

Virulence Constitution of Multi-Drug-Resistant *Pseudomonas aeruginosa* in Upper Egypt

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Purpose: Ventilator-associated pneumonia caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) is a major health-care problem. In this study, we explored the epidemiology of virulence determinants among multi-drug-resistant (MDR) clinical *P. aeruginosa* isolates from hospitalized patients with ventilator-associated pneumonia in intensive care units in Upper Egypt.

Patients and Methods: MDR *P. aeruginosa* isolates were screened for the presence of eight virulence factors and typed by ERIC-PCR.

Results: A total of 39 clinical MDR isolates were selected out of 173 isolated *P. aeruginosa* showing a combination of adhesion and cytotoxicity virulence patterns, with the detection of *aprA*, *exoU*, *exoS*, *lasB*, *algD*, *toxA* in 74.3%, 58.9%, 46.1%, 41.2%, 30.7%, 20.5% of the isolates, respectively. The MDR isolates were grouped into 13 different virulence profiles according to the pattern of virulence gene distribution. *exoU* genotype was more predominant among the *P. aeruginosa* isolates with more than 48% of the isolates harboring this gene alone, 7% harboring both *exoU* and *exoS* and 43.5% harboring *exoS* gene. An intermediate degree of diversity was detected by ERIC-PCR typing where the isolates were clustered in 7 major groups, indicating possible cross-infection within the hospital.

Conclusion: Our results highlight the increased frequency of virulent *P. aeruginosa* isolates with a shift to the more virulent cytotoxic *exoU* genotype. Further hospital infection-control measures are mandatory to control the hospital cross-transmission of these highly virulent isolates. This study could vastly be a help to develop efficient treatment policies against *P. aeruginosa* induced ventilator-associated pneumonia.

Keywords: *P. aeruginosa*, virulence, *exoU*, MDR

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most common causes of healthcare-associated infections being responsible for urinary tract, respiratory and surgical site infections.^{1,2} This opportunistic pathogen is considered as a major health hazard, especially in immunodeficient patients.³ Furthermore, infections caused by multi-drug-resistant (MDR) *P. aeruginosa* isolates are associated with longer duration of hospitalization, increased costs, as well as increased morbidity and mortality rates.⁴

P. aeruginosa has a repertoire of virulence factors that markedly contribute to its pathogenicity⁵ e.g., lipopolysaccharides, adhesion factor (pili type IV), flagella, exo-proteases (alkaline protease “AprA”, elastase, staphylolysin, protease IV), phospholipase C, exotoxins “Exo A”, exoenzymes S, T, and U, as well as sialidase.^{5–9} In addition, *P. aeruginosa* has the exquisite ability to form biofilms, which render it more resistant to antimicrobials.¹⁰

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Exoenzymes S, Y, T, and U are encoded by the genes *exoS*, *exoY*, *exoT*, and *exoU*, respectively, with *exoS* being the most predominant.¹¹ While former studies have shown that the existence of *exoS* is related to increased virulence in burn wounds and lung infections,¹² *ExoU* is 100 times more cytotoxic than *ExoS*.^{5,13} *LasB* elastase, a zinc metalloprotease encoded by the *lasB* gene, attacks eukaryotic proteins such as elastin and collagen.^{10,14} In addition, two phospholipases C encoded by *plcH* and *plcN* genes can hydrolyze phospholipids. The high frequency of virulence factor phospholipase C gene (*plcH*) in MDR *P. aeruginosa* clinical isolates probably plays an important role in the pathogenesis of this bacterium. The gene *algD* encodes the GDP-mannose dehydrogenase enzyme, which is the first element in the alginate biosynthetic cluster essential for alginate biosynthesis. Alginate is a linear exopolysaccharide, which protects the bacterium from antibiotics and the host's immune response.¹⁰

The increased invasiveness and pathogenicity as a result of harboring these virulence factors upsurge the morbidity despite the use of antibiotics.¹⁵ However, the arsenal of virulence mechanisms harbored by *P. aeruginosa* varies according to the settings and the environment of infection.¹⁶

In previous studies done in this region, a high incidence of MDR *P. aeruginosa* was observed among isolates from infected burn and surgical wounds.^{17,18} The identification of virulence genes' profile is crucial for developing efficient policies against *P. aeruginosa* infections; the aim of this study was to evaluate the distribution of different virulence genes among MDR *P. aeruginosa* isolated from different Egyptian ICUs.

