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ORIGINAL ARTICLE

Diversity of biofilm-specific antimicrobial resistance genes in *Pseudomonas aeruginosa* recovered from various clinical isolates

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ABSTRACT

Background and Objectives: The resistance of *Pseudomonas aeruginosa* to antibiotics offers a significant challenge in the treatment of patients. This study aimed to investigate the antimicrobial resistance profile, biofilm-specific antimicrobial resistance genes, and genetic diversity of *P. aeruginosa* recovered from clinical samples.

Materials and Methods: Totally 47 non-duplicate isolates of *P. aeruginosa* were recovered from various clinical samples. *toxA, algD, ndvB,* and *tssC1* genes were detected in biofilm-producing isolates. The DNA sequences of the *toxA* and *tssC1* genes were analyzed, by creating phylogenetic trees.

Results: The findings revealed that 30 (63.8%) of the isolates tested positive for Extended spectrum β-lactamase (ESBL), whereas 31 (65.9%) tested positive for Metallo-β-lactamase (MBL) and all of the isolates presented the *toxA* genes, and 19.1%,17%, 6.3% presented by *algD, ndvB* and *tssC1* genes. Besides, the phylogenetic trees of the *toxA* and *tssC1* gene isolates suggested a genotype that was closely aligned with others. Gene sequencing similarity revealed 99% identity with other isolates deposited in GenBank.

Conclusion: The occurrence of *toxA* was most prevalent. One isolate was recorded as a novel isolate in the global gene bank as a locally isolated strain from the city of Erbil that has never been identified in global isolates due to genetic variation.

Keywords: Biofilm; Drug resistance; Genetic variation; *Pseudomonas aeruginosa*; Virulence factors

INTRODUCTION

The rapid increase in the number of drug-resistant *P. aeruginosa* isolates in clinical settings poses a severe therapeutic challenge and has resulted in a higher mortality rate among patients with the infection (1). *P. aeruginosa* is characterized by intrinsic resistance to a broad range of antimicrobial drugs (2, 3). Biofilm-producing *P. aeruginosa* is the reason for a wide variety of serious infections such as post-surgical infections, otitis media, periodontitis, thermal burns, trauma, and cystic fibrosis (4). It is known that high levels of antibiotic resistance in biofilm producers are caused by a combination of variables that work together to result in a higher level of resistance than that found in planktonic cells (5). Due to the im-

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pact of the development and complexity of bacterial biofilms, many studies are trying to illuminate how these structures are designed (6). The expression of genes alters in biofilm cells, and most notably, genes associated with virulence factors and antibiotic resistance in *P. aeruginosa*, as well as genes associated with virulence factors and antibiotic resistance, exhibit differential expression in the biofilm growth modes (7). To identify novel mechanisms responsible for biofilm-specific antibiotic resistance, several well-characterized virulence factors of *P. aeruginosa* that facilitate the establishment of infections are *toxA, algD* gene, and *PlcH* (8). There are many genes involved in *P. aeruginosa* biofilm production; however, recent research has highlighted the importance of *tssC1* and *ndvB* (9). In biofilm-producing bacteria, *tssC1* and *ndvB* genes are expressed at greater levels. Biofilm-specific antibiotic resistance in *P. aeruginosa* is mediated in part by *tssC1*, a component of a type VI secretion system while *ndvB* plays a crucial role in the production of ethanol oxidation genes, which deplete antibiotics from biofilm-producing bacteria (10). The study aimed to explore antimicrobial susceptibility patterns, biofilm production, and frequency of *toxA, algD, ndvB,* and *tssC1* genes among *P. aeruginosa*, as well as perform DNA sequences of the biofilm specific antibiotic resistance locations of *toxA* and *tssc1* for detecting mutations in these genes and to find out the relationship among isolates by constructing phylogenetic trees.

MATERIALS AND METHODS

Identification of *Pseudomonas aeruginosa* isolates. A total of 47 non-duplicate isolates of *P. aeruginosa* from different clinical samples of urine, CSF, sputum, blood, and burn wounds were taken from patients suffering from infections. These samples were collected between September 2020 to March 2021, from patients at Rizgari Teaching Hospital, Erbil Laboratory Center, and Nanakaly Hospital in the Erbil-Kurdistan region. All of the isolates were identified using the VITEK-2 compact system (BioMérieux, Marcy L'Etoile, France). All isolates were kept at -70°C in tryptic soy broth (TSB) containing 20% glycerol until further analysis was carried out (11).

Ethical approval. This study was approved by the ethical committee of Hawler Medical University (HMUPH-EC 2020905-451). The participants were given information about the purpose of the study as well as information about secrecy. Parents were interviewed in the case of children. Finally, all of the participants in this study gave their verbal consent.

Phenotypic methods for ESBLs and MBL and biofilm production. All isolates of *P. aeruginosa* were tested for their ability to produce ESBL and MBL. The combined disk tests and the double disk method were employed to determine the presence of ESBL and the production of MBL (12, 13). A phenotypic evaluation of biofilm production was carried out by Congo red agar technique (14, 15).

Molecular detection of the genes. Virulence genes, including *toxA, algD, ndvB,* and *tssC1* were detected using specific primers as described in the previously published study (16).

