Characterization of biofilm formation, pyocyanin production, and antibiotic resistance mechanisms in drug-resistant *Pseudomonas aeruginosa* isolated from children in Egypt

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**ABSTRACT**

Children can be exposed to more bacterial infections, which has become of importance, especially when bacteria resist many of the antibiotics used today. Twelve *Pseudomonas aeruginosa* strains were collected and identified. Antibiotic sensitivity testing, biofilm formation, and pyocyanin production were evaluated. A cluster analysis was performed. Phenotypic and genotypic detection of efflux pump activity and Metallo-β-Lactamase (MβLs) resistance mechanisms were studied. A marked resistance (100%) resulted toward antibiotics including cefotaxime, cefoxitin, ceftriaxone, and meropenem. Imipenem and amikacin antibiotics showed sensitivity percentages of 41.7 and 33.3, respectively. 66.7% were able to form a biofilm at which they were categorized as moderate (25%) and weak (41.7%). Phenotypically, 41.67% of the isolates were MBL metallo-β-lactamase-positive, but genotypically, *bla*VIM gene was detected only in one isolate, while *blaIMP* was not detected in any isolate. MexR and MexZ genes were detected in all isolates (100%). NfxB gene and MexT gene were found in 27.27% and 45.45% of isolates, respectively. All the efflux genes were found collectively in three isolates. This study highlights the occurrence of antibiotic resistance, besides the production of important virulence factors (biofilm formation and pyocyanin production). Also, the gene occurrence of antibiotic resistance mechanisms was reported among our *P. aeruginosa*. This virulent bacterial behavior is alarming which needs attention to the way we use antibiotics.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a pathogen that can survive in different environments (Logan et al., 2017). It is also described as an important human pathogen, responsible for nosocomial infections (Ruiz-roldán et al., 2018). It is reported that multidrug-resistant (MDR) *P. aeruginosa* infections were associated with cost increase per patient in comparison with those nonresistant ones (Morales et al., 2012). Children are much more sensitive to disease-causing bacteria than adults, whereas no descriptions exist about characterization of *P. aeruginosa* isolates from children (Ruiz-roldán et al., 2018).

However, the treatment of those infections has become of importance as a result of its antibiotic resistance behavior (Pachori et al., 2019). Nowadays, *P. aeruginosa* displays resistance to a variety of antibiotics, including different classes (Alnour and Ahmed-Abakur, 2017), besides a large number of virulence factors (toxin A, alkaline protease, elastase, exoenzymes, and pyocyanin production) that play important roles in the pathogenicity process (Jazayeri et al., 2016). Bacterial biofilm formation in both medical and industrial fields causes big problems, as these structures resist the antibiotics and biocides treatments, besides reducing the host immune-responses action (Lima et al., 2018). *Pseudomonas aeruginosa* intrinsic resistance includes efflux pump expression that expels antibacterial agents outside the cell and produces antibiotic inactivating enzymes (Sachdeva et al., 2017). Because of all this, this study was aimed at studying antibiotic resistance profile, resistance mechanisms, and virulence factors in *P. aeruginosa* collected from children.
MATERIALS AND METHODS

Collection and identification P. aeruginosa strains
The study was conducted with 12 P. aeruginosa selected out of 136 Pseudomonas species that were collected from clinical samples routinely sent to the microbiology laboratory in a tertiary care pediatric Cairo University hospital in 3 months from October 2019 to December 2019. All the collected isolates were identified according to the standard procedures and confirmed by growing on Cetrimide agar and examined under ultraviolet to see the fluorescence of their colonies (Singh et al., 2015). The standard strain P. aeruginosa (American Type Culture Collection 15442) was used as control.

Antibiotics susceptibility tests
The bacterial antibiotic susceptibility profile was tested using Kirby–Bauer disk diffusion technique (Clinical and Laboratory Standards Institute (CLSI), 2019), using the commercial antibacterial agents (Oxoid, UK): ciprofloxacin (CIP, 5 µg), meropenem (MEM, 10 µg), gentamicin (CN, 10 µg), amikacin (AK, 30 µg), cefotaxime/cloxacillin (CTC, 40 µg), cefotaxime (CTX, 30 µg), cefoxitin (FOX, 30 µg), ceftriaxone (CRO, 30 µg), and cefepime (FEP, 30 µg).