Materials and Methods

In this cross-sectional study, samples were collected from respiratory tract infections over a period of 15 months between 2017 and 2019 from patients admitted to the different ICUs in Minia University Hospital (a tertiary care hospital). A written informed consent was obtained from every patient or his caregiver. The study was carried out as per the Helsinki declarations and was approved by the Ethical committee of the Faculty of Medicine, Minia University. A total of 173 *P. aeruginosa* isolates were phenotypically identified by routine cultural and biochemical methods. MDR isolates were selected for further characterization by antibiotic sensitivity testing using the disc-diffusion method according to CLSI 2017, using *P. aeruginosa* ATCC 27853 as a reference strain. MDR is identified as resistance to three or more groups of antimicrobials.

DNA Extraction, Identification and Virulence Gene Detection

Genomic DNA was extracted using a boiling method as described previously; one loopful of fresh bacteria (grown overnight on Brain-Heart Infusion agar plates) was picked up and suspended in 200 μ L of sterile DNase/RNase-free water and boiled for 10 min. The bacterial suspension was then centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatant was collected and kept at -20°C.¹⁹

Detection of the following virulence genes was carried out by conventional PCR: GDP-mannose dehydrogenase enzyme for alginate (*algD*), alkaline protease (*aprA*), elastase (*lasB*), exoenzyme S and U (*exoS* and *exoU*) and exotoxin A (*toxA*), hemolytic phospholipase C (*plcH*) and non-hemolytic phospholipase C (*plcN*), and the used primers are listed in Table 1. The amplification was carried in a 25 μ L volume, containing 12.5 μ L PCR Master mix (DreamTaq Green PCR master mix, Thermo scientific), 1 μ L of each primer (forward and reverse), 1 μ L of template DNA and nuclease-free water.

The PCR conditions for *lasB* and *aprA* comprised 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). The *exoS* and *exoU* amplification conditions included 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).^{11,20} For the rest of the genes, the conditions were as follows: preliminary 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.²¹ PCR products were run on 1.5% agarose gel and were afterward visualized under UV lamp.

Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR Typing)

ERIC PCR was performed to produce the repetitive sequence between the two primers.²² The PCR started with an initial denaturation at 94°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min and a final extension cycle at 72°C for 15 min. Amplicons were loaded in 1.5% agarose gel, which was run at 80 V for 3 hrs. Dendrogram was generated by using GelJ software using Dice Similarity method; UPGMA linkage.²³

Statistical Analysis

Statistical analysis was performed using Graph-pad Prism 6.

Table 1 Primers Used in This Study

Target Gene	Sequence (5' – 3')	Amplicon Size (bp)	Annealing Temperature
<i>toxA</i>	F: 5'GGTAACCAGCTCAGCCACAT 3' R: 5'TGATGTCCAGTCATGCTTC 3'	352	48°C
<i>plcN</i>	F: 5' GTTATCGCAACCAGCCCTAC 3' R: 5'AGGTCGAACACCTGGAACAC	466	48°C
<i>plcH</i>	F: 5'GAAGCCATGGGCTACTTCAA 3' R: 5'AGAGTGACGAGGAGCGGTAG 3'	307	48°C
<i>algD</i>	F:5' ATGCGAATCAGCATCTTTGGT 3' R:5' CTACCAGCAGATGCCCTCGGC 3'	1311	48°C
<i>lasB</i>	F:5'CCAGCCCCTGACCCACAAGCTGTA 3' R: 5'CATTCTTCCTGGAGTGCYRGCCG 3'	650	46°C
<i>aprA</i>	F:5' CCTGATCKGGCCGATAACTGCAAT 3' R:5' GGAAGACASCTATCAATTGCAACAG 3'	1580	46°C
<i>exoU</i>	F: 5' GGCACATATCTCCGGTTCCTTC 3' R: 5' TCAACTCAGCTGCCAACCATGC 3'	761	55°C
<i>exoS</i>	F: 5'ATGGCGTGTCCGAGTCA 3' R: 5' AGGTGTCGGTTCGTGACGTCT 3'	1587	55°C
ERIC primers	ERIC-1R, 5VCACTTAGGGGTCCTCGAATGTA-3V ERIC-2, 5V-AAGTAAGTGACTGGGGTGAGCG-3V		

