Original Article

Molecular characterization of virulence factors in *Pseudomonas aeruginosa* acquired from diverse clinical specimens in Erbil, Iraq

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ABSTRACT

Pseudomonas aeruginosa is a common opportunistic pathogen that can cause various infections. Many virulence factors, including exotoxin and exoenzyme genes, as well as the formation of a biofilm, may contribute to the pathogenicity of this organism. The PCR assay was conducted to evaluate the existence of *exo*T, *exo*A, *plch*, *pvda*, *las*B, *protease*, *phz*M, and ex*o*S virulence genes. On the other hand, this research comprises the detection of β-lactamase genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) and the detection of biofilm development and antibiotic susceptibility patterns among clinical isolates of *P. aeruginosa*. In this investigation, 75 isolates of the bacteria *P. aeruginosa* were obtained and tested from various clinical specimens. Isolates were found to have distinct antimicrobial susceptibility patterns. The virulence genes were identified with a PCR assay. It was determined whether biofilm creation was possible. A test for antibiotic susceptibility revealed that the highest resistance rate toward antibiotics was recorded against Amoxicillin-clavulanic acid (75, 100%), and the lowest resistance rate was Meropenem (18, 24%). Imipenem was shown to be the most effective antibiotic, with 100 percent of isolates responsive to it. The *phz*M and *exo*S genes were present in 90.67% and 82.67% of the samples. Both *exo*A and *pvda* genes were found in forty-three of the isolates tested (57.33%).

Eventually, this study revealed that *P. aeruginosa* exotoxin and exoenzyme genes, specifically the *exo*S gene, are the most common virulence factor in bacterial isolates from wound swab samples. When treating *P. aeruginosa* infection, biofilm is a severe obstacle to overcome.

Keywords: *Pseudomonas aeruginosa*, Virulence factors, Antimicrobial susceptibility, Biofilms

Introduction

Pseudomonas aeruginosa has emerged as an important nosocomial infection. This bacterium causes infection, especially in the hosts with compromised defense mechanisms, such as patients with severe burns and individuals with HIV infection (1). It causes many disorders, including septicemia, pneumonia, endocarditis, burn wounds, otitis, and keratitis (2). *P. aeruginosa* is responsible for mortality rates as high as 50%. It may quickly become problematic once introduced in a hospital because of its ability to adhere to medical devices such as catheters. This organism is often antibiotic-resistant and enters the blood, causing septicemia. *P. aeruginosa* appears to be related to producing many secretions and cell-associated virulence factors, including toxins, enzymes, and biofilm. Biofilm growth promotes bacterial survival. Once a biofilm is formed, it becomes complicated to be destroyed (3).

Biofilm is a complex aggregation of microorganisms in an exopolysaccharide matrix and is usually antibiotic-resistant. *P. aeruginosa* is a pathogen with innate resistance to many antibiotic classes, including aminoglycosides, carbapenems, antipseudomonal penicillins, quinolones, and cephalosporins. In addition, it has been known to acquire novel resistance genes via horizontal gene transfer (4).

P. aeruginosa also possesses a variety of virulence factors such as exotoxin A (encoded by *tox*A gene), exoenzyme S (encoded by *exo*S gene), and exoenzyme U (encoded by *exo*U gene). The *exo*A is the principal constituent of the type II secretion system (T2SS), which inhibits protein synthesis through the transfer of the adenosine diphosphate-ribosyl moiety from nicotinamide-adenine dinucleotide to elongation factor 2, resulting in the

inhibition of protein. Another essential virulence factor that was recently recognized is the type III secretion system (T3SS). T3SS is a contact-dependent protein secretion pathway that plays a significant role in the pathogenesis of severe *P. aeruginosa* infections. This system secretes effector proteins such as *exoS* and *exoU* (12). The *exoS* is a major cytotoxin required for colonization, invasion, and bacterial dissemination during infection (5). The *exo*U is a cytotoxin with phospholipase activity that affects epithelial cells and causes lung infection. In addition, *exoU* has a toxic effect on macrophages. One of these bacteria's most essential virulence determinants is the biofilm, a sessile population of microorganisms enclosed by the self-secreted extracellular polysaccharide matrix or slime layer. Biofilms are efficient barriers against antimicrobial agents (6-8). Multidrug-resistant forms of *P. aeruginosa* are a significant source of nosocomial infections (9). The increasing resistance of *P. aeruginosa* to numerous antibiotics because of excessive antibiotic administration is now leading to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of multidrug-resistant (MDR) forms of *P. aeruginosa (10)*.

The purpose of this study was to investigate the presence of virulence genes, the determination of biofilm production and antimicrobial susceptibility patterns among *P. aeruginosa* isolates and the correlation among them, and also to determine the prevalence of ESBLs and to detect the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} ESBL genes among clinical isolates of *P. aeruginosa*.

Materials and Methods

Specimen collection and bacterial identification

Between January and April 2022, 75 non-duplicate isolates of *P. aeruginosa* were collected from patients admitted to hospitals in Erbil City. Bacterial isolates were obtained from wounds, sputum, urine, burns, and stool. Each isolate was grown on MacConkey and Blood agar plates and incubated overnight at 37°C. Biochemical testing was used to identify isolates. Until further research, all bacterial isolates were kept at -20°C in a microtube containing tryptic soy broth (TSB), including 20% glycerol (11).

