Research Article

Virulence Genes in Pseudomonas Aeruginosa Strains Isolated at Suez Canal University Hospitals with Respect to the Site of Infection and Antimicrobial Resistance

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Keywords: Biofilm; Virulence genes; P. aeruginosa; Antimicrobial resistance; Egypt

Abstract

Background: *Pseudomonas aeruginosa* is one of the top five pathogens causing healthcare-associated infections. Biofilm formation is nowadays a major problem. Aim: The aim of this study was to examine the prevalence of virulence genes in clinical isolates of *Pseudomonas aeruginosa* at Suez Canal University Hospitals with respect to the site of infection and microbial resistance of the strains.

Materials and methods: A cross-sectional descriptive study was carried out on 47 *Pseudomonas aeruginosa* strains collected from hospitalized patients from December 2015 to August 2017. To detect biofilm formation, we used Tissue Culture Plate Method. The virulence genes (*toxA, algD, nan1, pslA* and *pelA)* were amplified using PCR technique.

Results: The highest sensitivity was to Imipenem and Ciprofloxacin (85.1% and 68.1% respectively).With respect to the virulence genes, *toxA* gene was detected in 45 isolates (95.7%), *algD* gene in 42 isolates (89.4%), *pslA* in 42 isolates (89.4%) %), *pelA* cted in 41 isolates (87.2%) and *nan1*gene was detected in 19 isolates (40.45%).

Conclusions and Recommendations: We conclude that there is relationship between virulence genes and biofilm formation in *Pseudomonas aeruginosa.* We recommend the expansion of work on a larger sample size in a longer period of time.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is considered one of the most prevalent nosocomial organisms associated with high mortality rates and one with the highest prevalence of antibiotic resistance. It is one of the top five pathogens causing Healthcare Associated Infection (HAI) [1]. In Egypt, it can be responsible for HAI in Intensive Care Unit (ICU) and Burn Unit: 17 % and 21.6% respectively [2,3].

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P. aeruginosa is considered multi-drug resistant (MDR) bacteria. As by definition, *P. aeruginosa* MDR was defined bacteria resistant at least to three drugs mainly aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones [4]. The mechanisms of drug resistance are intrinsic and acquired. This resistance is mediated through several mechanisms including multidrug efflux systems, enzyme production, outer membrane protein loss and target mutations [5]. For virulence, *P. aeruginosa* possesses extracellular virulence factors controlled by a complex regulatory circuit involving oquorum sensing (QS) thus producing these factors in a coordinated manner [6].

The biofilm formation which reflects a community of cells attached to either a biotic or an abiotic surface and enclosed in a complex exopolymeric substance is nowadays a major problem as it increases the potential of the pathogen to be resistant to antibiotics and disinfectants [7], is difficult to be eradicated and contributes to localized or systemic inflammation, which prolongs wound healing [8]. *P. aeruginosa* produces at least three polysaccharides {alg (alginate), Pel (pellicle), and Psl} which plays an important role in the stability of the biofilm structure [9]. To overcome this phenomenon, different strategies have been proposed in order to (i) avoid microbial attachment to a surface, (ii) disrupt biofilm development to increase the penetration of antimicrobials; and (iii) affect biofilm maturation [10].

The aim of this study is to examine the prevalence of virulence genes in clinical isolates of *P. aeruginosa* isolated from Suez Canal University Hospitals (SCUH) in respect to the site of infection and antimicrobial resistance of the strains.

Materials and Methods

A cross-sectional descriptive study was carried out from December 2015 to August 2017 at SCUH on 47 *P. aeruginosa* strains collected from hospitalized patients who were suffering from Urinary Tract Infection (UTI), Respiratory Tract Infection (RTI),burn infection, bed ulcers, wound infections and bacteremia in Suez Canal University Hospitals(SCUHs) in Ismailia.

Various clinical specimens were collected from hospitalized patients and processed in the Medical Microbiology and Immunology department SCUHs for the isolation and identification of *P. aeruginosa*.

