Original Article

Virulence Factors and Biofilm Formation in Multi-drug Resistant *Pseudomonas* Aeruginosa

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Abstract

Background: Multi-Drug resistant (MDR) Pseudomonas aeruginosa is the most common cause of of nosocomial infections, several virulence factors, including exoenzymes, exotoxins, and biofilm formation used by the organism to establish its pathogenicity.

Aim of the work: To evaluate the distribution of different virulence genes and biofilm formation among MDR Pseudomonas aeruginosa isolated from different clinical microbiological samples.

Patients and methods: A total of 117 Multi-Drug resistant Pseudomonas aeruginosa isolates were screened for the presence of eight virulence factors by using conventional PCR and for biofilm formation using colourimetric microtitre method.

Results: among MDR Pseudomonas Aeroginosa AprA gene was found in 53% isolates, the same percentage was recorded in Exo S gene. ExoU, ToxA, PlcN genes were recorded to present in 59.8%, 97.4% and 8.5% respectively. However, PlcH gene was found in 36.8% isolates and LasB in 47.9% of isolates. 113 isolates were biofilm producers, 69 isolates were strong biofilm producers, 34 isolates were moderate biofilm producers and 10 isolates were weak biofilm producers. Also we recorded that 29(25%) isolates were positive to more than 6 genes. Regarding genotypic pattern of biofilm producers in MDR Pseudomonas aueroginosa isolates, AprA gene was detected among 59(52.2%) isolates, ExoU, ExoS, AlgD genes were recorded to present in 66(58.4%), 61(53.9%) and 108(95.6%) isolates respectively.

Conclusion: AlgD, ToxA and ExoU genes were the most predominant among MDR p.aeuroginosa isolates. Moreover, they recorded the highest percentage among biofilm producer isolates. Antibiotic resistance against pefloxacin, rifampicin, minocyclin, gentamicin and aztereonam were high, while resistance against tigecyclin and colistin were very low.

Keywords: Virulence Factors, Biofilm Formation, Pseudomonas Aeruginosa

INTRODUCTION

Pseudomonas aeruginosa has become a leading cause of nosocomial infections People with severe burns or HIV infection are more vulnerable to infection by this bacterium. **(1).**

Ventilator-associated pneumonia, cystic fibrosis, meningitis, abscess, skin and soft tissue, urinary tract, bone and joint, bacteraemia, corneal, and other infections are all caused by *P. aeruginosa*. In some cases, it causes fatality rates of 50% or higher. P. aeruginosa is already resistant to a wide variety of antibiotics, and it can acquire additional resistance by actively mutating its genetic makeup. **(2).**

P. aeruginosa produces many virulence factors, such as enzymes, poisons, and the ability to build biofilms. In order to confer resistance to immune clearance and antibacterial therapy, bacteria form biofilms, which are sessile microbial territories covered by an extracellular polysaccharide substance that allows the cells' permanent attachment to the substructure or each other. **(3).**

At least three different exopolysaccharides make up P. aeruginosa's biofilm components; alginate, a linear polymer, plays a crucial role in the structural stability and protection of biofilm. Alginate production and transcription of the Alg proteins are regulated through the algD gene. **(4).**

Exotoxin A, exoenzyme S, exoenzyme U, alkaline protease, and las B elastase are all produced by P. aeruginosa and are thought to be very important virulence factors. These proteins are encoded by the *toxA, exoS, exoU, aprA, and lasB* genes. Phospholipases C, which are encoded by plcH and plcN genes, can also hydrolyze phospholipids. **(5).**

ExoA is the primary inhibitor of protein production by the type II secretion system (T2SS). ExoS is a key cytotoxin essential for bacterial colonisation, invasion, and infection spread. Lung infection is caused by exoU because it is a cytotoxin with

phospholipase activity that targets epithelial cells. **(6)**.
For example, the zinc metalloprotease lasB elastase assaults eukaryotic proteins like elastin and collagen, while alkaline protease is crucial for bacterial survival in tissues because it inhibits Toll-like receptor 5 activation and degrades host proteins including cytokines and complements. **(7)**.
The purpose of a recent study was to assess the prevalence of several virulence genes among multidrug-resistant P. aeruginosa isolated from a variety of clinical microbiological samples.
The aim of this study was to evaluate the distribution of different virulence genes and biofilm formation among MDR Pseudomonas aeruginosa isolated from different clinical microbiological samples.

MATERIALS AND METHODS

This study was cross sectional study done in Clinical Pathology Department in Zagazig University Hospitals, 117 Egyptian Patients were included. There were 59 (50.4%) male patients and 58(49.6%) female patients with age range from 31 to 70 years old for male patients and from 30 to 69 years old in female patients.

