could be explained by the importance of the ExoS toxin in the spread and pathogenicity of bacterial isolates, as it is known to be an invasive factor. Our results contribute to understanding the complex interplay between genotypic variation and biofilm-forming capabilities in P. aeruginosa and suggest that biofilm formation is a complex trait influenced by multiple factors, including, but not limited to, genotype, as discussed in several studies.[36,37]

Antibiotic susceptibility patterns and correlation with exoU/exoS genotype

The sensitivity of P. aeruginosa to various antibiotics has been the subject of numerous studies owing to the bacterium's capacity to acquire resistance 38. Ten different antibiotic agents belong to five groups; two antibiotics from each group were used to evaluate antibiotic susceptibility patterns and investigate the potential correlation between antibiotics and different (exoU/exoS) genotypes in P. aeruginosa isolates. Based on the results illustrated in Figure 3, the isolates were found to have a broad spectrum of resistance from 86.3% against each of Ceftazidime and Cefepime to 18.8% against each of Ciprofloxacin and Levofloxacin; a wide range of sensitivity from 78.8% against Ciprofloxacin to 13.8% against each of Ceftazidime and Cefepime; and a wide range of intermediate susceptibility from 28.8% against Ticarcillin-clavulanate to 0% against each of Ceftazidime and Cefepime.

Biofilm Genotypes

As shown in the figure above, most of the bacterial isolates were resistant to the Cephalosporin group of antibiotics (Ceftazidime and Cefepime, with a resistance rate of 86.3%). These results are in line with a previous local study in Iraq that reported that isolated P. aeruginosa from burns were resistant to Ceftazidime and Cefepime at rates of 84.50% and 85.92%, respectively.[39] However, an international investigation showed the opposite results regarding Ceftazidime and Cefepime; it has been reported that P. aeruginosa isolated from burns was sensitive to Ceftazidime and Cefepime at rates of 59.3% and 77.8%, respectively.[40] The resistance of P. aeruginosa to cephalosporin antibiotics can result from the induction of chromosomal β-lactamases upon exposure to these antibiotics.[41]

It can be seen that all isolates showed different sensitivity patterns to the antibiotics under investigation. The highest sensitivity rates were 78.8%, 75%, and 68.8% for Ciprofloxacin, Piperacillin-tazobactam, and Levofloxacin, respectively. These findings are similar to those reported by Abdi et al., who found that Piperacillin-tazobactam and Ciprofloxacin had high sensitivity rates of 74.1% and 66.67%, respectively 40. These antibiotics can be recommended as effective antibacterials for the treatment of burn wound infections caused by P. aeruginosa.

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Table 5.Comparison of biofilm types with (ExoU/ExoS) genotype in P. aerugenosa isolates

Biofilm U(+)/S(+) Positive Negative			Genotypes							
			U(-)/S(-)		U(+)/S(-)		U(-)/S(+)			
			Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
	Non	N	1	2	0	3	0	3	2	1
		%	9.1%	2.9%	0.0%	3.9%	0.0%	4%	3.3%	5%
	Weak	N	2	18	0	20	3	17	15	5
		%	18.2%	26.1%	0.0%	26.3%	60%	22.7%	25%	25%
	Moderate	N	4	23	3	24	0	27	20	7
		%	36.4%	33.3%	75%	31.6%	0.0%	36%	33.3%	35%
	Strong	N	4	26	1	29	2	28	23	7
		%	36.4%	37.7%	25%	38.2%	40%	37.3%	38.3%	35%
Total %		N	11	69	4	76	5	75	60	20
		100%	100%	100%	100%	100%	100%	100%	100%	
P value			P>0.05		P>0.05		P>0.05		P>0.05	
Total Biofilm Producers			10		4		5		58	
%			12.98 %		5.1%		6.49%		75.32%	
P value			P>0.05		P>0.05		P>0.05		P>0.05**	

Other antibiotics under investigation, including Imipenem, Amikacin, Gentamicin, Meropenem, and Ticarcillin-clavulanate, showed a wide range of resistance, sensitivity, and intermediate susceptibility. These differences in antibiotic susceptibility patterns between all the antibiotics under study, as well as in other studies, are a result of the fact that several factors affect antibiotic susceptibility, such as the presence of resistant genes, efflux pumps, biofilm, its exogenous DNA, and other 42,43. Furthermore, there are three fundamental mechanisms of antimicrobial resistance (1) enzymatic degradation of antibacterial drugs, (2) alteration of bacterial proteins that are antimicrobial targets, and (3) changes in membrane permeability to antibiotics 31. These factors and mechanisms vary among bacterial isolates, resulting in different responses to antibiotics.

The susceptibility of P. aeruginosa to various antibiotics and its correlation with other virulence factors has been a topic of interest in many studies, given the bacterium's ability to develop resistance to antibiotics44,45,38. However, only two genotypes (exoU+\exoS- and exoU+\exoS+) have been found to correlate with the antibiotics under investigation, while other genotypes (exoU-\exoS- and exoU-\exoS+) showed no correlation.