The collected specimens were inoculated onto blood agar, MacConkey's agar and Pseudomonas agar P plates. Then the plates were incubated aerobically at 35± 2°C for 24 hours. Colonies on blood agar and MacConkey's agar suspected to be *P. aeruginosa* (by their colonial morphology, being non-lactose fermenter on MacConkey's agar and gram negative bacilli by gram stain) were confirmed to be *P. aeruginosa* by oxidase test and by production of the blue phenazine pigment pyocyanin on Pseudomonas Agar P which is absolute confirmation of a strain as *P. aeruginosa* [11].

We used quality control strain P. aeruginosa ATCC 27853. According to Clinical and Laboratory Standards Institute (CLSI) [12], the following antibiotic discs were used as follows: Pipracillin-Tazobactam 100/10μg as β- Lactamase inhibitors, Ceftazidime 30μg and Cefepime 30μg (third and fourth generation Cephalosporins respectively), Azetronam 30μg as Monbactams, Imepenem 10μg and Merpenem 10μg for Carbapenems, Gentamycin 10μg and Amikacin 30μg for Aminoglycosides, Ciprofloxacin 5μg and Levofloxacin 5μg as second and third generation Fluoroquinolones respectively and Colisitin 10μg for Lipopeptides.

To detect biofilm formation, we used Tissue Culture Plate Method (TCP) [13] as follows: overnight isolates from fresh agar plate (24 hours at 37°C) were diluted 100 folds in trypticase soy broth. Then 200 μL of this suspension were inoculated into a 96-well flat-bottomed polystyrene plate, covered and incubated overnight at 37°C. Each well was washed four times with 200 μl of phosphate buffer saline (pH 7.2) in

order to remove free-floating planktonic bacteria. For the non-adherent bacteria, the plates were severely shaken to get rid of it. After drying the plates, the wells were stained with 200 µL of crystal violet for 15 minutes (dye was dissolved with 200 µL of ethanol 95%). The optical density (OD) at 630nm was recorded and the results were interpreted [14].

Table 1 shows the mean Optical density (OD) to detect the biofilm formation by using TCP method.

The virulence genes (*toxA, algD, nan1, pslA* and *pelA)* were amplified by PCR using a specific set of primers listed in table 2.

Bacterial DNA for the PCR analysis was prepared using the bacterial DNA extraction kit (Sigma) following the manufacturer's instructions. PCR was carried out with 2 µL template DNA, 0.25 µM of each primer, 0.2 mM deoxyribonucleoside triphosphates, 1x reaction buffer, 2 mM $MgCl_2$ and 1.5U Prime Taq DNA polymerase in a total volume of 25 µL.

For *toxA, nan1* and *algD* genes, the DNA was amplified using the following protocol: initial denaturation (94 ºC for 5 minutes) followed by 30 cycles of denaturation (94 ºC for 40 seconds), annealing (55 ºC, from 45 seconds to 1 minute) and extension (72 ºC, from 45 seconds to 1 minute 35 seconds), with a single final extension of 7 minutes at 72 ºC [6].

For *pelA* and *pslA* genes, the DNA was amplified using the following protocol: initial denaturation (5 minutes at 94°C) followed by 30 seconds-35 cycles of denaturation at 94°C, 40 seconds of annealing at 52°C and 50 seconds of extension at 72°C.The amplified products were held at -20°C until analysis [15].

PCR products were separated in 1% agarose gel for 50–110 minutes at 120 volts, stained with ethidium bromide (0.5g/ml) and detected by ultraviolet trans-illumination (wavelength 312 nm).

Ethical considerations

The study work obtained approval from the Ethics Committee of Faculty of Medicine, Suez Canal University (FOMSCU), Ismailia, Egypt.

Table 1: Classification of bacterial adherence and biofilm formation by TCP method.

Mean OD value at 630nm	Biofilm production
< 0.120	Non/weak
0.120 - 0.240	Moderate
> 0.240	Strong

Table 2: Primers that were used for amplification of virulence genes..