Ethical Issues

The study protocol was approved by the Institutional Review Board of Zagazig University (approval no.IRB#:6078-21-4-2020 Samples were collected over the period of 24 months between May 2020 and May 2022. A written informed consent was obtained from every patient or his caregiver. The study was approved by the ethical committee of faculty of medicine, Zagazig university. A total of 117 Multi-Drug resistant (MDR) Pseudomonas aeruginosa isolates collected from 83 urine samples, 14 wound samples, 12 sputum samples, 4 blood samples, 2 CSF samples, 1 peritoneal fluid sample and 1 pleural fluid sample. Patients receiving antibiotic treatment were excluded . The collected samples were subjected to :

1- Routine culture on blood and macConkey agar
2- Isolation and Identification of *Pseudomonas aeruginosa* by Vitek 2 compact (BioMerieux Ltd)
3- Detection of MDR *Pseudomonas aeruginosa* isolates by the automated broth microdilution system (Vitek 2 compact, BioMerieux Ltd).
4- Detection of toxA, exoU, exoS, aprA, lasB, plcH and plcN genes by conventional PCR through the following steps:
5- Detection of biofilm forming isolates using a colorimetric microtitre method.
6- Detection of algD gene by conventional PCR.

Molecular detection of different virulent genes from different microbiological samples:

DNA Extraction, Identification and Virulence Gene Detection **(8)**

Using a boiling method, genomic DNA was extracted as follow; Bacteria cultured overnight on Blood and Mackonkey agar plates, one loopful of fresh bacterial isolate was taken and suspended in 200 μL of sterile DNase/RNase-free water and boiled for 10 min. Then, suspension of bacteria was centrifuged at 12,000 rpm at 4°C for 10 min, followed by collection of the supernatant and storing it at −20°C **(9).** By using conventional PCR, the following virulence genes were detected: alkaline protease (aprA), elastase (lasB), GDPmannose dehydrogenase enzyme for alginate (algD), exoenzyme S (exoS), exoenzyme U (exoU), exotoxin A (toxA), hemolytic phospholipase C (plcH) and non-hemolytic phospholipase C (plcN). Primers used are listed in Table 1. The step of amplification was done in a 25μL volume, containing 12.5 μL PCR Master mix (DreamTaq Green PCR master mix, Thermo scientific), 1μL of each primer (forward and reverse), 1 μL of template DNA and nuclease-free water. Conditions of PCR for lasB and aprA were optimized to be as follow: Denaturation at 94°C (4 mins), followed by 25 cycles of: 94°C (1 min), 46°C (40 s), 72°C (1 min) and a final extension at 72°C (2 mins). While, amplification conditions of exoS and exoU comprised denaturation at 95°C (5 mins), followed by 35 cycles of 95°C (1 min), 55°C (1 min), 72°C (1 min) followed by final extension at 72°C (10 mins) **(10,11).** As regard remaining genes, the conditions were as follows: primary denaturation at 94°C (5 min), 35 cycles of denaturation at 94°C (60 s), annealing at 48°C (60 s) and extension at 72°C (90 s), with a last extension cycle at 72°C for 10 min **(12).** PCR yields were run on 1.5% agarose gel and then they were visualized under UV lamb.

Amplification of the extracted DNA for identification of different virulent genes :

Table (1): Sequences of primers used in PCR:

Product Size	Annealing NEB	Annealing Thermo	Sequence	Primer
352	52	56.7	F: 5 GGTAACCAGCTCAGCCACAT 3 R: 5 TGATGTCCAGGTCATGCTTC 3	ToxA
466	53	57.5	F: 5 GTTATCGCAACCAGCCCTAC 3 R : 5 AGGTCGAACACCTGGAACAC 3	PlcN
307	52	56.9	F : 5 GAAGCCATGGGCTACTTCAA 3 R : 5 AGAGTGACGAGGAGCGGTAG 3	PlcH
1311	52	58.9	F : 5 ATGCGAATCAGCATCTTTGGT 3 R : 5 CTACCAGCAGATGCCCTCGGC 3	AlgD
665	60	67.7	F : 5 CCAGCCCGCTGACCCACAAGCTGTA 3 R : 5 CATTCCTTCCTGGAGTGCYRGCCG 3	LasB
1609	52	58.3	F : 5 CCTGATCKGGCCGATAACTGCAAT 3 R : 5 GGAAGACASCTATCAATTCGAACAG 3	AprA
761	55	62.7	F : 5 GGCACATATCTCCGGTTCCTTC 3 R : 5 TCAACTCAGCTGCCAACCATGC 3	ExoU
1587	54	58.7	F : 5 ATGGCGTGTTCCGAGTCA 3 R : 5 AGGTGTCGGTTCGTGACGTCT 3	ExoS