Susceptibility patterns for antibiotics

Susceptibility to antimicrobial agents was tested for the isolates using the Kirby-Bauer disk diffusion method on Muller-Hinton agar following Clinical and Laboratory Standards Institute (CLSI) guidelines (12). To summarize, a suspension of each isolate was adjusted to a turbidity of 0.5 McFarland and then inoculated onto a Muller-Hinton agar plate. Amikacin (30 µg), Amoxicillin-clavulanic acid (20+10 µg), Aztreonam (30 µg), Chloramphenicol (30 µg), Ceftazidime (30 µg), Ciprofloxacillin (5 µg), Meropenem (10 µg), Imipenem (10 µg), Gentamicin (10 µg), Cefotaxime (30 µg), Tetracycline (30 µg), Tetracycline (75 µg), and Tobramycin (10 µg) were used. After overnight incubation at 35°C, the plates were evaluated as susceptible, intermediate, or resistant using the CLSI-recommended criteria.

Phenotypic detection of ESBL

Extended-spectrum β-lactamases production in *P. aeruginosa* was detected by the double disk synergy test (DDST) described by Begum, Salam (13). Mueller Hinton agar was inoculated using a sterile cotton swab with standardized inoculum (corresponding to a 0.5 McFarland tube). An Augmentin (20 µg Amoxicillin and 10 µg of Clavulanic acid AMC) disk was placed in the center of the plate. Test disks of 3rd generation Cephalosporins (Ceftazidime CAZ 30 µg, Ceftriaxone CRO 30 µg, Cefotaxime CTX 30 µg) and Aztreonam (ATM 30 µg) disks were placed at 20 mm distance (center to center) from the Amoxicillin-Clavulanic acid disk before incubation. The plate was incubated overnight at 35°C. Enhancement of the zone of inhibition of any of the four-drug disks toward Amoxicillin-Clavulanic acid suggested the presence of ESBL. An increase of ≥5 mm in the inhibition zones of either cephalosporin in combination with clavulanic acid compared to the cephalosporin alone was interpreted as ESBL production (14).

Molecular identification of *P. aeruginosa*.

To confirm bacterial isolates as *P. aeruginosa*, all isolates were screened using specific primers listed in Table 1 for the presence of the 16S rRNA gene. The final volume of the PCR reaction was 25 µL, containing two µL of genomic DNA template, a 2× PCR master mix (AMPLIQON, Denmark). The 16S rRNA gene was amplified using the following protocol: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a single final extension of 7 min at 72°C. Amplification was performed with 10 µL of PCR products, separated in 1.5% agarose gel for 30 min at 120V.

DNA extraction and PCR

Genomic DNA from *P. aeruginosa* isolates was extracted from pure cultures grown in LB medium through the GeneAll® Exgene™ for Cell SV mini kit (Songpa-gu, Seoul, KOREA). By incubating the culture for 12–24 hours at 37°C with dynamic shaking until the cells enter the log phase, it is acceptable to develop bacterial cells. The collected bacterial cells could be used immediately for genomic DNA extraction. Electrophoretic analysis through a 1.5 % agarose gel examined the quality of extracted DNA from samples (15, 16).

Detection of virulence genes by PCR

The multiplex PCR assay was performed for amplification of the *phz*M, *exo*T, *exo*A, *exo*S, *plch*, *pvda*, *las*B, and *protease* genes performed in a 25 µL reaction mixture containing 12.5 µL of 2× PCR master mix (AMPLIQON, Denmark), 1.0 µL of each primer (10 pmol), 1.5 µL of genomic DNA template. The volume was completed to 25 µL with free nuclease water. Each gene was amplified separately. PCR products were visualized by electrophoresis using a 1.5% agarose gel stained with Red Safe dye (17).

Table 1. The primer sequences used in this study

Primer target	Oligonucleotide Sequence (5′-3′)	Amplicon size (bp)	T_m (°C)	References
16S rRNA	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	50	(18)
*exo*T	AATCGCCGTCCAACTGCA TGCG TGTTCGCCGAGGTACTGCTC	152	55	(19)
*exo*A	AACCAGCTCAGCCACATGTC CGCTGGCCCATTCGCTCCAGCGCT	207	55	(20)
plch	GAAGCCATGGGCTACTTCAA AGAGTGACGAGGAGCGGTAG	407	55	(20)
pvda	GACTCAGGCAACTGCAAC TTCAGGTGCTGGTACAGG	1281	55	(21)
*las*B	GGAATGAACGAAGCGTTCTC GGTCCAGTAGTAGCGGTTGG	300	55	(20)
protease	TATTTCGCCGACTCCCTGTA GAATAGACGCCGCTGAAATC	752	55	(20)
*phz*M	GCCTTCCATTGAGATCCCCAG CGAGATGGTTCGCTCGATCA	363	56	(20)
*exo*S	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504	58	(22)
bla_{TEM}	TCGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	60	(23)
bla_{SHV}	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	747	60	(24)
bla_{CTX-M}	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	593	60	(25)

Detection of ESBL genotypes by multiplex PCR

Multiplex PCR detected the ESBL bla_{SHV}, bla_{CTX-M}, and bla_{TEM} genes. Primers for the ESBL gene are shown in Table 1. All PCR reactions were performed by using a two µL DNA template (10 ng/µL), 1.5 µL of each primer, 25 µL of Master Mix, and 14 µL water nuclease-free in a final volume of 50 µL. For detection of ESBL genotypes by multiplex PCR, the program was carried out in a thermocycler according to the following protocol: initial denaturation (94 °C for 10 min) followed by 35 cycles of denaturation (94 °C for 30 sec), annealing (60 °C for 30 sec), extension (72 °C for 120 sec), and a final cycle of extension at 72 °C for 10 min (25). The PCR products were loaded on a 2% agarose gel at 70 V for one hr., and the banding patterns were visualized under ultraviolet illumination.