Abbreviations: *toxA*, exotoxin A; *plcH*, hemolytic phospholipase C; *plcN*, non-hemolytic phospholipase C; *algD*, GDP-mannose dehydrogenase enzyme for alginate; *lasB*, elastase; *aprA*, alkaline protease; *exoU*, exoenzyme U; *exoS*, exoenzyme S; ERIC, Enterobacterial Repetitive Intergenic Consensus-PCR.

Results

Identification and Virulence-Profiling

This study included 39 (22.5%) MDR isolates from a total of 173 *P. aeruginosa* isolates (the antimicrobial-susceptibility profile of the MDR isolates to different anti-pseudomonal drugs is illustrated in [Supplementary Figure 1](#)). The prevalence of eight virulence genes among MDR *P. aeruginosa* nosocomial isolates was investigated, and the frequencies of *toxA*, *plcN*; *plcH*; *algD*; *lasB*; *aprA*; *exoU* and *exoS* genes among the MDR isolates were 9 (23%); 0 (0%); 5 (12.8%); 12 (30.7%); 17 (43.5%); 27 (67.2%); 22 (50.4%) and 20 (51.3%), respectively ([Figure 1](#)). None of the isolates harbored *plcN* gene. Only three isolates (7.6%) showed a simultaneous presence of *exoU* and *exoS* genes ([Table 2](#)).

The majority of the isolates (23 isolates) had between 1 and 4 virulence genes, and only two isolates showed 6 virulence genes altogether, while no isolates possessed more than 6 virulence genes. There were 13 different virulence profiles of MDR *P. aeruginosa* isolates with types 1, 2 and 3 being the most common, while the rarest were types 10–13 ([Table 3](#)). Furthermore, due to the danger of *exoU*-harboring isolates, the isolates were classified into three categories

according to the presence or absence of *exoU* and *exoS* genes ([Figure 2](#)), where *exoS*⁻/*exoU*⁺ genotype was the commonest genotype (48.7%).

Genotyping by ERIC-PCR

This study revealed intermediate diversity of ERIC fingerprints, with 2 major clusters (1 and 2) enclosing 22

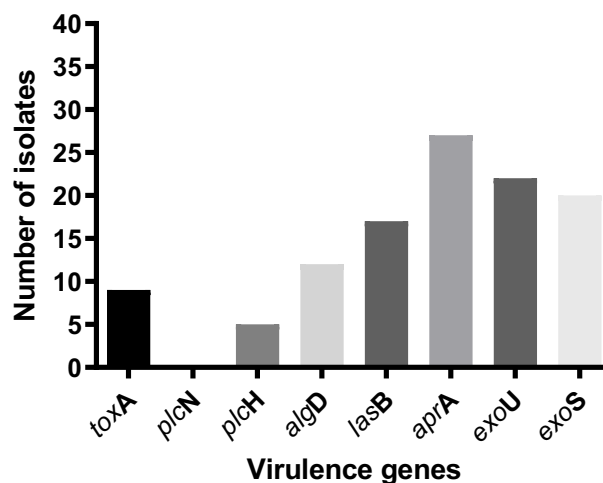
**Figure 1** Frequency of virulence genes in MDR *P. aeruginosa*.