Biofilm Formation

Using a colorimetric microtiter technique, the ability to produce biofilm was assessed in all isolates. Bacterial colonies were cultivated overnight at 37°C in Tryptic Soy Broth (TSB) in order to quantify biofilm development (Merck Darmstadt, Germany). A fresh TSB medium was used to dilute the bacterial suspensions (1:100), and 150 μL of this solution was used to inoculate the sterile flat-bottomed 96-well polystyrene microtiter plates. The wells were gently cleaned three times with distilled water after being incubated for 24 hours at 37°C. The wells were dried inverted at room temperature before being stained for 10 to 15 minutes with 125 L of a 0.1% crystal violet solution (CV) in water. Crystal violet was thrown away, and additional crystal violet was removed by washing the wells three times. Finally, 125 μL of 30% acetic acid was added to liberate the bound CV. A new sterile plate was inoculated with 125 μL distaining solutions in each well. Using an ELISA reader, the optical density (OD) absorbance of each well was determined at 550 nm (Biotek elx800). All procedures were repeated three times. The background OD was calculated using the uninoculated media as a control. The cut-off OD (ODc) was known as three standard deviations above the mean OD of the negative control. Regarding the results of the microtiter plate test, the isolates were divided into four groups based on the optical density: non-biofilm producers (OD test <ODc), weak biofilm producers (ODc< OD < 2× ODc), moderate biofilm producers (2× ODc< OD < 4X ODc) and strong biofilms pro-ducers (4× ODc< OD) **(13)**

Statistical Analysis

Data were collected throughout history and basic clinical examination; outcome measures were coded, entered, and analyzed using Microsoft Excel software. Data were then imported into the Statistical Package for the Social Sciences (SPSS version 20.0) (Statistical Package for the Social Sciences) software for analysis.

RESULTS

This study included 117 Multi-Drug resistant (MDR) Pseudomonas aeruginosa isolates. The antimicrobial susceptibility profile of the MDR isolates to different antibiotics is illustrated in figures 1,2.

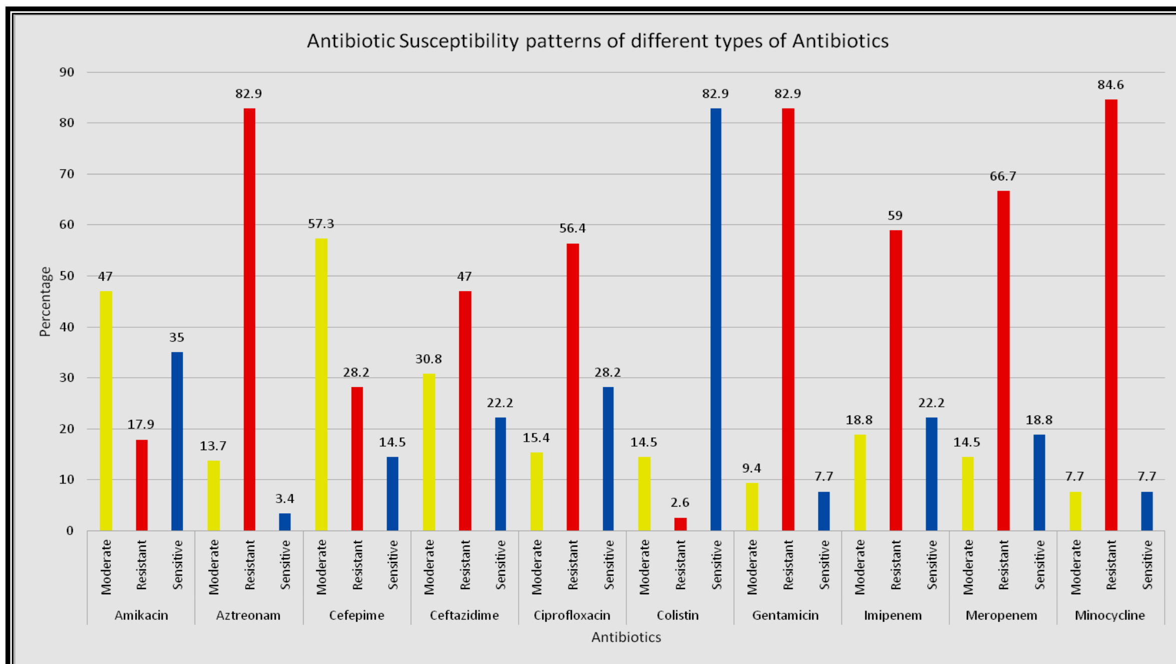

Figure (1): Antibiotic susceptibility patterns of Pseudomonas aueroginosa in clinical isolates