Detection of virulence factors

Biofilm ability using microtiter plate assay
Biofilm formation
Pseudomonas aeruginosa isolates were screened for their biofilms formation ability by microtiter plate according to the method described (Mohamed et al., 2018c). 100 µl P. aeruginosa suspension of 0.5 McFarland was added to sterile brain heart infusion broth (100 µl) in wells of 96-well microtiter plate (Cellstar®, Greiner Bio-One) and then incubated at 37°C. The assay was done in triplicate.

Biofilm quantification using crystal violet (CV) assay
After 18 hours incubation period, wells were evacuated and gently washed. Pseudomonas aeruginosa biofilms formed on the walls were dried and stained with 1% CV for 20 minutes. After properly washing and drying, the stained P. aeruginosa biofilm was reconstituted in absolute ethanol, and its absorbance was measured at 630 nm using microplate reader (Stat Fax-2100; GMI, Inc.,). Optical density cut-off value (O.Dc) was calculated as mentioned by (Mohamed et al., 2020b).

Pyocyanin production
Phenotypic visual detection
Phenotypic detection was done as described with minor modifications. Briefly, the bacterial suspension (0.5 McFarland) was swabbed on Pseudomonas-Agar P media plates supplemented with 1.5% glycerol. After incubation for 24 hours, agar was cut into small pieces, and pyocyanin was extracted with chloroform after shaking. The chloroform color changed to dark green or blue and then was re-extracted into 1 ml of HCl (0.2N) to give a pink to a deep red solution, which was considered positive results (Feghali and Nawas, 2018).

Quantitative of pyocyanin
Pigment quantification was done depending on the absorbance of pyocyanin in acidic solution, at which solution was measured, and the concentrations expressed as µg/mL of pigment produced by the culture supernatant were calculated by using the optical density reading at 520 nm multiplying by 17.072 (Khadim and Marjani, 2019).

Cluster analysis
Based on the phenotypic traits like antibiotics susceptibility results and virulence factors (biofilm formation ability and pyocyanin production) of different P. aeruginosa strains, hierarchical cluster analysis was performed using Statistical Package for the Social Sciences software (SPSS Inc. v. 12). The antibiotic susceptibility results for each were coded as “1” resistant and “0” nonresistant. The positive results of biofilm formation and pyocyanin production for each strain were coded as “1” whereas the negative ones were coded “0.” The analysis was presented graphically to find the similarity among strains, based on average linkage, and the branch length represents the distance between the strains (Mohamed et al., 2018a).

Phenotypic detection of efflux pump activity
Minimum inhibitory concentration (MIC)
Resistance to CIP was confirmed by MIC evaluation using an antibiotic agar dilution test (Al Rashed et al., 2020). CIP stock solution was prepared according to manufacturer recommendation. Antibiotic final concentration range of 2 mg/l up to 512 mg/l and 0.5 McFarland standard bacterial turbidity were used. A volume of 10 µl of each inoculum was pipetted into serial agar plates. After incubation for 18 hours at 37°C, MIC was read. The interpretation of MIC values was done according to CLSI recommendations (Clinical and Laboratory Standards Institute (CLSI), 2019).

Ethidium bromide-(EtBr-) agar cartwheel method
Tryptic soy agar plates containing EtBr concentrations (Sigma-Aldrich) of 0–3 mg/l were swabbed with bacterial suspension (0.5 MacFarland), making cartwheel pattern (Osman et al., 2018). All plates were examined under the UV light, after the incubation period of 18 hours at 37°C. EtBr concentration at which the swabbed culture produces fluorescence was recorded. The higher the concentration of EtBr required for the appearance of fluorescence considered, the greater the EP activity. P. aeruginosa standard strain is considered a negative control.

Detection of MβLs
This test was done using the combined imipenem/ethylenediaminetetraacetic acid (EDTA) disk method (Alkhudhairy and Al-Shammari, 2020). Single imipenem disk and imipenem disk (10 µg) supplemented with 10 µl EDTA (750 µg) were placed on the plate and incubated for 18 hours period at 37°C. A 7 mm inhibition increase of the imipenem/EDTA disk than the single imipenem disk was considered a positive result.
Genotypic detection of resistance mechanisms

Total bacterial DNA extraction was done as mentioned by Mohamed and Khalil (2020). Detection of resistance genes was done using primers mentioned in Table 1. Cycling conditions for the efflux pump regulatory genes and both blaIM and blaVIM genes were performed as mentioned. The amplified DNA was electrophoresed using 1.6% agarose gel stained with EtBr, and the bands were visualized under ultraviolet trans-illuminator (Alkhudairy and Al-Shammari, 2020; Osman et al., 2018).