The exoU+\exoS- genotypes showed a statistically significant correlation with susceptibility against fluoroquinolone antibiotic groups (Ciprofloxacin and Levofloxacin). This genotype showed a high resistance rate against these antibiotics, which was 60% for both compared with other genotypes that showed a lower resistance rate against the same antibiotics. For Ciprofloxacin, the resistant rates were 0%, 25%, and 18.3% for exoU+\exoS+, exoU-\exoS-, and exoU-\exoS+ genotypes, respectively, and for Levofloxacin, the resistant rates were 0%, 0%, and 20% for exoU+\exoS+, exoU-\exoS-, and exoU-\exoS+ genotypes, respectively, as shown in Figure 4. Similar findings were reported by Sawa et al.; the presence of the exoU+ genotype correlates with a fluoroquinolone resistance phenotype 46. According to Subedi et al., all isolates that harbored the exoU gene exhibited higher resistance to fluoroquinolones47. Furthermore, these isolates showed high resistance to all antibiotics under study. Figure 4 clearly shows that the exoU+\exoS- genotypes exhibited higher resistance to most antibiotics under investigation than other genotypes. This is a concerning sign; the cytotoxic genotype is more resistant to antibiotics, especially with the possibility of transferring the exoU gene between bacterial isolates, which could increase antibiotic resistance rates. These findings align with other investigations that revealed the

presence of the exoU gene associated with high antibiotic resistance. The exoU gene is often found amongst MDR P. aeruginosa strains 16. Higher antimicrobial resistance is generally observed in P. aeruginosa isolates possessing the exoU gene.[48]

Interestingly, a statistically significant correlation between the genotype exoU+\exoS+ and the Cephalosporin antibiotic groups (Ceftazidime and Cefepime) has been observed, as shown in Figure 4; this is the first report of such a correlation. The sensitivity rate was 36.4% for the above antibiotics, whereas other genotypes (exoU+\exoS- and exoU-\exoS-) showed 0% sensitivity to the same antibiotics. Interestingly, the genotype exoU-\exoS+ showed an 11.7% sensitivity rate. Based on the above results, the presence of the exoS gene may affect P. aeruginosa's susceptibility to the cephalosporin antibiotic groups under investigation.

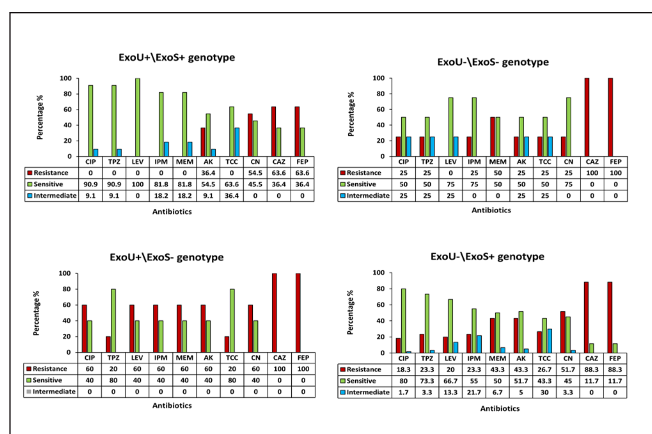

Figure 4.Antibiotic susceptibility pattern of ten different antibiotics tested against different genotypes of P. aeruginosa isolates. Amikacin (AK), Gentamycin (GN), Imipenem (IMP), Meropenem (MEM), Ceftazidime (CAZ), Cefepime (FEP), Ciprofloxacin (CIP), Levofloxacin (LEV), Piperacillin-tazobactam (TPZ) and Ticarcillin-clavulanate (TCC)

The association of the exoU+ genotype with fluoroquinolones has been found to be linked with quinolone resistance-determining regions (QRDRs), topoisomerase II (gryA and gyrB), and topoisomerase IV (parC and parE), mutations in these genes have been shown to increase fluoroquinolone resistance in P. aeruginosa.[49] Furthermore, it was noted that fluoroquinolone resistance in ExoU strains was associated with a specific combination of mutations in gyrA and parC. Sequence analysis revealed that six out of eight exoU strains exhibited at least one mutation in either gyrA (T83I) or parC (S87L)47. Regarding cephalosporin correlation, as reported here for the first time, the association with cephalosporin groups is unclear. Therefore, further investigation is needed to understand the correlation better. It is worth noting that antibiotic susceptibility can be influenced by many factors, including the presence of resistance genes, the expression of efflux pumps, and the ability to form biofilms.[50] Therefore, the correlation between exoU/exoS genotypes and antibiotic susceptibility is complex, given the number of genes and factors involved.

Conclusion

Different genotypes of P. aeruginosa are present in burn wound infections in Iraq, as indicated by the presence of Type III secretion system toxins, with the exoS+\exoU- genotype (invasion genotype) being highly prevalent. The most virulent genotype (exoS+\exoU+, which is invasive and cytotoxic) is more sensitive to antibiotics than other genotypes. Therefore, the elimination of this genotype could be controlled. While the exoS-\exoU+ genotype (cytotoxic genotype) is more resistant to antibiotics than other genotypes, this is a worrisome sign, especially when exoU can transfer via horizontal gene transfer, which, in turn, raises antibiotic resistance in P. aeruginosa.

Ethical Approval

All participants agreed to provide the investigator with the specimens. The Ethics Committee of the College of Science at the University of Baghdad authorized this work (Ref. CSEC/0123/0008). All participants provided informed consent in accordance with the Declaration of Helsinki.

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