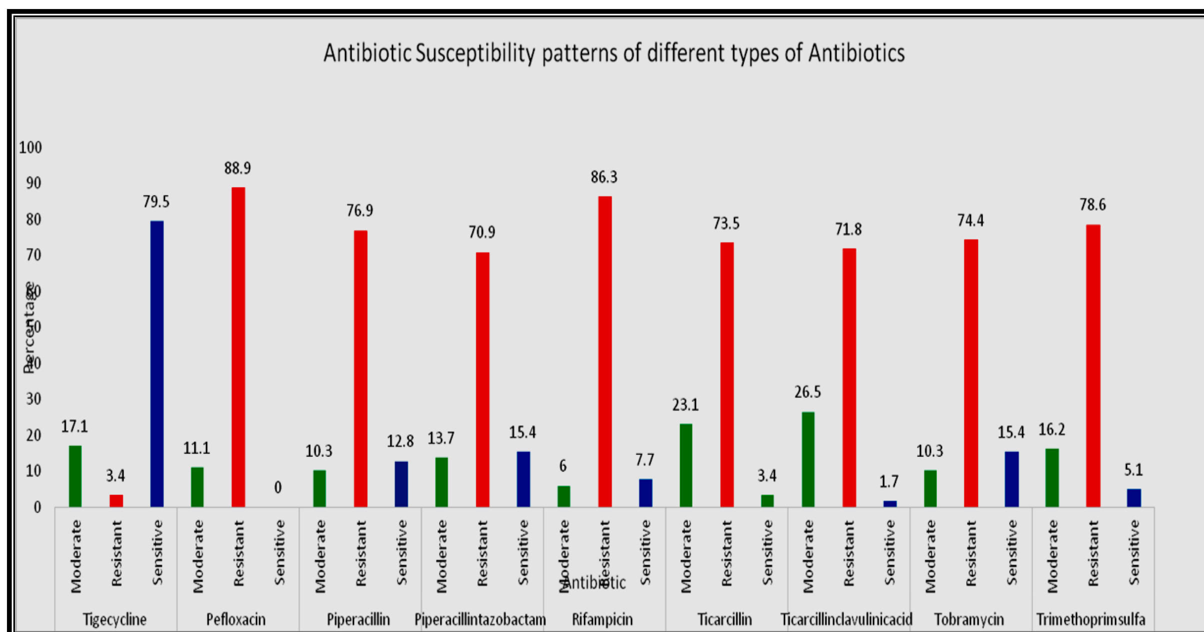

Figure (2): Antibiotic susceptibility patterns of Pseudomonas aueroginosa in clinical isolates.

Regarding antimicrobial resistance pattern of Pseudomonas Aueroginosa, we found that isolates from urine samples (No=83) showed the highest resistance to pefloxacin (90.4%), followed by rifampicin (84.3%), both gentamicin and minocycline (83.1%) each, however, the lowest resistance was to tigecycline (2.4%), colistin (3.6%) and amikacin (19.3%).

Referring to samples obtained from wounds (No=14), Pseudomonas Aueroginosa showed the highest resistance to piperacillin/azobactam (92.9%) as well as rifampicin (92.9%) and aztereonam (92.9%), while the lowest resistance was to tigecycline (7.1%) and amikacin (21.4%).

Pseudomonas Aueroginosa isolated from the two CSF samples showed 100% resistance to trimethoprim/sulfa, tobramycin, ticarcillin/ avulinicacid, rifampicin, piperacillin, piperacillin/azobactam, pefloxacin and aztereonam, however, no resistance was detected to tigecyclin, cefepime, ceftazidime, colistin and amikacin. While isolates obtained from sputum samples (N=14) showed the highest resistance to gentamicin and minocycline (100%) each followed by aztereonam (91.75%), while the lowest

resistance was to tigecycline (8.3%). No resistance was detected to amikacin.
Referring to samples obtained from blood (No=4), Pseudomonas Aueroginosa showed the highest resistance to minocycline, pefloxacin, rifampicin and trimethoprim/sulfa (100%), while no resistance was detected to colistin and tigecycline.
As regard peritoneal fluid, there was only one isolate and showed resistance to all tested antibiotics except amikacin, profloxacin, colistin, tigecycline and ticarcillin for which no resistance were detected. While pleural fluid single isolate was resistant to all tested antibiotics except cefepime, ceftazidime, ciprofloxacin, colistin, tigecycline and pefloxacin for which no resistance were detected. Antibiotics with the highest resistance were pefloxacin (88.9%), rifampicin (86.3%) and minocycline (84.6%) followed by gentamicin and aztreonam (82.9%) each, while antibiotics to which most isolates were sensitive were colistin (82.9%) and tigecycline (82.9%).