Table 2 Characteristics of MDR *P.aeruginosa* Isolates from Different ICUs in Egypt

Isolate No.	toxA	plcN	plcH	algD	lasB	aprA	exoU	exoS	Number of Virulence Genes
PA-M1	-	-	-	-	-	+	+	-	2
PA-M2	-	-	-	-	-	+	+	-	2
PA-M3	-	-	-	-	+	+	-	+	3
PA-M4	-	-	-	-	-	-	-	+	1
PA-M5	-	-	-	-	+	+	+	-	3
PA-M6	-	-	-	-	-	-	+	+	2
PA-M7	-	-	-	-	-	-	+	-	1
PA-M8	-	-	-	-	+	+	-	+	3
PA-M9	-	-	-	-	-	-	-	+	1
PA-M10	-	-	+	+	-	-	+	-	3
PA-M11	-	-	-	+	+	+	+	-	4
PA-M12	-	-	-	-	+	+	-	+	3
PA-M13	-	-	-	-	+	+	+	-	3
PA-M14	-	-	-	-	+	+	+	-	3
PA-M15	+	-	+	+	+	+	-	+	6
PA-M16	-	-	-	-	-	+	+	-	2
PA-M17	+	-	-	+	-	+	-	+	4
PA-M18	+	-	-	+	-	+	+	-	4
PA-M19	+	-	-	+	-	+	-	+	4
PA-M20	-	-	-	-	-	+	+	-	2
PA-M21	-	-	-	-	-	+	+	-	2
PA-M22	-	-	-	-	+	+	-	+	3
PA-M23	-	-	-	-	-	-	-	+	1
PA-M24	-	-	-	-	-	-	-	+	1
PA-M25	-	-	-	-	-	-	+	+	2
PA-M26	-	-	-	-	-	-	+	-	1
PA-M27	+	-	+	-	+	-	+	-	4
PA-M28	-	-	-	-	+	+	+	-	3
PA-M29	-	-	+	+	-	-	+	-	3
PA-M30	-	-	-	+	-	+	+	+	4
PA-M31	-	-	-	-	+	+	-	+	3
PA-M32	+	-	-	+	+	-	-	+	4
PA-M33	-	-	-	-	+	+	+	-	3
PA-M34	+	-	+	+	+	+	-	+	6
PA-M35	-	-	-	-	-	+	+	-	2
PA-M36	-	-	-	-	+	+	+	-	3
PA-M37	-	-	-	-	+	+	-	+	3
PA-M38	+	-	-	+	-	+	-	+	4
PA-M39	+	-	-	+	-	+	-	+	4

Abbreviations: PA, *P. aeruginosa*; toxA, exotoxin A; plcH, hemolytic phospholipase C; plcN, non-hemolytic phospholipase C; algD, GDP-mannose dehydrogenase enzyme for alginate; lasB, elastase; aprA, alkaline protease; exoU, exoenzyme U; exoS, exoenzyme S; ERIC, Enterobacterial Repetitive Intergenic Consensus-PCR.

isolates, three groups with 3 isolates and two groups with 2 closely related isolates. Four isolates showed a unique pattern (9, 10, 15, and 25), while groups (3 and 7) were composed of 2 isolates (Figure 3). The difference in the ERIC regions is not strictly related to the occurrence of virulence genes. Furthermore, isolates with shared ERIC types displayed different virulence factor arrays.

Discussion

One of the very important pathogens causing opportunistic health-care-acquired infections is *P. aeruginosa*, because of its capacity to invade tissues using a myriad of enzymes and toxins.²⁴ It is contemplated as a threat to health particularly in patients with deficient immune response: as those in intensive care units (ICU) and

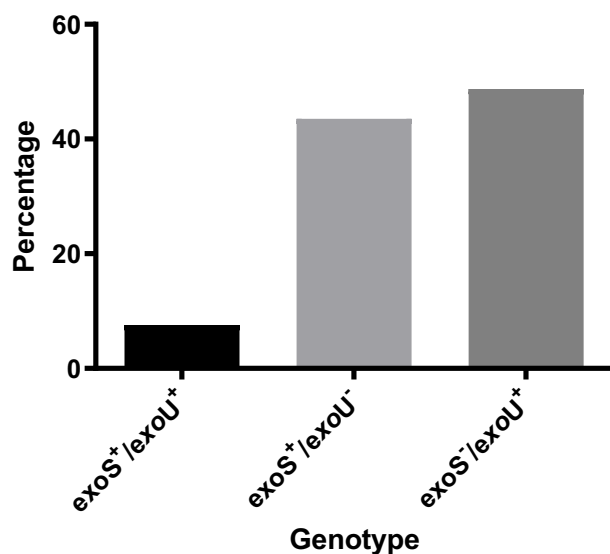
Table 3 Virulence Profiles of MDR *P. aeruginosa* Isolates