Phenotypic detection biofilm formation

A colorimetric microtiter plate-based assay was used for the evaluation of the formation of biofilm by a method described in detail by (26). Briefly, *P. aeruginosa* isolates were grown at 37 °C in TSB supplied with 1% glucose for 24 hrs. Then, a new TSB medium was used to dilute the bacterial suspensions (1:100). Next, 100 µL of the obtained dilution was added to each well flat-bottomed 96-well polystyrene microtiter plate and then incubated at 37 °C for 24 hrs. Following overnight incubation, phosphate-buffered saline (PBS) was applied to the washing wells, and the washing operation was repeated with PBS three times. Then, 100 µL of methanol was used to fix the solution. After 10 minutes, the wells were stained with 1% crystal violet (w/v) for 5 minutes. Afterward, 100 µL of absolute ethanol was added to the washed wells, and the biofilm formation was quantified by measuring optical density (OD_{570}) using an ELISA reader (BioTek TS800). For each isolate tested, biofilm assays were performed in triplicate, and the mean biofilm absorbance value was determined. TSB without bacteria was used as the negative control. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. According to the results of the microtiter plate test, the isolates were classified into the following four categories based on the optical density: non-biofilm producers (OD test<ODc), weak biofilm producers (ODc<OD<2×ODc), moderate biofilm producers (2×ODc<OD<4×ODc) and strong biofilms producers (4×ODc<OD) (27).

Statistical analysis

The GraphPad Prism software (San Diego, CA, USA version 9.0) was performed for statistical analysis. The correlation between the prevalence of the virulence gene, antibiotic resistance patterns, and biofilm production was determined using $Chi-square\ test$. A *p*-value less than 0.05 was considered statistically significant.

Results and Discussion

Pseudomonas aeruginosa, as an opportunistic pathogen, has different virulence factors that aid the bacteria in colonizing different niches in their host. The bacteria are a leading cause of nosocomial and community-acquired infections worldwide (28).

Bacterial isolates and epidemiological data

Seventy-five isolates of *P. aeruginosa* were recorded from 193 samples taken from the clinical specimens, including wounds, sputum, urine, burn, and stool, from patients admitted to different hospitals in Erbil city. Isolates were identified using cultural, morphological, and biochemical tests. In addition, the identities of the isolates were confirmed through PCR assay. The PCR technique was initiated by amplifying 16S rRNA, and the product was 956 bp, which confirmed *P. aeruginosa* isolates (Figure 1). The distribution of *P. aeruginosa* was isolated from samples according to sources of infection as follows: wounds (26; 34.67%), sputum (19; 25.33%), urine (13; 17.33%), burn (12; 16%), and stool (5; 6.67%). Amoon, Abdallha (29) identified 40 clinical isolates as *P. aeruginosa* by conventional methods, including growth characteristics, colony morphology, and biochemical tests. All tested isolates were confirmed by PCR assay using 16S rRNA species-specific genes.

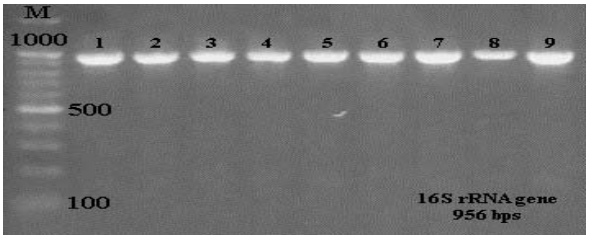

Figure 1. Electrophoresis image of 16S rRNA gene amplification (956 bps) for molecular identification of *P. aeruginosa* isolates from clinical specimens. M: DNA ladder (100 bp), 1-22: positive samples for 16S rRNA gene.

The assessment of the antibiotic susceptibility of the *P. aeruginosa* isolates

The current study has tested antimicrobial resistance against 12 different antimicrobial agents. The results of the antimicrobial susceptibility test against isolates of *P. aeruginosa* demonstrated that isolated bacteria differed in their susceptibility to the tested antimicrobials. Table 2 shows the patterns of antibiotic susceptibility of *P. aeruginosa* isolated from clinical specimens. The highest resistance was to Amoxicillin-clavulanate (100% in clinical isolates). The lowest resistance was to Meropenem (24.0%), Aztreonam (32.0%), Amikacin, and Gentamicin (33.0%) in clinical samples. Results indicated that there was sensitivity toward Imipenem in all clinical isolates.