Target Gene	Oligonucleotide Sequence (5'-3')	Amplicon Size (bp)*
toxA [55]		150 bp
-F**	GGAGCGCAACTATCCCACT	
-R***	TGGTAGCCGACGAACACATA	
nan1 [46]		1317 bp
-F	ATG AAT ACT TAT TTT GAT AT	
-R	CTA AAT CCA TGC TCT GAC CC	
algD [53]		1310 bp
-F	ATG CGA ATC AGC ATC TTT GGT	
-R	CTA CCA GCA GAT GCC CTC GGC	
pelA [54]		118 bp
-F	CCTTCAGCCATCCGTTCTTCT	
-R	TCGCGTACGAAGTCGACCTT	
pslA [15]		656 bp
-F	TCCCTACCTCAGCAGCAAGC	
-R	TGTTGTAGCCGTAGCGTTTCTG	

Statistical analysis

The data collected were entered into a database file. Statistical analysis was performed by using the SPSS 22 software statistical package. Qualitative data was summarized in frequencies. For a subsequent analysis of data, chi square test was used to detect the difference between qualitative data. The outcome variables included sex, type of specimen, hospital wards, biofilm formation, drug susceptibility and MDR. Statistical significance was considered at *p*. value ≤ 0.05.

Results

This study was carried out on 47 *P. aeruginosa* isolates that were collected from 296 patients who had HAIs after 24-48 hours of admission in SCUHs in Ismailia during the period from December 2015 to August 2017.

P. aeruginosa was defined. In regard to its percentage in relation to sex, it was found out that it was higher among males than females (55.3% versus 44.7%). With reference to its percentage among different age groups, it was the highest among age group ≥50 years (21.3%) and the lowest from the age group 10 - 40 years (8.5%).

With regard to the percentage of *P. aeruginosa* in different hospital wards, the highest percentage was found in cases in the ICU (29.8%), while the lowest percentage was in pediatrics department (6.4%).

The highest percentage of *P. aeruginosa* was isolated from pus of the wounds and burns (38.3%), then 31.9% from urine, 19.1% from sputum and 10.6% from blood samples.

Testing the antibiotic susceptibility pattern of the isolated strains showed that the highest sensitivity was to Imipenem and Ciprofloxacin (85.1% and 68.1% respectively), while the highest prevalence of resistance was to Cefepime and Ceftazidime (68.1% for each of them) (Table 3).

Twenty eight strains were MDR (59.6%) and 19 strains were non MDR (40.4%) out of 47 *P. aeurginosa* strains.

Testing the resistance pattern of the MDR and non MDR strains showed that; the highest prevalence of resistance for MDR strains was to Azetronam and Cefepime (92.85% for each) and the highest prevalence of resistance for non MDR strains was to Levofloxacin and Meropenem (47.36% for each) (Table 4).

For biofilm production, results showed that 13 strains out of 47 (27.7%) were strong biofilm producers, 10 strains (19.1%) moderate biofilm producers and 24 strains (51.1%) weak or non-biofilm producers .

Testing the relation between biofilm production and different sites of infection showed that the percentage of biofilm formation is higher among sputum and blood specimens than others (Table 5).

According to the antibiotic susceptibility pattern of both biofilm- producing and nonbiofilm- producing isolates, it was found that biofilm- producing strains had high prevalence of resistance to Ciprofloxacin (80%), followed by Azetronam (70%), Gentamicin (66.6%), Amikacin (64.7%), Pipracillin-Tazobactam (64%) and Cefepime and Ceftazidime (59.4% for each). The resistance pattern to Ciprofloxacin, Gentamicin, Cefepime, Ceftazidime, Azetronam and Pipracillin-Tazobactam was significantly higher (*p.* value ≤0.05) among biofilm producers than non-biofilm producers as shown in table 6.

The percentages of the virulence genes among the isolated strains were determined, *toxA* gene was detected in 45 isolates (95.7%), while *algD* gene in 42 isolates (89.4%), *pslA* in 42 isolates (89.4%) %), *pelA* in 41 isolates (87.2%) and *nan1*gene in 19 isolates (40.4) (Table 7).