RESULTS

In this study, 12 P. aeruginosa strains were included, at which endotracheal aspirate (50%) was the most predominant source among our isolates, followed by sputum (33.3%) and urine (16.67%). High resistance rates (100%) toward cephems antibiotics (including CTX, FOX, and CRO) and carbapenems antibiotic (MEM) were observed (Table 2). On the other hand, imipenem and AK were the most effective antibiotics by sensitivity percentages of 41.7 and 33.3, respectively. Out of 12 strains, 8 (66.7%) were able to form a biofilm at which they were categorized as moderate (25%) and weak (41.7%); besides, 4 isolates (33.3%) were found non-biofilm formers. Three isolates showed positive results through the visual pyocyanin test (Table 3), and by spectrophotometer, pyocyanin concentrations of 1.23, 1.1, and 0.836 μg/ml were detected.

Cluster analysis of converted antimicrobial susceptibility and virulence factors production data was performed and presented in a dendrogram (Fig. 1) showing the difference between the used P. aeruginosa strains.

CIP susceptibility was confirmed among all isolates using agar dilution method, at which isolates were categorized at breakpoint recommended by CLSI, one isolate was categorized as not resistant with MIC value <2 μg/ml, one isolate was categorized as resistant with MIC value 4 μg/ml, three isolates were categorized as resistant with MIC value 16 μg/ml, two isolates were categorized as resistant with MIC value 32 μg/ml, two isolates were categorized as resistant with MIC value 128 μg/ml, and three isolates were categorized as resistant with MIC value 256 μg/ml. By using the EtBr-agar Cartwheel method, results showed that isolates express efflux pump activity by different levels. The ATCC strain was found to fluoresce at EtBr concentration of 0.5 mg/l (negative control). The minimum concentration at which the adapted isolates showed fluorescence was 1 mg/l for isolates P1, P2, P3, and P4, while it was 1.5 mg/l for isolate P10, but isolate P8 was 2.5 mg/l, and isolates P11 and P12 were 3 mg/l; however, the isolates P6, P7, and P9 were more than 3 mg/l. The previous results revealed that isolates P6, P7, and P9 have a high efflux pump activity. Seven isolates (58.3%) were defined as imipenem resistance using the cut-off value of 1.5 cm according to CLSI recommendation using disk diffusion method. By using imipenem/EDTA combined disk test, the increase in inhibition zone with the imipenem/EDTA disk 7 mm or more than the imipenem disk alone was observed in five isolates [positive metallo-[β-lactamase (MBL)] (Table 4).

All CIP-resistant isolates (n = 11) were subjected to genotypic detection for efflux pump genes presented in Table 1. Both MexR and MexZ genes were detected in all isolates (n = 11). NfxB gene and MexT gene were found in 3 isolates and 5 isolates, respectively. All genes were found collectively in three isolates (P3, P4, and P11). Concerning carbapenem genes, seven isolates (Imipenem-resistant) including five isolates (positive MBL) that previously showed positive results on phenotypic screening and

### Table 2. Resistance percentages of P. aeruginosa toward different antibiotics.

| Antibiotic family | Antibiotic | Resistance pattern (n = 12) |
|-------------------|------------|-----------------------------|
|                   |            | Resistant | Nonresistant |
|                   |            | No. | %    | No. | %    |
| Carbapenems       | MEM        | 12  | 100  | 0   | 0    |
|                   | IPM        | 7   | 58.3 | 5   | 41.7 |
| Aminoglycosides   | AK         | 8   | 66.7 | 4   | 33.3 |
| Fluoroquinolones  | CIP        | 11  | 91.7 | 1   | 8.3  |
| Cephems           | CRO        | 12  | 100  | 0   | 0    |
|                   | FOX        | 12  | 100  | 0   | 0    |
| B-lactam combination | CTC    | 9   | 75   | 3   | 25   |

### Table 1. Primer sequences used in this study.

| Resistance mechanism | Gene   | Product size (bp) | Sequence |
|----------------------|--------|-------------------|----------|
| Efflux pump activity | MexAB-OprM | MexR | 637 | F: CGCCATGGCACCAGTATTTGAG |
|                      | MexCD-OprJ | NfxB | 939 | R: GCCATCCGCCAGTAAGCAG |
|                      | MexEF-OprN | MexT | 997 | F: CGATTCCTTCCTATTGCACG |
|                      | MexXY-OprM | MexZ | 781 | R: CGAGGAGCGACAGTAGCATA |
| Carbapenems          | blaIM    | 232  | F: CGAATGCGCAGTATTTGAG |
|                      | blaVIM   | 390  | R: CGAATGCGCAGCAGGAG |

Osman et al., 2018

Alkhudairy and Al-Shammari, 2020
two isolates (negative MBL) were subjected to *bla*VIM and *bla*IMP genes detection. Results showed that *bla*VIM gene was found only in one isolate (Table 4).