A broad group of *P. aeruginosa* strains is resistant to various antibiotics or antibacterial agents, making it difficult to control the infection (30). According to the results of Bahador, Shoja (31), a high rate of resistance was observed for tetracycline (32.85%) and Ofloxacin (30%). A low resistance was observed for Colistin (1.42%). One isolate (1.42%) was resistant to all the tested antibiotics. In addition, 58.6% of the isolates were sensitive to all the antimicrobial agents. In total, 24.3% of the isolates showed resistance to at least three different classes of antimicrobial agents and were identified as MDR. Zarei, Shokoohizadeh (32) stated that the isolates of *Pseudomonas aeruginosa* showed high-level resistance to many antimicrobial agents. The high rates of clinical and environmental *P. aeruginosa* isolates show the multidrug resistance (MDR) phenotype. The antibiotic susceptibility pattern of *P. aeruginosa* showed that 45% and 37.5% of clinical and environmental isolates were resistant to more than three antibiotics from different classes. However, if more antibiotics were checked, MDR isolates also would have been increased. According to our results, carbapenems (e.g., Imipenem and Meropenem), ciprofloxacin, gentamicin, and piperacillin did not have an effective activity against *P. aeruginosa* isolates.

Table 2. Antibiotic susceptibility patterns of clinical *P. aeruginosa* isolates.

Antibiotics	Symbol	Susceptibility rate of isolates *n*. = 75	
		S *n*. (%)	R* *n*. (%)
Amikacin	AK	42(56.0)	33(44.0)
Amoxicillin-clavulanic acid	AMC	0(0.0)	75(100.0)
Aztreonam	AZT	51(68.0)	24(32.0)
Cefotaxime	CFX	18(24.0)	57(76.0)
Ceftazidime	CTZ	24(32.0)	51(68.0)
Chloramphenicol	C	2(2.67)	73(97.33)
Ciprofloxacin	CIP	28(37.33)	47(62.67)
Gentamycin	CN	42(56.0)	33(44.0)
Imipenem	IMP	75(100.0)	0(0.0)
Meropenem	MEM	57(76.0)	18(24.0)
Tetracycline	TE	15(20.0)	60(80.0)
Tobramycin	TOB	40(53.33)	35(46.67)

*R: resistant; S: susceptible.

Based on the CLSI interpretive criteria (12), the resistance rate among *P. aeruginosa* isolates to antibiotics tested was as follows: Imipenem 22.5% (n = 18), Meropenem 15% (*n*. = 12), Gentamicin 18.75% (*n*. = 15), Teicoplanin 16.25% (*n*. = 13), Amikacin 12.5% (*n*. = 10), Ciprofloxacin 20% (*n*. = 16), Levofloxacin 23.75 (*n*. = 19), Ceftazidime 17.5% (*n*. = 14), and Piperacillin/Tazobactam 12.5% (*n*. = 10). The prevalence of MDR-PA and non-MDR-PA was 20% (*n*. = 16) and 80% (*n*. = 64), respectively.

Detection of ESBL by double-disc synergy test (DDST)

Extended-spectrum β-lactamase (ESBL) was achieved for all isolates with the use of antibiotics (Amoxicillin-clavulanic acid, Cefotaxime, Ceftazidime) in the double-disc synergy test (DDST). Of 75 *P. aeruginosa* isolates, ESBL producers represented 52 (69.33%), while non-ESBL producers accounted for 23 (30.67%). The prevalence of ESBL-producing isolates among sources of isolates was different; the highest rate was in wound isolates (*n*. = 19, 73.08%). In contrast, the lowest rate was recorded among the stool isolates (*n*. = 3, 60%), as in Table 3.

Laudy, Róg (33) tested the ESBL test through the DDST method among the 900 *P. aeruginosa* isolates studied. Initially, they were described as resistant to third-generation cephalosporins in the case of 720 isolates (80%). The disc diffusion method confirmed resistance to Ceftazidime and Cefepime. In 180 out of 900 (20%) isolates, the inhibition zones around the discs with cephalosporins were similar to the zone diameter breakpoints. In at least one of the phenotypic assays, ESBL-type enzyme production was detected in 110 out of 720 isolates (15%). The most significant number of ESBL-positive isolates was found using the DDS-SAM test (92 out of 110 isolates) and CDT with Ceftazidime, DDS-AMC, and DDS-IMP (77, 76, and 75 isolates, respectively). They found that the largest group of 79 out of 110 ESBL-positive isolates were resistant to three antibiotics: CAZ, FEP, and ATM. The second group, 21 out of 110, consisted of isolates resistant to CAZ and FEP and simultaneously sensitive to ATM.

Table 3. Rate of detection of *P. aeruginosa* and ESBL-positive strains from clinical specimens

Specimens	*P.* aeruginosa *n*. (%)	ESBL-positive *n*. (%)
Wound swab	26 (34.67)	19 (73.08)
Sputum	19 (25.33)	13 (68.42)
Urine	13 (17.33)	8 (61.54)
Burn	12 (16)	9 (75)
Stool	5 (6.67)	3 (60)
Total	**75**	**52 (69.33)**