Profile	Virulence Gens	Number/%
(Profile 1)	<i>lasB-aprA-exoS</i>	6 (15%)
(Profile 2)	<i>lasB-aprA-exoU</i>	6 (15%)
(Profile 3)	<i>aprA-exoU</i>	6 (15%)
(Profile 4)	<i>toxA-algD-aprA-exoS</i>	5(12.8%)
(Profile 5)	<i>exoS</i>	4(10%)
(Profile 6)	<i>exoU</i>	2 (5%)
(Profile 7)	<i>exoU/exoS</i>	2(5%)
(Profile 8)	<i>toxA-plcH-algD-lasB-aprA-exoS</i>	2(5%)
(Profile 9)	<i>plcH-algD-exoU</i>	2(5%)
(Profile 10)	<i>toxA-algD-aprA-exoU</i>	1(2%)
(Profile 11)	<i>toxA-plcH-lasB-exoU</i>	1(2%)
(Profile 12)	<i>algD-lasB-aprA-exoU</i>	1(2%)
(Profile 13)	<i>algD-aprA-exoU-exoS</i>	1(2%)

Abbreviations: *toxA*, exotoxin A; *plcH*, hemolytic phospholipase C; *plcN*, non-hemolytic phospholipase C; *algD*, GDP-mannose dehydrogenase enzyme for alginate; *lasB*, elastase; *aprA*, alkaline protease; *exoU*, exoenzyme U; *exoS*, exoenzyme S; ERIC, Enterobacterial Repetitive Intergenic Consensus-PCR.

burn units.³ In fact, *P. aeruginosa* is the most commonly isolated pathogen from patients with ventilator-associated pneumonia (VAP) in ICUs.²⁵ Epidemiological evaluation of *P. aeruginosa* on the molecular level is extremely important so as to comprehend the potential risk elements related to the infection process, assist in finding epidemic strain(s) in a particular population of patients, and recognize virulent strain(s).²⁶

In the current study, MDR *Paeruginosa* showed a frequency of 22.5%, which is far less than previous results found in the same region.¹⁸ These could be due to

**Figure 2** Distribution of *exoU*, *exoS* genotypes among MDR *P. aeruginosa* isolates.

differences in the nature of infection with different associated risk factors.²⁷ In addition to the fact that the epidemiology of MDR microorganism infections fluctuates from year to year between hospitals, wards, departments in addition to the geographical zone.²⁸ Furthermore, a better implementation of antimicrobial stewardship in different ICUs is another reason for this decrease in MDR rate.

Identification of the *P. aeruginosa* armor of virulence factors is crucial to understand the pathogenesis of this opportunistic pathogen and is important in exploring new antimicrobial objectives especially in MDR strains. In this study, most of the isolates (23 isolates) had at least one virulence gene with a maximum of 6 virulence genes detected in 2 isolates only. The virulence phenotype was a mixture of adhesion and cytotoxicity, where *aprA* (encoding alkaline metalloproteinase) detected in 74.3% of the isolates; *exoU* (encoding exoenzyme U) in 58.9%; *exoS* (encoding exoenzyme S) in 46.1%; *lasB* (encoding elastase LasB) in 41.2%; *algD* (encoding GDP-mannose 6-dehydrogenase) in 30.7%; *toxA* (encoding exotoxin A) in 20.5% of the isolates and *plcH* (encoding hemolytic phospholipase C precursor) in 10.2% of the isolates.