Table (2): Distribution of different virulent genes among MDR Pseudomonas aeuroginosa.

Variables		N =117	%
AprA	**Negative**	55	47.0
	Positive	62	53.0
ExoS	**Negative**	55	47.0
	Positive	62	53.0
AlgD	**Negative**	6	5.1
	Positive	111	94.9
ExoU	**Negative**	47	40.2
	Positive	70	59.8
ToxA	**Negative**	3	2.6
	Positive	114	97.4
PlcN	**Negative**	107	91.5
	Positive	10	8.5
PlcH	**Negative**	74	63.2
	Positive	43	36.8
LasB	**Negative**	61	52.1
	Positive	56	47.9

The prevalence of eight virulence genes among MDR pseudomonas aeruginosa isolates was investigated and the results were :
AprA gene was found in 53% isolates, the same percentage was recorded in Exo S gene. While Alg D gene was detected in 94.9% isolates.
ExoU, ToxA, PlcN genes were recorded to present in 59.8%, 97.4% and 8.5% respectively. However, PlcH gene was found in 36.8% isolates. Finally, we detected LasB in 47.9% of isolates.
ToxA gene was the highest gene detected among isolates (97.4%) followed by AlgD (94.9%). However, PlcN was the gene detected least among isolates (8.5%).

Table (3): Biofilm production among MDR isolates

Biofilm							
Non-biofilm producer		**Strong biofilm producer**		**Moderate biofilm producer**		**Weak biofilm producer**	
N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
4	3.4	69	59	34	29.1	10	8.5

Regarding to biofilm production among MDR isolates, there were 4(3.4%) non-biofilm producers isolates, while 69(59%) isolates were strong biofilm producers. In addition, 34(29.1%) isolates were recorded to be moderate biofilm producers. However, 10(8.5%) isolates were weak biofilm producers. (Table 3).

Table (4): Genotypic pattern of Non biofilm producers in MDR Pseudomonas aueroginosa isolates

	AprA	**ExoU**	**ExoS**	**AlgD**	**ToxA**	**PlcN**	**PlcH**	**LasB**
	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)	N(%)
Non Biofilm Producer (4)	3 (75%)	4 (100%)	1 (1%)	3 (75%)	2 (50%)	0 (0%)	3 (75%)	3 (75%)

The genotypic pattern of the four non-biofilm producers in MDR Pseudomonas aueroginosa isolates., AprA gene was detected

among 3 isolates, ExoU, ExoS, AlgD genes were recorded to present in 4, 1 and 3 isolates respectively. However, ToxA, PlcH and LasB genes were recorded among 2, 3 and 3 isolates respectively. PlcN gene was not detected among any isolate.

Table 5: Genotypic Pattern of the three types of Biofilm Producers in MDR Pseudomonas aeruginosa isolates.

	AprA	ExoU	ExoS	AlgD	ToxA	PlcN	PlcH	LasB
	N	N	N	N	N	N	N	N
Strong Biofilm Producer (69)	35	38	36	65	69	7	26	30
	AprA	**ExoU**	**ExoS**	**AlgD**	**ToxA**	**PlcN**	**PlcH**	**LasB**
	N	N	N	N	N	N	N	N
Moderate Biofilm Producer (34)	22	25	22	33	33	3	14	20
	AprA	**ExoU**	**ExoS**	**AlgD**	**ToxA**	**PlcN**	**PlcH**	**LasB**
	N	N	N	N	N	N	N	N
Weak Biofilm Producers (10)	2	3	3	10	10	0	0	3

The genotypic pattern of the sixty-nine 69 strong biofilm producers in MDR Pseudomonas aeruginosa isolates : AprA gene was detected among 35 isolates, ExoU, ExoS, AlgD genes were recorded to present in 38, 36 and 65 isolates respectively. However, ToxA, PlcN, PlcH and LasB genes were recorded among 69, 7, 26 and 30 isolates respectively. The genotypic pattern of the thirty four moderate biofilm producers in MDR Pseudomonas aueroginosa isolates, AprA gene was detected among 22 isolates, ExoU, ExoS, AlgD genes were recorded to present in 25, 22 and 33 isolates respectively. However, ToxA, PlcN, PlcH and LasB genes were recorded among 33, 3, 14 and 20 isolates respectively, AprA gene was detected among 2 weak biofilm producer isolates, ExoU, ExoS, AlgD genes were recorded to present in 3, 3 and 10 isolates respectively. However, ToxA and LasB genes were recorded among10 and 3 isolates respectively. PlcN and PlcH genes were not detected among any isolate (table 5).

Table (6): Number of non biofilm producer and type of antibiotic resistance.