During the seven years, two hundred and forty *P. aeruginosa* isolates were recovered from hospitalized patients in the affiliated Jilin region by Chen, Niu (34). Among all isolates, 91 strains were isolated from wounds at burn wards and 149 from surgical wards. They found that 210 strains (87.5%, 210/240) produced ESBLs, and 30 strains (12.5%, 30/240) did not produce ESBLs. In another study in a tertiary care hospital in China, 63.5% of *P. aeruginosa* were ESBL producers. In contrast, the frequency of ESBL production by *P. aeruginosa* in the hospital

was more than those isolates, nearly to Woodford reporter (35). In a survey by Yu et al. in 2007 in a general hospital in China, 59.2% of isolates were ESBLs positive, and all isolates were susceptible to Imipenem (36); our finding also showed all strains had sensitivity toward Imipenem. Among the 900 isolates of *P. aeruginosa* studied by Laudy, Róg (33), initially described as resistant to third-generation cephalosporins, in the case of 720 isolates (80%), resistance to Ceftazidime or/and Cefepime was confirmed by the disc diffusion method. In 180 out of 900 (20%) isolates, the inhibition zones around the discs with cephalosporins were similar to the zone diameter breakpoints. In at least one of the phenotypic assays, ESBL-type enzyme production was detected in 110 out of 720 isolates (15%). The most significant number of ESBL-positive isolates was found using the DDS-SAM test (92 out of 110 isolates) and CDT with Ceftazidime, DDS-AMC, and DDS-IMP (77, 76, and 75 isolates, respectively). The information describes the detection of ESBL-positive *P. aeruginosa* isolates by different phenotypic tests concerning the resistance profiles of the studied strains. The largest group, 79 out of 110 ESBL-positive isolates, were resistant to three antibiotics: CAZ, FEP, and ATM. The second group, 21 out of 110, consisted of isolates resistant to CAZ and FEP and, at the same time, sensitive to ATM. The most significant percentage of ESBL-positive strains between these two groups were detected using the DDS-SAM test, 93.7% (74 out of 79) and 71.4% (15 out of 21) isolates. Interestingly, for some of the first group of strains resistant to CAZ, FEP and ATM, it was necessary to conduct tests in the presence of boronic acid, especially in the case of DDS-AMC (31.6%, 18 out of 57 ESBL-positive isolates) and CDT with Cefepime (56.3%, 18 out of 32). Generally, for most ESBL-positive isolates, a clean extension of the inhibition zone of Ceftazidime and Cefepime towards discs with AMC, SAM, TZP, or IPM was observed. However, unlike Cefepime, when using discs with Ceftazidime in the extended CLSI confirmatory disc diffusion test, a large percentage of ESBL-positive isolates, 82.3% (65 out of 79) in the first group and 52.4% (11 out of 21) in the second group was observed.

Detection of virulence genes by PCR

This research was conducted for 75 isolates of *P. aeruginosa*, which were recovered from different clinical specimens for molecular detection of eight virulence genes (Table 4), two of which were performed using conventional PCR to test the existence (*phz*M and *exo*S) of amplicon-sized genomic DNA (363 bp and 504 bp), respectively. The findings showed that *phz*M is represented in 68 (90.67%) isolates, and *exo*S is expressed as virulence genes in 62 (82.67%), as demonstrated in Figure 2. The other six genes were screened by using multiplex PCR to check the presence of (*exo*T, *exo*A, *plch, pvda, Las*B, *and protease)* as virulence genes with amplicon sizes (152 bp, 207 bp, 407 bp, 1281 bp, 300 bp, and 752 bp), respectively. The results showed that *pvda* was (43, 57.33%, *las*B (58. 77.33%), *protease* (49, 65.33%), *exo*A (43, 57.33), *exo*T (53, 70.76%), *and plch* (38, 50.67%), as shown in Figure 3.

Table 4. Distribution of virulence genes among different clinical specimens of *P. aeruginosa* isolates.

Specimens	Virulence gene n. (%)							
	*exo*T	*exo*A	*plch*	*pvda*	*las*B	*protease*	*phz*M	*exo*S
Wound swab (26)	14 (53.85)	18 (69.23)	11 (42.31)	13 (50.0)	20 (76.92)	15 (57.69)	24 (92.31)	22 (84.62)
Sputum (19)	16 (84.21)	11 (57.89)	9 (47.37)	12 (63.16)	16 (84.21)	13 (68.42)	17 (89.47)	18 (94.74)
Urine (13)	11 (84.62)	5 (38.46)	7 (53.85)	10 (76.92)	10 (76.92)	9 (69.23)	11 (84.62)	8 (61.54)
Burn (12)	7 (58.33)	6 (50.0)	9 (75.0)	5 (41.67)	8 (66.67)	11 (91.67)	11 (91.67)	9 (75.0)
Stool (5)	5 (100.0)	3 (60.0)	2 (40.0)	3 (60.0)	4 (80.0)	1 (20.0)	5 (100.0)	5 (100.0)
Total (75)	53 (70.67)	43 (57.33)	38 (50.67)	43 (57.33)	58 (77.33)	49 (65.33)	68 (90.67)	62 (82.67)

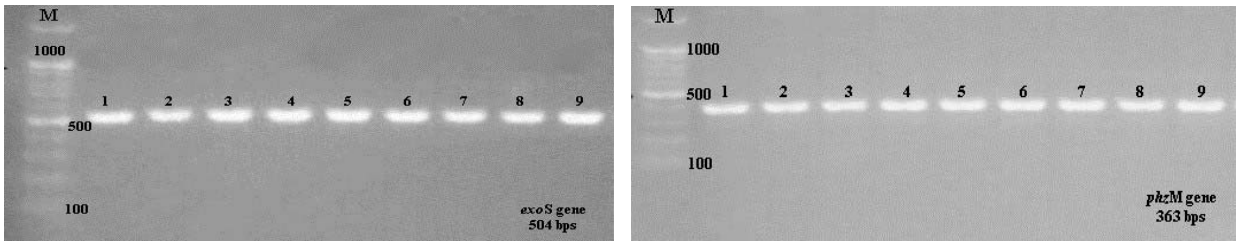

Figure 2. PCR amplification of *exo*S *and phz*M *virulence* genes of *P. aeruginosa*, lanes 1-9 represent the amplified product (504 and 363 bps) of *P. aeruginosa* isolates, M: ladder 100 bp.