The high incidence of *aprA* among our isolates is consistent with other studies,^{21,29} as this enzyme is essential for bacterial survival in tissues by diminishing the activation of Toll-like receptor 5 (TLR-5) and degrading host proteins as cytokines and complement.³⁰

We found a high frequency of *exoU* in our isolates indicating that more than half of the isolates display the cytotoxic phenotype,³¹ which is associated with more pulmonary damage than invasive phenotypes.³² This finding is consistent with Rodulfo et al,³³ who found a high frequency of *exoU* in MDR *P. aeruginosa* isolates. On the other hand, *exoS* was detected in a relatively similar frequency as *exoU*; with 17 isolates (43%) harboring *exoS* gene alone and 3 isolates possessing both genes (7.6%). These results are different from those obtained earlier by our team, where *exoS* genotype was much more frequent than *exoU*.³⁴ This could be due to the difference in sample source as in the earlier study samples were from infected surgical wounds.

Elastase *lasB*, an important virulence attribute detected in a relatively high frequency in our isolates, is known to evade the immune response and particularly target alveolar macrophages.^{20,32} Our results are concurring with several studies showing a high frequency of *lasB* in isolates from different sources.^{35–37}

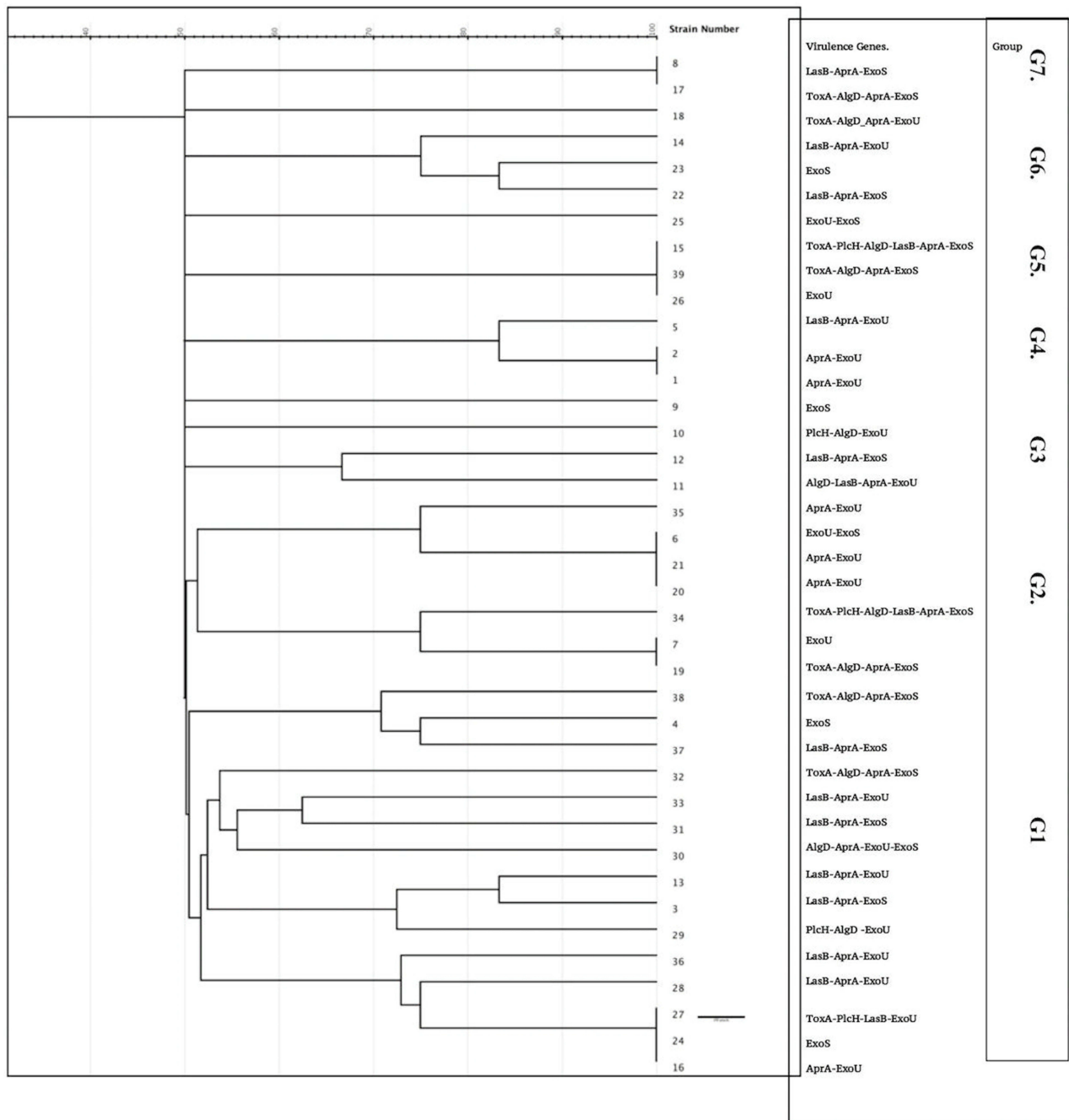


Figure 3 Dendrogram illustrating the genetic relationship among MDR *P. aeruginosa* isolates.

GDP-mannose dehydrogenase enzyme encoded by *algD* (which contributes to bacterial adhesion) was not very frequent in our isolates. These findings are not in agreement with Al Dawodeyah et al who found a very high frequency of *algD* among their respiratory isolates.³⁷ As this polysaccharide is a biofilm-related one, this might indicate the absence or lack of biofilm

formation by our isolates, which needs further confirmation in future work.

Exotoxin A (a very potent and is the most toxic virulence determinant of *P. aeruginosa*) was detected in a smaller number of our isolates. This finding is not concurring with a study carried by Nikbin et al or Khattab et al, who found that almost all of their pulmonary isolates harbored *toxA*.^{38,39}

Two phospholipases C determined by *plcH* and *plcN* genes are known to hydrolyze the phospholipids contained in pulmonary surfactants. We did not find any *plcN* in our isolates; however, *plcH* was detected in about 10% of MDR *P. aeruginosa*. This adds more threat, as *plcH* increases the virulence (causing organ injury and increased mortality) compared to *plcN*, which has no pathogenic effect.²¹ Our results are comparable to those done by Fadhil et al,²⁴ while higher incidence of both genes was reported in different studies involving *P. aeruginosa* from various hospital sources.^{6,21}