Resistant Antibiotc	Non biofilm producer (4)
Amikacin	1
Aztreonam	4
Cefepime	2
Ceftazidime	2
Ciprofloxacin	2
Colistin	0
Gentamicin	4
Imipenem	2
Meropenem	2
Minocycline	4
Tigecycline	0
Pefloxacin	4
Piperacillin	1
Piperacillintazobactam	1
Rifampicin	2
Ticarcillin	1
Ticarcillinclavulinicacid	3
Tobramycin	1

Trimethoprimsulfa	4

According to results, we found that there was 1 non-biofilm producer with amikacin resistance. Howevere, regarding resistance to aztreonam, cefepime, ceftazidime,ciprofloxacin, gentamicin, imipenem, meropenem and minocycline, number of non-biofilm producers were 4,2, 2,2, 4, 2, 2 and 4 respectively. Also number of non-biofilm producers that showed resistance to pefloxacin, piperacillin, piperacillin/tazobactam, rifampicin, ticarcillin, ticarcillin/clavulinicacid, tobramycin and trimethoprim/sulfa were 4, 1, 1, 2, 1, 3, 1 and 4 respectively. No non-biofilm producer was detected to be resistant to colistin and tigecycline (table 6).

Table (7): Number and percentage of biofilm producers and type of antibiotic resistance.

Resistant Antibiotc	Biofilm producers (113)
Amikacin	20 (95%)
Aztreonam	93 (59.9%)
Cefepime	31(93.9%)
Ceftazidime	53(96.4%)
Ciprofloxacin	64(96.9%)
Colistin	3(100%)
Gentamicin	93(95.9%)
Imipenem	67(97.1%)
Meropenem	76(97.4%)
Minocycline	95(95.9%)
Tigecycline	4(100%)
Pefloxacin	100(96.2%)
Piperacillin	89(98.9)
Piperacillintazobactam	82(98.8%)
Rifampicin	99(99%)
Ticarcillin	85(98.8%)
Ticarcillinclavulinicacid	81(96.4%)
Tobramycin	86(98.9%)
Trimethoprimsulfa	88(95.7%)

Regarding results shown in table 7, we found that there are 20(95.2%) biofilm producers with amikacin resistance. Howevere, regarding resistance to aztreonam, cefepime, ceftazidime,ciprofloxacin, colistin, gentamicin, imipenem, meropenem, minocycline and tigecycline, number of biofilm producers were 93,31, 53,64, 3, 93, 67, 76, 95 and 4 respectively. Also number of biofilm producers that showed resistance to pefloxacin, piperacillin, piperacillin/tazobactam, rifampicin, ticarcillin, ticarcillin/clavulinicacid, tobramycin and trimethoprim/sulfa were 100, 89, 82, 99, 85, 81, 86 and 88 respectively .

DISCUSSION

Multidrug-resistant (MDR) infections, especially Gram-negative bacteria, have been on the rise worldwide during the past few decades (GNB). Multiple-drug-resistant Pseudomonas aeruginosa is a major cause of morbidity and mortality due to its role in healthcare-associated illnesse. (**18).**

The study included 117 patients. Male patients represented (50.4%), while female patients represented (49. 6%).This is in contrast to **Chand *et al.,* (15)** who found that the distribution of *P. aeruginosa* was higher in male patients, 56 (64.36%), than in female patients, 31 (35.63%). The possible reasons may be males have routine outdoor work and they are frequently at risk of infection from the infected environments **(16)**.

The age of patients was from 30 to 70 years old with mean age (50.61±18.63). This is in agreement with **Derakhshan *et al.,* (17)** who reported that the age of their patients were between 47-66 years old and explained that Pseudomonas aeruginosa infections occur in people in the hospital and/or with weakened immune system.

In this study, Urine samples represented the highest percentage 70.9%, followed by samples obtained from wounds (12%), sputum samples (10.3%), blood samples (3.45), CSF(1.7%), while samples represented the least percentage were peritoneal fluid and pleural fluid(0.9%) each. This is in agreement with **Chand *et al.,* (15)** who reported that the maximum number of MDR *P. aeruginosa* (MDRPA) was isolated from urine 24 (60.00%) followed by sputum 8 (33.33%), pus 4 (30.77%), and body fluids 2 (25.00%).