In the study of Faraji, Mahzounieh (2), they screened clinical isolates for the prevalence of different virulence genes of *P. aeruginosa*. Interestingly, *tox*A, *las*B, and *exo*S genes had a higher occurrence in *P. aeruginosa* isolated from patients with CF. Forty-one (63.1%) and 21 (36.9%) of *P. aeruginosa*, isolated from CF and burn wound infections, possessed the *tox*A gene. Sixthly two (95/ 4%) and 47 (82%) of the isolated bacteria from CF and burn wounds had the *las*B gene, and 46 (70/8%) and 12 (21/1%) of *P. aeruginosa* isolated from CF and burn wounds possessed *exo*S gene. The PCR results of Al-Dahmoshi, Al-Khafaji (37) for virulence factor genes among clinical isolates of *P. aeruginosa* and the occurrence of their works showed that *exo*A was present

among 12 (46.15%), *opr*L was 11 (42.3%), *opr*I was 22 (84.61%), *las*I was 14 (53.84%), and *las*B was 18 (69.23%). At least ten virulence genes were studied by Alonso, Fernández-Barat (38), and they recorded that *alg*D, *las*B, *plc*N, *plc*H, and *exo*T were present in all the strains, while *Apr, las*I, *las*R, and *exo*A were found in 99, 98, 97, and 93% of the strains, respectively. A lower incidence was obtained for *rhII* (82%), *rhRI* (81%), *algU* (82%), and *exoS* (76%). The *exoU* was present in only 28 (31.1%) strains. One strain presented *lasI* but not *lasR*. The same phenomenon was observed in another strain where *rhII* was present, although its regulatory gene was absent. The distribution of virulence genes did not differ significantly between cases and controls (p-value > 0.05). The outcomes of Benie, Dadie (39) revealed that all the virulence genes studied were detected in the 100 strains of *P. aeruginosa*. According to their findings, the *las*B gene, with 89.0%, has been most detected, followed by *exo*S (84.0%). The prevalence of *alg*D and *plch* genes was 73.0% and 71.0%, respectively.

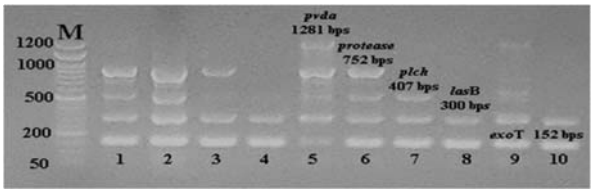

Figure 3. Multiplex PCR amplification of *exo*T, *exo*A, *plch, pvda, Las*B, *and protease virulence* genes of *P. aeruginosa*, lanes 1-10 represent the amplified product (152, 207, 407, 1281, 300, and 752 bps) of *P. aeruginosa* isolates, M: ladder 100 bp.

Genotypic detection of ESBL by multiplex PCR amplification

In PCR detection of ESBL genotypes, it was found that all of the ESBL screening positive *P. aeruginosa* isolates had one or more ESBL genes tested in the present study. Overall, all ESBL genes were beard by isolates of *P. aeruginosa* and were distributed in variable ranges among clinical specimens of isolates (Figure 4).

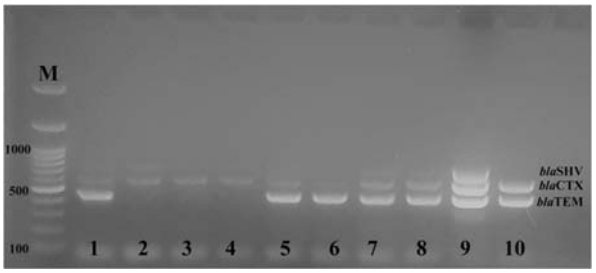

Figure 4. Multiplex PCR assays for screening of ESBL encoding genes from *P. aeruginosa* isolates.

The multiplex PCR assay results indicated that the ESBL genes among wound isolates were (11/26) 42.31% bla_{CTX-M} genes (593 bp), (6/26) 23.08% bla_{SHV} genes (747 bp), and (14/26) 53.85% of bla_{TEM} genes (445 bp) were detected in the *P. aeruginosa* isolates, which revealed that the most abundant distributed sources of ESBL genes followed by sputum, burn, urine and the lowest distributed sources of ESBL genes was the stool source (Table 5).