Table 3: The results of antibiotic susceptibility pattern of the isolated strains:

No.	Antibiotic	Resistance No. (%)
1	Imipenem	7 (14.9%)
2	Ciprofloxacin	15 (31.9%)
3	Amikacin	17 (36.2%)
4	Meropenem	18 (38.3%)
5	Colistin	18 (38.3%)
6	Gentamicin	21 (44.7%)
7	Levofloxacin	22 (46.8%)
8	Pipracillin\ tazobactam	25 (53.2%)
9	Azetronam	27 (57.4%)
10	Ceftazidime	32 (68.1%)
11	Cefepime	32 (68.1%)

Table 4: The resistance pattern of the Multi Drug Resistant and Non Multi Drug Resistant strains among the studied population (n=47):

No.	Antibiotic	Multi Drug Resistant (N=28) No. (%)	Non Multi Drug Resistant (n=19) No. (%)
1	Azetronam	26 (92.85%)	1 (0.05%)
2	Cefepime	26 (92.85%)	6 (31.57%)
3	Ceftazidime	25 (89.28%)	7 (36.84%)
4	Pipracillin-tazobactam	23 (82.14%)	2 (10.5%)
5	Gentamicin	16 (57.14%)	5 (26.31%)
6	Levofloxacin	13 (46.4%)	9 (47.36%)
7	Amikacin	13 (46.4%)	4 (21.1%)
8	Coilstin	11 (39.28%)	7 (36.84%)
9	Ciprofloxacin	10 (35.71%)	5 (26.31%)
10	Meropenem	9 (32.14%)	9 (47.36%)
11	Imipenem	4 (14.28%)	3 (15.78%)

Table 5: The relation between biofilm production and different sites of infection.

Biofilm production	The different sites of infection				
	Pus	Urine	Sputum	Blood	*P value*
Strong biofilm producer No. (%)	4 (22.2%)	4 (26.66%)	3 (33.33%)	2 (40%)	0.850
Moderate biofilm producer No. (%)	2 (11.11%)	4 (26.66%)	2 (22.22%)	2 (40%)	0.490
Non biofilm producer No. (%)	12 (66.66%)	7 (46.66%)	4 (44.44%)	1 (20%)	0.147
Total No. (%)	18(38.3%)	15 (31.9%)	9 (19.1%)	5 (10.6%)	

P value<0.05 significant

Table 6: The antibiotic susceptibility pattern of both biofilm producing and non-biofilm producing isolates.

No.	Antibiotic	Biofilm producer (n=23) No. (%)	Non biofilm producer (n=24) No. (%)	*P* value
1	Ciprofloxacin	12 (52.17%)	3 (12.5%)	0.005
2	Levofloxacin	12 (52.17%)	10 (41.67%)	0.564
3	Meropenem	7(30.43%)	11 (45.83%)	0.371
4	Imipenem	4 (17.39%)	3 (12.5%)	0.701
5	Gentamicin	14 (60.86%)	7 (29.16%)	0.041
6	Amikacin	11 (47.82%)	6 (25%)	0.135
7	Pipracillin-tazobactam	16 (69.56%)	9 (37.5%)	0.041
8	Cefepime	19 (82.60%)	13 (54.16%)	0.050
9	Ceftazidime	19 (82.60%)	13 (54.16%)	0.050
10	Azetronam	20 (86.95%)	9 (37.5%)	0.001
11	Coilstin	8 (34.78%)	10 (41.6%)	0.766

P value<0.05 significant

Table 7: The percentages of the virulence genes among the isolated strains:

No.	Genes	Positive No. (%)	Negative No. (%)
1	*toxA*	45 (95.7%)	2 (4.3%)
2	*algD*	42 (89.4%)	5 (10.6%)
3	*pslA*	42 (89.4%)	5 (10.6%)
4	*pelA*	41 (87.2%)	6 (12.8%)
5	*nan1*	19 (40.4%)	28 (59.6%)

The percentage of virulence genes in different hospital wards were determined. It was higher in ICU, surgery and Burn Unit than other wards with statistically insignificant *P.* value (Table 8).

The percentages of virulence genes in terms of the site of infection were determined. It was higher in pus of the wounds and followed by urine, sputum and blood (Table 9).

It was found out that certain biofilm-producing strains were specifically connected to certain virulence genes. Ninteen strains out of 23 (82.6%) express *pelA* gene, while all the strains express *pslA* gene with statisticaly significant *P*. value (*P* ≤0.05) (Table 10).