Bacterial phenotypic typing methods as phage typing and serotyping are now replaced by molecular methods as pulse-field gel electrophoresis (PFGE), ribotyping, and different PCR-based methods such as ERIC-PCR. ERIC-PCR is a technique that uses the difference in the position and number of ERIC sequences as an indicator of bacterial diversity.⁴⁰ Bacterial genotyping using ERIC-PCR is of tremendous importance in epidemiological studies carried on *P. aeruginosa* to evaluate genetic relations, particularly in health-care-acquired infections. In the current study, we found intermediate diversity of ERIC patterns, with no close relation to the presence of different virulence genes. In addition, we found that sharing the same ERIC group did not imply having the same virulence determinant pattern. Great genetic heterogeneity in *P. aeruginosa* isolates was reported by several studies using ERIC-PCR, RAPD, and PFGE.^{3,36,41} We used ERIC-PCR as it is cheap, reliable and has a good discriminatory power for *P. aeruginosa* genotyping.⁴² Our results suggest that nosocomial transmission of the isolates in this study and its cross-infection is relatively limited due to the relative degree of diversity among the ERIC-patterns apart from isolates in the first cluster, where cross-acquisition may have occurred. *P. aeruginosa* cross-infection is reported in several studies done in ICUs with variable rates between different hospitals.^{43–45}

Conclusion

In conclusion, pulmonary isolates obtained from ICUs in Upper Egypt possess a wide array of virulence determinants in addition to its MDR attributes. Furthermore, we report here a high incidence of the virulent *exoU* genotype, the presence of which in MDR *P. aeruginosa* found in our ICUs is of tremendous significance due to the harmful blend of increased cytotoxicity and limited treatment options for these patients.

Ethics Approval and Consent to Participate

A written consent was obtained from each patient or his/her caregiver and the study was ethically approved by the Ethical Committee of the Faculty of Medicine, Minia University.

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Disclosure

The authors declare no conflicts of interest in this work.

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