**DISCUSSION**

*Pseudomonas aeruginosa* is commonly known to cause healthcare-acquired infections showing high rates of mortality. Recently, many studies had been conducted to study bacterial infections among children, especially *P. aeruginosa* (Bitsori et al., 2012; Hassuna et al., 2015; Logan et al., 2017). We found that the frequency of *P. aeruginosa* in children infections reported differs according to the region. In Omani’s study published in 2015, among children uropathogens, the low frequency of *P. aeruginosa* was reported (2.86%) (Sharef et al., 2015). Similar low frequencies of 2.3% and 5.9% were also reported in Saudi Arabia among children with urinary tract infection (Alshamsam et al., 2009; Hameed et al., 2019) and 11.54% among children diagnosed with respiratory tract infections (Walid et al., 2016). In the United Arab Emirates, *P. aeruginosa* (36%) was the most common bacteria in CF children (Redha and Panickar, 2016). In 2018, a study conducted on children patients revealed that *P. aeruginosa* was the most prevalent by frequently 16.67%; those bacteria were found to be MDR. The authors suggest that this may be due to the high prevalence of the use of invasive devices during the period of hospitalization (El-Nawawy et al., 2018).

### Table 3. Source and virulence factors distribution among *P. aeruginosa* strains.

| Isolates | Source   | Biofilm interpretation | Pyocyanin production |
|----------|----------|------------------------|----------------------|
|          |          |                        | Visual detection     | Concentration (µg/ml) |
| P1       | ETA      | ++                     | –                    | –                     |
| P2       | ETA      | ++                     | –                    | –                     |
| P3       | ETA      | ++                     | –                    | –                     |
| P4       | ETA      | –                      | +                    | 1.23                  |
| P5       | Urine    | +                      | +                    | 1.10                  |
| P6       | Urine    | –                      | –                    | –                     |
| P7       | ETA      | +                      | –                    | –                     |
| P8       | ETA      | –                      | –                    | –                     |
| P9       | Sputum   | –                      | –                    | –                     |
| P10      | Sputum   | –                      | +                    | 0.836                 |
| P11      | Sputum   | +                      | –                    | –                     |
| P12      | Sputum   | +                      | –                    | –                     |

For biofilm interpretation: - = nonbiofilm; + = weak biofilm; ++ = moderate biofilm; ETA = endotracheal aspirate. For pyocyanin production: - = negative and +: positive.

### Table 4. MBL screening using imipenem/EDTA combined disk test (CDT) and the occurrence of *bla*VIM and *bla*IMP genes.

| Isolates | MBL screening |
|----------|---------------|
|          | EDTA          | IPM | IPM/EDTA |
| P1       | 0.9           | –   | 3        |
| P2       | –             | 1.4 | 2.6      |
| P4       | 2             | 1.4 | 1.9      |
| P8       | 1.7           | 1.3 | 1.9      |
| P9       | 0.8           | –   | 2.2      |
| P10      | 1             | –   | 2.7      |
| P12      | 0.9           | –   | 2.3      |
| Control  | 2             | 2.2 | 2.5      |

For inhibition zones (cm): nd = not determined.

**Figure 1.** Hierarchical cluster analysis of different strains of *Pseudomonas aeruginosa.*
Besides the fact that \textit{P. aeruginosa} is naturally resistant to many antimicrobial agents, they easily acquire resistance to new ones (Alnour and Ahmed-Abakur, 2017). This antimicrobial behavior significantly limits therapeutic options (Hirsch and Tam, 2010). Herein, high percentages of resistance to several antibiotics were detected (Table 2), at which a 100% resistance toward cephems antibiotics was observed; our results are in line with those reported about children (Hassuna et al., 2015; Pourakbari et al., 2016), but opposite to those reported among Spanish children (Ruiz-roldán et al., 2018) and Saudi children at which \textit{P. aeruginosa} was found to be more sensitive to a range of antibiotics (Hameed et al., 2019).