The percentage of virulence genes among MDR and non-MDR isolates were determined. Twenty seven strains of 28 total MDR bacteria were positive to *toxA*, *algD* and *pslA* genes (96.4%) with statistically significant *P.* value (*P.* ≤0.05) for *algD* and *pslA* genes. Also, 25 strains (89.3%) were positive to *pelA* and 13 strains were positive to *nan1* gene (46.4%) (Table 11).

In regard to the relation between MDR, biofilm production and virulence genes of *P. aeruginos,* MDR and biofilm producer strains were found the highest groups which carried virulence genes (Table 12).

Table 8: The percentage of virulence genes in different hospital wards.

Genes	Different hospital wards							
	ICU No. (%)	Burn unit No. (%)	Surgry No. (%)	NICU No. (%)	Internal medicine No. (%)	Pediatric department No. (%)	Total	*P* value
toxA	14 (31.1%)	7 (15.5%)	9 (20%)	5 (11.1%)	7(15.5%)	3 (6.66%)	45	0.486
nan1	12 (28.5%)	6 (14.28%)	10 (23.8%)	5 (11.9%)	6 (14.28%)	3 (7.14%)	42	0.825
algD	8 (42.1%)	5 (26.31%)	4 (21.05%)	1 (5.26%)	1 (5.26%)	0 (0%)	19	0.080
pelA	13 (31.7%)	5 (12.19%)	9 (21.95%)	5 (12.19%)	6 (14.63%)	3 (7.32%)	41	0.431
pslA	13 (30.9%)	7 (16.6%)	9(1.42%)	4 (9.52%)	6 (14.28%)	3 (7.14%)	42	0.766

Table 9: The percentages of virulence genes according to the different sites of infection.

Genes	The different sites of infection				
	Pus	Urine	Sputum	Blood	*P value*
toxA positive No. (%) negative No. (%)	17 (94.4%) 1 (5.55%)	14 (93.3%) 1 (6.66%)	9 (100%) 0 (0%)	5 (100%) 0 (0%)	0.050
algD positive No. (%) negative No. (%)	17 (94.4%) 1 (5.55%)	13 (86.66%) 2 (13.33%)	8 (88.8%) 1 (11.1%)	4 (80%) 1 (20%)	0.025
nan1 positive No. (%) negative No. (%)	10 (55.5%) 8 (44.4%)	8 (53.33%) 7 (46.66%)	1 (11.1%) 8 (88.8%)	0 (0%) 5 (100%)	0.785
pelA positive No. (%) negative No. (%)	17 (94.4%) 1 (5.55%)	15 (100%) 0 (0%)	6 (66.66%) 3 (33.33%)	4 (80%) 1 (20%)	0.050
pslA positive No. (%) negative No. (%)	16 (88.8%) 2 (11.1%)	13 (86.66%) 2 (13.33%)	8 (88.8%) 1 (11.1%)	4 (80%) 1 (20%)	0.959
Total No. (%)	18 (38.3%)	15 (31.9%)	9 (19.1%)	5 (10.6%)	

P value<0.05 significant

Table 10: The percentages of pelA and pslA genes among biofilm producer and biofilm non producer strains:

Genes	Biofilm producer n=23	Non biofilm producer n=24	*P value*
pelA: positive No. (%) negative No. (%)	19 (82.6%) 4 (17.3%)	23 (95.8%) 1 (4.1%)	0.142
pslA: positive No. (%) negative No. (%)	23 (100%) 0 (0%)	19 (79.1%) 5 (20.8%)	0.021
Total No. (%)	23 (48.9%)	24 (51.1%)	

P value<0.05 significant

Table 11: The percentage of virulence genes among Multi Drug Resistant and Non- Multi Drug Resistant isolates.

Genes	Multi Drug Resistant No. (%)	Non Multi Drug Resistant No. (%)	Total No.	*P* value
toxA	27 (96.4%)	18 (94.7%)	45	0.778
PslA	27 (96.4%)	15 (78.9%)	42	0.050
algD	27 (96.4%)	15 (78.9%)	42	0.050
pelA	25 (89.3%)	16 (84.2%)	41	0.609
nan1	13 (46.4%)	6 (31.6%)	19	0.309
Total	28	19	47	

P value<0.05 significant

Table 12: The relation between Multi Drug Resistant, biofilm production and virulence genes.