Table 5. Distribution of ESBL genotypes among isolates of *P. aeruginosa*

Specimen sources (n.)	ESBL-positive n. (%)		
	bla_{TEM}	bla_{SHV}	bla_{CTX-M}
Wound swab (26)	14 (53.85)	6 (23.08)	11 (42.31)
Sputum (19)	9 (47.37)	3 (11.54)	4 (15.38)
Urine (13)	5 (38.46)	2 (7.69)	6 (23.08)
Burn (12)	5 (41.67)	2 (7.69)	7 (26.92)
Stool (5)	2 (40.0)	1 (3.85)	3 (11.54)
Total (75)	35 (46.67)	14 (18.67)	31 (41.33)

Of 204 *P. aeruginosa* isolates were collected by Hosu, Vasaikar (40), and 82 isolates were tested for genotypic detection of ESBL and MBL. The consequences revealed that ESBL—genotypic resistance is driven by bla_{TEM} (79.3%), followed by bla_{SHV} (69.5%), and lastly, bla_{CTX-M} (31.7%). The most common ESBL-genotype combination among *P. aeruginosa* was a combination of $bla_{TEM} + bla_{SHV}$ (40.5%). To confirm the phenotypic method-based identification of β-lactamases, Nasser, Ogaili (41) performed a multiplex PCR-based amplification for the detection of ESβLs, MβLs, Amp-C genes undertaken using specific primers of each β-lactamase. Of the 65 studied, MDR *P. aeruginosa* isolates 43% (n = 28), 30.7% (n = 20), 30.7% (n = 20), 24.6% (n = 16), and 12.3% (n = 8) isolates were identified with VEB, GES, CTX-M, OXA-10, TEM, and SHV genes, respectively.

The frequencies of occurrence of virulence genes in all studied strains were done by Mitov, Strateva (42) and included (n=202) were as follows: *alg*D – 91.1%, *pil*B – 23.8%, *nan1* – 21.3%, *las*B – 100%, *plch* – 91.6%, *exo*S – 62.4%, and *exo*U – 30.2%. Elmouaden, Laglaoui (43) performed PCR analysis to screen five virulence-encoding genes (*las*B, *alg*D, *plch*, *exo*A, and *exo*S). The results highlighted that *las*B (98.7%) and *exo*S (98.7%) were the most frequent virulence genes in *P. aeruginosa* strains, followed by *plc*h (96.1%) and *alg*D (87.7%). The least commonly detected virulence factor gene was *exo*A (74.2%).

Phenotypic detection biofilm formation

The mean of OD_{570} in the microplate readings after crystal violet staining ranged from 0.359 to 0.914. The mean of NC was 0.0717, and the ODc of biofilm formation was recorded as 0.101. The strains were divided into four groups: non–biofilm producer (−), $OD_{570} \leq 0.101$; weak biofilm producer (+), $0.101 < OD_{570} \leq 0.203$; moderate biofilm producer (++), $0.203 < OD_{570} \leq 0.407$; strong biofilm producer (+++), $0.407 \leq OD_{570}$. Our outcomes revealed that 89.33% of all *P. aeruginosa* isolates were positive for biofilms production, of which 50.67% were recorded as strong producers of biofilms (n=38), 22.67% as moderate producers of biofilms (n=17) and 16% as a weak producer of biofilms (n=12). Only 10.67% (n=8) of isolates were stated as non-biofilm producers (Table 6).

Table 6. Screening of *P. aeruginosa* isolates from biofilm production by MTP assay

Specimen sources (n.)	Biofilm status			
	Strong former	Moderate former	Weak former	None former
Wound swab (26)	15 (57.69)	7 (26.92)	3 (11.54)	1 (3.85)
Sputum (19)	10 (52.63)	4 (21.05)	2 (10.53)	3 (15.79)
Urine (13)	6 (46.15)	3 (23.08)	4 (30.77)	0 (0.0)
Burn (12)	5 (41.67)	2 (16.67)	3 (25.0)	2 (16.67)
Stool (5)	2 (40.0)	1 (20.0)	0 (0.0)	2 (40.0)
Total (75)	**38 (50.67)**	**17 (22.67)**	**12 (16)**	**8 (10.67)**

The prevalence rates of virulence genes among biofilm-producing isolates of *P. aeruginosa* are listed in Table 7. The positive rates of virulence genes identified in the biofilm-producing isolates were significantly higher than those in the non-producing isolates (p-value=0.005). Furthermore, except for *exo*T and *pvda* genes, the six remaining genes tested in the moderate biofilm-producing isolates were higher than those in the strong and weak biofilm-producing isolates. All eight genes tested in this research showed the lowest prevalence rates among the non-producers. Biofilm production was significantly associated with the expression of all virulence genes investigated in this work. In addition to biofilm formation, the principal virulence factors of *P. aeruginosa* are elastase, phospholipase C, protease A, exotoxins and cytotoxins, flagella and pili, pigment production, and QS regulatory system proteins, which regulate both virulence factor transcription and biofilm formation (44). Although it is well established that these proteins cause lung damage during infection with *P. aeruginosa*, their importance as virulence factors in VAP is unknown (45).

The antibiotic susceptibility patterns of the biofilm-producing and non-producing isolates of *P. aeruginosa* are shown in Table 8. Both biofilm producers and non-producers were highly resistant to Amoxicillin-clavulanic acid and moderately resistant to cefotaxime. Of the 12 antibiotics, Amikacin, Ciprofloxacin, Tetracycline, Cefotaxime, and Gentamicin were resistant to non-biofilm producers. A resistance of 58.67% was observed for the biofilm-producing isolates to Amoxicillin-clavulanic acid, whereas a resistance of 100.0% was noticed for the non-producing isolates of Amoxicillin-clavulanic acid. Isolates showing resistance to Amikacin, Aztreonam, Ciprofloxacin, Tetracycline, Cefotaxime, and Ceftriaxone produced more biofilm than strains that did not make a biofilm that exhibited resistance to these antibiotics. Biofilm production was significantly associated with resistance to antibiotics (p-value=0.001).