Genetically, \textit{P. aeruginosa} virulence is known to be multifactorial and combinatorial (Wendt et al., 2017). Such bacterial pathogens always share common mechanisms for their abilities to adhere, invade, survive host defenses, and cause infection (Wilson et al., 2002). The pathogenesis of \textit{P. aeruginosa} infection is somehow related to its ability to synthesize some virulence factors (pyocyanin; proteases) and to form biofilms (Maiser et al., 2020). In this study, biofilm formation ability was studied as one factor of the important virulence factors presented in \textit{P. aeruginosa}, and as a result, 66.7% were able to form a biofilm at which their ability is ranging between moderate and weak. During our research journey, only a few types of research studied the biofilm formation among \textit{P. aeruginosa} isolates from children's infections. Ralte et al. (2019) reported that, among different bacterial species isolated from children, the \textit{P. aeruginosa} strain showed a strong ability to produce a biofilm. Biofilm formation \textit{P. Aeruginosa} ability is an important process for bacterial colonization for persistence (Vallet et al., 2004), making it a problematic issue especially for patients requiring mechanical ventilation and catheterization (Alnour and Ahmed-Abakur, 2017). Inside those biofilms, bacteria became more resistant and difficult to eradicate than the planktonic ones (Mohamed et al., 2018b, 2020a); therefore, this mode of bacterial growth is strongly linked to various diseases at which many bacterial infections are biofilm-related (Mohamed et al., 2019, 2020c).

Three of our isolates are shown to produce pyocyanin pigment after 24 hours with different concentrations (Table 3). \textit{P. aeruginosa} synthesizes a characteristic chloroform-soluble blue redox-active secondary metabolite called pyocyanin (Khadim and Marjani, 2019), which is considered an important virulence factor since it has a role to induce oxidative stress (Jazayeri et al., 2016). Cluster analysis was applied to detect the similarity of our \textit{P. aeruginosa} strains phenotypic traits (Fig. 1). This analysis was performed to survey the prevalence of bacterial antibiotic susceptibility and complete phenotypic classification (Berrazeg et al., 2013).

It is reported that rates of antibiotic resistance among children \textit{P. aeruginosa} infection are rising nationally (Logan et al., 2017). This bacteria can acquire new resistance mechanisms (Ruiz-roldán et al., 2018); the main ones for \textit{P. aeruginosa} were mutation in target sites, changes in membrane permeability, the transmission of plasmid resistance genes, and efflux mechanism (Bejestani et al., 2015). In our study, two resistance mechanisms (efflux pump and MBL) were studied phenotypically and some genes have been detected genotypically. All CIP-resistant isolates showed higher MIC for EtBr than the standard strain using the phenotypic method. Reports of efflux pump percentages among children's \textit{P. aeruginosa} infections were also found. Among children's \textit{P. Aeruginosa} infected by Pourakbari et al. (2016), 62% showed an increased expression level of efflux pump genes. Helmy and Kashef (2017) reported that 23.5% of \textit{P. aeruginosa} isolates showed multidrug efflux-mediated resistance. Many phenotypic methods have been used nowadays to detect MBL producing bacteria, which are majorly based on the ability to inhibit MBLs using metal chelators (Sachdeva et al., 2017). Our data identified that 41.67% of the \textit{P. aeruginosa} isolates are MBL-positive using the combined imipenem/EDTA disk test method. In the United States, 11.3% of \textit{Pseudomonas} was found to be carbapenem-resistant (Logan et al., 2017). Bejestani et al. (2015) reported that the frequency of imp gene was detected in 3.3% \textit{P. aeruginosa} isolates; on the other hand, the vim gene was not detected in any of MBL-positive isolates (0%), at which this low frequency comes in line with our results.

**CONCLUSION**

This study highlights the high frequency of antibiotic resistance, besides the production of important virulence factors (biofilm formation and pyocyanin production). Also, the gene occurrence of antibiotic resistance mechanisms was reported among our \textit{P. aeruginosa} from children. This virulent bacterial behavior is alarming which needs attention to the way we use antibiotics. However, studies with a larger number of \textit{P. aeruginosa} children isolates are recommended.

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**ETHICAL APPROVAL**

This study involved microbes isolated in microbiology laboratory as a part of continuous routine laboratory work and was determined not to be human subject’s research. The study did not involve clinical data for patients and did not involve laboratory animals or invasive procedures.

**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest.

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