Genes	Multi Drug Resistant & biofilm producer No. (%)	Multi Drug Resistant & non biofilm producer No. (%)	Non Multi Drug Resistant & biofilm producer No. (%)	Non Multi Drug Resistant & non biofilm producer No. (%)	Total	*P* value
toxA	18 (40%)	9 (20%)	4 (8.88%)	14 (31.1%)	45	0.006
algD	18 (42.85%)	9 (21.42%)	4 (9.5%)	11 (26.2%)	42	0.050
pslA	18 (42.85%)	9 (21.42%)	5 (11.9%)	10(23.8%)	42	0.009
pelA	16 (39%)	9 (21.95%)	3 (7.31%)	13 (31.7%)	41	0.050
nan1	9 (47.3%)	4 (21%)	2 (10.52%)	4 (21%)	19	0.141

P value<0.05 significant

Discussion

P. aeruginosa is a *Gram*-negative bacterium possessing pili, flagella, (lipopolysaccharide) LPS [16]. It is difficult to be eradicated due to its ability to produce biofilm [17]. It infects the pulmonary tract, urinary tract, burn and becomes a major cause of HAI worldwide [18]. Eradication of *P. aeruginosa* has become increasingly difficult due to its remarkable capacity to resist antibiotics. *P. aeruginosa* strains are known to utilize their high levels of intrinsic and acquired resistance mechanisms to counter most antibiotics. In addition, adaptive antibiotic resistance of *P. aeruginosa* is a recently-characterized mechanism [(4)] which includes biofilm-mediated resistance and formation of multi-drug-tolerant cells, and is responsible for the relapse of infections. The discovery and development of alternative therapeutic strategies that present novel avenues against *P. aeruginosa* infections demand a more increasing attention [19].

This study aimed at determining the prevalence of certain virulence genes in clinical isolates of *P. aeruginosa* and to correlate the presence of these genes in different sites of infection with antimicrobial resistance.

A total of 296 specimens were collected from patients with nosocomial infections in SCUH. From the specimens, 47 *P. aeruginosa* strains were isolated (15.9%). In the study of Mahmoud et al. [20] at Menofia University hospital, *P. aeruginosa* was found to account for 19.8% of nosocomial infections. Wassef et al. [21] in Cairo, Egypt, isolated *P. aeruginosa* with a prevalence rate of 20.7%. Lower isolation rate (6.67%), was reported by a number of studies such as Khan et al. [22] in Pakistan. The percentage of *P. aeruginosa* is variable in various studies in literature. This might be attributed to drug overuse and hospital policy in management of such cases. Moreover, geographic climatic and hygienic factors may also be correlated with the relative variability of results among different areas [19].

In this study, the highest percentages of *P. aeruginosa* were from ICU, Surgery Department and Burn Unit (29.8%, 21.3% and 14.5% respectively). This is comparable with several studies such as Ikeno et al., Gad et al. and Pourshafie et al [23-25], which can be seen as ringing danger alarms for the widespread organism. This can be interpreted in terms of anaerobic growth of the bacteria obtaining energy from oxidation of sugars thus rendering difficulty of eradication [21]. Amany et al. 2017 [26], found that acquired infection rate in ICU was higher than other hospital wards. The ubiquitous nature, including the ability to survive in a moist environment and resistance to many antibiotics, makes *P. aeruginosa* a common pathogen in the ICUs of hospitals.

From a different perspective, the cause of the widespread existence of *P. aeruginosa* in the Burn Unit was due to impairment of the skin barrier in burn victims, debridement and manipulation of the burn site [27,28,29,30,31]. Also, it can be attributed to the production of proteases that can alter the host's physical barriers by splitting proteins with the production of amino-acids that allow the deep infiltration of the bacteria. Exotoxin A halts the synthesis of proteins and the hemolysins break down lipids in epithelial cells in order to permit the bacteria for more penetration and spreading [30].