Antibiotics with the highest resistance were pefloxacin (88.9%), rifampicin (86.3%) and minocycline (84.6%) followed by

gentamicin and aztreonam (82.9%) each, this is in agreement with **El-Far *et al.,* (18) Sid Ahmed *et al.,* (19)** who found that aminoglycoside and aztereonam resistance were 97% and 78.7% among pseudomonas isolates respectively.
As regard distribution of different virulent genes among MDR Pseudomonas Aeroginosa, we recorded the following results, AprA gene was found in 53% isolates, the same percentage was recorded in Exo S gene, this is in consistant with **Fazeli and Momtaz, (20),** who found that Exo S gene was detected in 67.64% of isolates. While Alg D gene was detected in 94.9% isolates, which is similar to results recorded by **Gholami *et al.,* (9)**, who found AlgD gene in 98% isolates.
ExoU, ToxA, PlcN genes were recorded to present in 59.8%, 97.4% and 8.5% respectively. However, PlcH gene was found in 36.8% isolates. Finally, we detected LasB in 47.9% of isolates.This is in agreement with **Hassuna *et al.,* (8)**, who reported that frequency of exoU and lasB genes among isolates were 50.4% and 43.5% respectively. **Bogiel *et al.,* (21) and Chand *et al.,* (15)** recorded that 96.3% and 95.4% of MDR *P. aeruginosa* isolates had *toxA* gene respectively, which is in consistent with our results, thus, may lead, additionally, to their increased virulence. However**, Hassuna *et al.,* (8)** recorded a percentage of 23% of *toxA* gene. Also, in contrast to our results, **Bazghandi *et al.,* (22)** found that plcH and plcN genes among pseudomonas isolates represented 86.9% each.
Derakhshan *et al.,* (17) reported that exo U in their study was 8.6 and explained that The lower prevalence of exo genes in their study may be due to differences in the source of isolates or geographical regions. Many of the P. aeruginosa virulence factors are variable traits and they are found in some isolates, but not in others. For example, **Choy et al**., **(23)** reported that the exoU is commonly found in P. aeruginosa strains isolated from keratitis, whereas it occurs at low prevalence in the nonocular isolates or the prevalence of exoS was significantly higher in isolates from blood than those obtained from respiratory infections. In addition, it is possible that strains of **Derkhshan et al., (17)** were isolated from chronic infections. The expression of Type three secretion system T3SS has been found to be downregulated in isolates from chronic phase of infection, which is consistent with the notion that persistence of bacteria in the host requires the down-regulation of many virulence factors.
ExU, ExoS, ToxA genes present in 83 urine isolates as 46 (55.4%), 40 (48.2%), and 81 (97.5%) respectively., in 12 sputum isolates as 6 (50%), 4 (33.3%), and 11 (91.6%) respectively, in 14 wound isolates as 14 (100%), 14 (100%), 14 (100%) respectively. This is in agreement with **Bahador *et al.,* (13)** who reported that ExoU and ExoS genes were significantly prevalent in urine and wound isolates. Also **Bahador *et al.,* (13)** reported that 100% of P. aeruginosa isolates recovered from eye and wound samples were positive for ToxA gene.
The Number of Pseudomonas Aueroginosa isolates that harboured ExoU only was 70, while number of isolates harboured ExoS gene only was 62. Coexistence of ExoU and ExoS genes was detected among 39 (33.3%) isolates while **Bahador *et al.,* (13)**found that coexistence of both genes was detected among 4.28% and **Hassuna *et al.,* (8)** found coexistence of both genes among 7.6% of MDR isolates.
We detected 33(28%) isolates that were positive to 1-4 genes, while, 55(47%) isolates harboured from 5 to 6 genes. In addition, we recorded that 29(25%) isolates were positive to more than 6 genes. This is in contrast to **Hassuna *et al.,*(8)** who reported that 2 out of 39 isolates had 6 virulence genes altogether, while no isolates possessed more than 6 virulence genes.
Biofilms are sessile microbial territories covered by an extracellular polysaccharide material, which facilitates irreversible attachment of microbial cells to the substructure or each other so it can confer resistance to immune clearance and antibacterial treatment **(24 , 3)**.
Regarding biofilm production among MDR isolates, we recorded 4(3.4%) non-biofilm producers isolates, 10(8.5%) isolates were weak biofilm producers. While 69 (59%) isolates were strong biofilm producers. In addition, 34(29.1%) isolates were recorded to be moderate biofilm producers. This is in agreement with **Gajdács *et al.,* (25)** who found that 69.93% of MDR isolates were strong biofilm producers, also, in agreement with our results, **Behzadi *et al.,* (26)** who found that 27.71% of pseudomonas isolates were moderate biofilm producers, while, **Shukla *et al.,* (27)** found that non-biofilm producer isolates were 1.6% among MDR *p. aeuroginosa.*

Regarding genotypic pattern of non-biofilm producers in MDR Pseudomonas aueroginosa isolates, AprA gene was detected among 3(75%) isolates, ExoU, ExoS, AlgD genes were recorded to present in 4(100%), 1 (25%) and 3(75%) isolates respectively. Similarly, **Rajabi *et al.,* (28)** found that AlgD gene was detected among 51.8% of non-biofilm producers. However, ToxA, PlcH and LasB genes were recorded among 2(50%), 3(75%) and 3(75%) isolates respectively. PlcN gene was not detected among any isolate.