Table 7. Prevalence of virulence genes within biofilm-forming isolates of *P. aeruginosa*.

Virulence genes	Biofilm forming status: Strong biofilm producer $n = 38$	Moderate biofilm producer $n = 17$	Weak biofilm producer $n = 12$	Non-biofilm producer $n = 8$	p-value
*exo*T	29 (76.32)	12 (70.59)	8 (66/67)	4 (50.0)	0.005
*exo*A	22 (57.89)	11 (64.71)	6 (50.0)	4 (50.0)	
plch	20 (52.63)	9 (52.94)	5 (41.67)	4 (50.0)	
pvda	25 (65.79)	11 (64.71)	5 (41.67)	2 (25.0)	
*las*B	35 (92.11)	16 (94.12)	6 (50.0)	1 (12.5)	
protease	26 (68.42)	14 (82.35)	7 (58.33)	2 (25.0)	
*phz*M	35 (92.11)	17 (100.0)	11 (91.67)	5 (62.5)	
*exo*S	31 (81.58)	15 (88.24)	10 (83.33)	6 (75.0)	

The frequency rates of MBL- and ESBL-producing isolates in the study of Rahimi, Asgari (46) were 56 (65.9%) and 60 (70.6%), respectively. Among all the isolates, 81 (95.3%) had at least one of the studied genes. Data analysis showed that the presence of the ESBL genes, namely bla_{TEM}, bla_{SHV}, and bla_{CTX}, this association was observed for cephalosporins (p-value < 0.007), including Cefepime, Cefotaxime, and Ceftazidime. On the other hand, the frequency of these genes among *P. aeruginosa* strains isolated from the surgery and internal wards was low. Moreover, the frequency of bla_{VIM} and bla_{TEM} genes among *P. aeruginosa* strains isolated from wound and urine samples was high.

Table 8. Antibiotic resistance pattern of the biofilm-producing and non-producing *P. aeruginosa* isolates

Antibiotic	Resistant of biofilm producer (n = 67)	Resistant of non-biofilm producer (n = 8)	p-value
Amikacin	41 (54.67)	6 (75.0)	0.001
Amoxicillin-clavulanic acid	44 (58.67)	8 (100)	
Aztreonam	39 (52.0)	2 (25)	
Cefotaxime	36 (48.0)	5 (62.5)	
Ceftazidime	27 (36.0)	3 (37.5)	
Chloramphenicol	29 (38.67)	3 (37.5)	
Ciprofloxacin	42 (56.0)	6 (75.0)	
Gentamicin	24 (32.0)	5 (62.5)	
Imipenem	17 (22,67)	0 (0.0)	
Meropenem	19 (25.33)	1 (12.5)	
Tetracycline	39 (52.0)	7 (87.5)	
Tobramycin	18 (24.0)	3 (37.5)	

Of the 90 strains studied by Alonso, Fernández-Barat (38), they found that 76 (84.4%), 13 (14.5%), and 1 (1.1%) strains were high, moderate, and low biomass producers, respectively. Quantitative biofilm determination using the microtiter assay (Ghanbarzadeh Corehtash et al. (2015) for detection of biofilm formation. The obtained data revealed that 133 isolates (92.4%) were biofilm, and the remaining 11 were non-biofilm producers.

The statistical analysis to examine the link between antibiotic resistance and biofilm formation showed that the biofilm production in MDR *P. aeruginosa* isolates was significantly higher than that in the non–MDR *P. aeruginosa* isolates (p-value<0.001). Ratajczak, Kamińska (47) analyzed the prevalence of different resistance phenotypes among the strains with varying biofilm-forming abilities. They revealed that all strains with the resistance phenotype MDR belonged to the group of strong biofilm producers. They accounted for 22.2% of strains with strong biofilm-forming capacity. Furthermore, strains with the resistance phenotype LDR represented 63.0%, and strains showing sensitivity to all antibiotics – 14.8%. Regarding the group of moderate biofilm producers, 58.3% of strains represented the resistance phenotype LDR, while 41.7% were sensitive to all antibiotics studied. Among the weak biofilm producers, 42.9% of strains had the LDR phenotype, while 57.1% were susceptible to all antibiotics. The results were found to be statistically significant (p-value=0.0215).

Conclusion

Based on the correlation of study findings, it was observed that the biofilm-forming ability was significantly higher among strains with the resistance phenotype. It was also found that among the ESBL-producing strains, there was only one weak biofilm producer, while the remaining strains exhibited a strong biofilm-forming ability. Resistance to antimicrobial agents and the ability to grow as a biofilm are the main problems in treating infections triggered by *P. aeruginosa*. The high degree of this resistance, growth in the biofilm form, and various virulence factors are the reasons for difficulties in managing infections caused by *P. aeruginosa*. The ability of *P. aeruginosa* to grow as a biofilm is believed to explain the weak relationship between antibiotic sensitivity under *in vitro* conditions and clinical response. A better understanding of the genes and mechanisms involved in biofilm formation by *P. aeruginosa* strains and gaining insights into its structure can assist in developing new therapies to eliminate biofilm formation.

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