Infections caused by *P. aeruginosa* are often severe due to limited antibiotic susceptibility and emergence of antibiotic resistance [30]. NNIS data (i.e. within the period from 1998 to 2003) [33] showed the highest prevalence of resistance rates of *P. aeruginosa* against antibiotics was to Imipenem, Ciprofloxacin, and Ceftazidime by 15%, 9%, and 20%, respectively. Also, there was evidence that the highest rates were against Cefepime and Ceftazidime (68.1% for each) which was in convenience with Mahmoud et al. and Oni et al [20,34]. This is explained by Cefepime which has reliable activity against *P. aeruginosa* because of the drug chemical structure allowing binding to penicillin-binding proteins and penetrating through the outer membrane of *Gram*-negative bacteria more rapidly than most Cephalosporins. Moreover, Cefepime is also stabler to β-lactamase hydrolysis [35].

From the previous explanation, we had expected to find high sensitivity level to Cefepime, but we found a high level of susceptibility to the drug. This may be attributed to the production of high levels of AmpC β-lactamases by some strains that become fully Cefepime-susceptible. This phenotype is usually found among ICU patients who frequently receive multiple treatment courses of expanded-spectrum β-lactam antibiotics for prolonged periods.

For the Ceftazidime that has a $C=N-OCH_3$ group in its chemical structure which provides stability against β-, acts as a penicillin-binding proteins inhibitor [37]. *P. aeruginosa* resistance against Ceftazidime arise from the horizontal acquisition of β-lactamases, altered expression of class C β-lactamase AmpC [36].

In the current study, Imipenem and Ciprofloxacin were the most effective drugs against *P. aeruginosa*. The sensitivity of Imipenem and Ciprofloxacin were 85.1% and 68.1% respectively due to their ability in producing several different porins as outer membrane porin D (OprD) so they can cross the outer membrane of *P. aeruginosa* [37].

The variations in the results of the antibiotic resistance might be referred to the difference in the pattern of drug use in different parts of the world and due to the several mechanisms that have been reported for *P. aeruginosa*, including: 1) Reduced expression or loss of OprD porin causing reduced antibiotic permeability, 2) Over-expression of MexAB-OprM pump which increases antibiotic efflux, 3) Production of β-lactams and aminoglycosides inactivating enzymes, 4) Mutations of gyrases and topoisomerases which cause fluoroquinolone resistance. These mechanisms in combination lead to multiple drug resistance [38,39].

What adds to the problem of *P. aeruginosa* causing HAIs is the emergence of MDR strains. In this study, a high prevalence of MDR *P. aeruginosa* strains (59.6%) was reported, and the highest prevalence of resistance for MDR strains was to Azetronam and Cefepime (92.85% for each). Similarly, a high rate of MDR was reported in a number of studies. For instance, in Turkey, Ünan and Gnsern [40] reported that 60% of their *P. aeruginosa* isolates were MDR; in Egypt, Mahmoud et al. [20] found that MDR *P. aeruginosa* were (52%) among their isolates.

The evolution of numerous MDR *P. aeruginosa* can be explained by the ability of the bacteria to acquire antibiotic resistance through horizontal gene transfer and spontaneous mutation [41].

The TCP assay is a simple and rapid method to quantify biofilm formation. We found that 13 strains out of 47 (27.7%) were strong biofilm producers, 10 (19.1%) moderate and 24 (51.1%) weak or non-biofilm producers.

It is noteworthy that literature shows results quite consistent with the present ones. In Egypt, Hisham et al. [42], found that 16 isolates (80%) were strong biofilm producers; 2 isolates (10%) were moderate and another 2(10%) were weak. Also, Abd El-Galil et al. [43]. found that 42 isolates (84%) were strong biofilm producers; 4 isolates (8%) were moderate ones and 4 isolates (8%) were weak ones. With no much difference from the present study results, Maita and Boonbumrung found that 60% of strains were strong biofilm producers from a total of 136 strains; 11% were moderate and 22% non-producers.