Regarding genotypic pattern of biofilm producers in MDR Pseudomonas aueroginosa isolates, AprA gene was detected among 59(52.2%) isolates, ExoU, ExoS, AlgD genes were recorded to present in 66(58.4%), 61(53.9%) and 108(95.6%) isolates respectively.This is in agreement with **Shahbazzadeh *et al.,*(29)** who recorded that AlgD gene was detected among 100% of biofilm producing P. aeruginosa isolates. However, in contrast to our results, **Azimi *et al.,* (1)** recorded that 2.5% of isolates that harboured *ExoU* and *ExoS* genes, were biofilm producers. However, *ToxA*, *PlcN*, *PlcH* and *LasB* genes were recorded among 112(**99.1**%), 10(**7.8**%), 40(**35.4**%) and 53(**46.9**%) isolates respectively

. Strong and moderate biofilm producers show high prevalence for ToxA, ExoU, and ExoS genes. This is in agreement with

Bahador ***et al.,*** **(13)** who reported that these proteins modify host cell functions which are important in cytoskeletal organization and signal transduction. Also ExoS is a bi-functional toxin exhibiting ADP ribosyl-transferase and GTPase activating activity. ExoU exhibits phospholipase activity and disrupt eukaryotic membranes following its delivery into cytoplasm.

We found that there was 1(%4.8) non-biofilm producers with amikacin resistance. Howevere, regarding resistance to aztreonam, cefepime, ceftazidime,ciprofloxacin, gentamicin, imipenem, meropenem and minocycline, number of non-biofilm producers were 4(4.1%),2(6.1%), 2(3.1%),2(3%), 4(4.1%), 2(2.9%), 2(2.6%) and 4(4%) respectively. Also number of non-biofilm producers that showed resistance to pefloxacin, piperacillin, piperacillin/tazobactam, rifa rifampicin, ticarcillin, ticarcillin/clavulinic acid, tobramycin and trimethoprim/sulfa were 4(3.9%), 1(1.1%), 1(1.2%), 2(2%), 1(1.5%), 3(3.6%), 1(1.1%) and 4(4.3%) respectively. In contrast to our results, **Shukla** ***et al.,*** **(27)**, recorded that percentage of non-biofilm producers with resistance to amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, piperacillin/tazobactam, ticarcillin/clavulanic acid were 34.7%, 39.1%, 62.5%, 55.5%, 37.5%, 37.2%, 27.2%, 20%, 19.4%, 17.3% and 24.1% respectively .

No non-biofilm producer was detected to be resistant to colistin and tigecycline in this study, this is in agreement with **Shukla** ***et al.,*** **(27)**, who found no non-biofilm producers among colistin resistant strains.

We found that there are 20(95.2%) biofilm producers with amikacin resistance. Howevere, regarding resistance to aztreonam, cefepime, ceftazidime,ciprofloxacin, colistin, gentamicin, imipenem, meropenem, minocycline and tigecycline, number of biofilm producers were 93(95.9%), 31(93.9%), 53(96.4%), 64(96.9%), 3(100%), 93(95.9%), 67(97.1%), 76(97.4%), 95(95.9%) and 4(100%) respectively. This is in agreement with **Heidari** ***et al.,*** **(30)**, who found that biofilm production among carbapenem resistant pseudomonas aeuroginosa isolates represented (94.0%). Also number of biofilm producers that showed resistance to pefloxacin, piperacillin, piperacillin/tazobactam, rifampicin, ticarcillin, ticarcillin/clavulinicacid, tobramycin and trimethoprim/sulfa were 100(96.2%), 89(98.9%), 82(98.8%), 99(99%), 85(98.8%), 81(96.4%), 86(98.9%) and 88(95.7%) respectively. This is in agreement with **Shahbazzadeh et al.,(29)**, who stated that biofilm producers among amikacin resistant strains were 100%, while percentages of biofilm producers among ceftazidime, ciprofloxacin, gentamicin, meropenem, imipenem, piperacillin and trimethoprim/sulfa, were 93.3%, 80.2%, 100%, 94.2%, 100%, 100% and 87.6% respectively.

Conclusion

Our study revealed that AlgD, ToxA and ExoU genes were the most predominant among MDR p.aeuroginosa isolates. Moreover, they recorded the highest percentage among biofilm producer isolates. Antibiotic resistance against pefloxacin, rifampicin, minocyclin, gentamicin and aztereonam were high, while resistance against tigecyclin and colistin were very low, so, prescription of them can be effective in the treatment of MDR p.aeuroginosa isolates.

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