In the present study, biofilm production was higher among blood and sputum than other specimens. The cause can be ascribed by the observation that biofilm-colonizing devices implanted inside the body or forming a connection between inner and outer surface of the body where a normal microbial flora is present, are to blame. This type of infections is particularly associated with orthopedic devices and intravenous catheters.

Statistical analysis of this study showed significant association (P value ≤0.05) between biofilm production and MDR. 40.7% were MDR and biofilm producers and 19.1% MDR and non-producers.

Previous studies have shown that biofilm formation is higher in MDR strains [44-46]. This may be referred to the protective nature of the biofilm that makes the bacteria (i.e. growing intrinsically) resistant to many antibiotics up to 1000 times higher than normal levels. Another reason is the slow growth rate of the bacteria in the presence of antibiotic degradation mechanisms.

In this study, the resistance pattern to Ciprofloxacin, Gentamicin, Cefepime, Ceftazidime, Azetronam and Pipracillin-Tazobactam was significantly higher (p value ≤0.05) to biofilm than non-biofilm-producing strains.

It is worth mentioning that Maita and Boonbumrung found that the antibiotic resistance to Amikacin, Gentamicin, Ceftazidime, Cefepime, Imipenem, Meropenem, Cefoperazone/Sulbactam and Piperacillin/Sulbactam was higher among biofilms producing *P. aeruginosa* than that which was non-producers. However, Levofloxacin and Ciprofloxacin were found to exhibit similar resistance in both biofilm producers and non-producers. In the case of the biofilm-producing strains, relating results in the present study found an increase more than 50% of the resistance to Ceftazidime (52.8%), Levofloxacin (51.9%), Ciprofloxacin (51.9%) and Cefoperazone/Sulbactam (55.6%).

In the present study, the percentages of a number of *P. aeruginosa* virulence genes (*toxA, nan1, algD, pelA* and *pslA* genes) and their relation to the site of infection were detected. It is known *toxA* gene encodes exotoxin A that acts as a major virulence factor of *P. aeruginosa*. The gene was detected in 45 isolates (95.7%). Other studies reported the same results as Qin et al., Lavenir et al. and Nikbin et al. [47-49].

As for *algD* gene, it encodes GDP-mannose 6-dehydrogenase enzyme which catalyzes the oxidation GDP-D-mannose to GDP-D-mannuronic acid, a precursor for alginate polymerization. The alginate layer causes a mucoid phenotype and provides a protective barrier against host immune defenses and antibiotics. In the present study, evidently, it was detected in 42 isolates (89.4%). Al-Dahmoshi et al. [14], and Ra'oof revealed that all isolates had the *algD* gene and showed high capacity of alginate biofilm formation which interfered with response of the *P.aeruginosa* isolates to antibiotics.

PelA gene, which is necessarily associated to the polysaccharide stage of biofilm

development and maintenance, was detected in 41 isolates (87.2%) and in 19 biofilm- producing strains out of 23 (82.6%). This result corresponds to Sharma and Choudhury's [50].

Equally important, we found *pslA* in 42 isolates (89.4%). Moreover, all 23 biofilm-producing strains expressed *pslA* gene with statisticaly significant *P.* value (*P* ≤0.05) in a manner comparable with Maita and Boonbumrung.

Several studies suggest that the *nan1* gene encodes neuraminidase which has a role in enhancing bacterial adhesion. *Nan1*gene was detected in 19 isolates (40.4%), e.g. Strateva, Mitov and Ra'oof [6,51,52].

The percentages of all virulence genes were high in ICU, surgery and Burn Unit. The differences in the distributions of virulence factor genes in the populations strengthen the probability that some *P. aeruginosa* strains are better adapted to the specific conditions found in specific infectious sites [53].

The study results were limited to a sample of 47 strains. The reason behind this was due to insufficient financial support and the short duration of the study period. However, we anticipate the expansion of the work on a large sample size in a longer period.

We conclude that *P. aeruginosa* is seen an extremely versatile micro-organism. It will continue to surprise us yet with unappreciated modes of niche adaptation, lifestyle, and pathogenicity. We conclude that there is relationship between virulence genes and biofilm formation in *P. aeruginosa.* We advise the expansion of work on a large sample size in a longer period of time in order to study other virulence genes.

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