DNA sequencing. Sequencing of the target PCR products was performed by the National Instrumentation Center for Environmental Management (Seoul, Korea). *toxA* and *tssc1* sequences were compared with the existing sequence data using BLAST searches, against the GenBank database (National Center for Biotechnology Information,) to identify the sequences. Multiple sequence alignment was manually performed with closely related reference sequences from other *P. aeruginosa* isolates, available in GenBank using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov). To do multiple sequence alignment in Clustal W and to determine the greatest likelihood between bacterial isolate groups, the BioEdit program (V.7.2) was employed. The phylogenetic study was conducted with the aid of the MEGA7 software, which employed the neighborhood joining tree approach. A total of 1000 bootstraps were utilized to determine the probability of nod branching.

Statistical analysis. GraphPad Prism was used to conduct analyses of data (version 5; GraphPad Software, San Diego, CA). A p-value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Throughout the study period, 47 isolates of *P. aerugi-*

nosa were obtained from different clinical specimens that were admitted to various hospitals in Erbil city. The isolates were identified with the Vitek II Compact system. 27 isolates (57.4%) from male patients and 20 isolates (42.6%) from female patients were isolated. As shown in Table 1, patients' ages ranged from (≤15 to ≥61) years. Regarding the distribution of *P. aeruginosa* in collected samples, 9 were from Urinary tract infection (19.1%), 19 from pulmonary infection (40.4%), 11 from systematic infection (23.4%), 6 from meningitis (12.8%), and 2 from sepsis (4.3%). Statistical analysis revealed that the distribution of samples differed significantly ($P < 0.001$).

Table 1. Frequency distribution of *Pseudomonas aeruginosa* in different types of clinical samples (n=47).

Features	Frequency	Percentage
Gender		
Male	27	(57.4%)
Female	20	(42.6%)
Age group		
≤15	5	(10.6%)
16-30	6	(12.8 %)
31-45	7	(14.9%)
46-60	11	(23.4%)
≥61	18	(38.3%)
Clinical features		
Urinary Tract Infection	9	(19.1%)
Pulmonary Infection	19	(40.4%)
Systematic Infection	11	(23.4%)
Meningitis	6	(12.8%)
Sepsis	2	(4.3%)

Standard deviation ≤15=4.97, 16-30= 4.926, 31-45= 4.282, 46-60= 1.371 and ≥61= 6.166

The lowest rates of resistance among the isolates were seen for amikacin, tobramycin then netilmicin. While the most common forms of resistance were to the antibiotics piperacillin (87.2%) and pep/tazobactam (85.1%). As shown in Table 2.

According to the data in Table 3, the prevalence of ESBL and MBL among the tested strains was as follows: ESBL was detected in 30 (63.8%) of the tested strains, while MBL was detected in 31 (65.9%) of the tested strains. However, 39 (82.9%) examined strains were biofilm producers. The valuation of the virulence genes revealed a wide distribution of diversity. The incidence of virulence genes amongst the examined strains was as follows: the *toxA* gene was detected in all of the examined strains, whereas the *algD, ndvB,* and *tssC1* genes were detected in 9 (19.1%), 8 (17%), and 3 (6.3%) respectively.

The target PCR products were sequenced for both genes. The results were evaluated and compared with the reference strains available in the NCBI database's GenBank using the BLAST algorithm. The sequencing results revealed that the strains were 99% compatible with each other as shown in Figs. 1 and 2.

DISCUSSION

The incidence of *P. aeruginosa* infection differs depending on the site of infection and affects areas that lack normal defenses, such as skin disruption and an intravenous or urinary catheter (17). The results enrolled 47 isolates of *P. aeruginosa*, of which (57.4%) were male and (42.6%) were female. A comparable study conducted in Estonian hospitals and recognized that 61% were male (18). The isolate distributions shown in Table 1 were in agreement with those that described that most common *P. aeruginosa* isolates derived from purulent specimens (19). Several categories of antibiotics, including piperacillin, piperacillin/tazobactam, ceftazidime, and cefepime, were found to be ineffective against most of the tested strains, similar results have been reported (20). This is due to intrinsic resistance of *P. aeruginosa* to antibiotics (21-23). Regarding phenotypic detection of biofilm formation in this study, 39 (82.9%) of the isolates were biofilm producers. Similar results were reported, where 84.3% and 83.75% of isolates formed biofilm, respectively (24, 25). These results were consistent with other described studies (26) found that very high rates of biofilm formation were detected in MBL and ESBL *P. aeruginosa* strains. According to the findings, the correlation between biofilm and antimicrobial resistance is significant for all drug categories. A higher proportion of antibiotic resistance in biofilm producers has been revealed in many studies (14, 27). Resistance of *P. aeruginosa* to many antimicrobial agents is a significant challenge in controlling its infections (22, 25). In this investigation, the vast majority of the bacteria that were identified possessed at least one virulence gene. Virulence was manifested as a combination of adhesion and cytotoxicity, where *toxA* was found in 100%; *algD* in 19.1%; *ndvB* in 17% and *tssC1* in 6.3% of the

Table 2. Antimicrobial resistant properties in *P. aeruginosa* isolated from clinical infections

Antibiotics	Urine n=9 (19.1%)	Sputum n=19 (40.4%)	Pus and Wound n= 11 (23.4%)	CSF n=6 (12.8%)	Blood n= 2 (4.3%)	Total n= 47
Amikacin	0	7 (14.9%)	5 (10.6%)	1 (2.1%)	0	13 (27.6%)
Gentamicin	1 (2.1%)	8 (17%)	4 (8.5%)	1 (2.1%)	0	14 (29.7%)
Netilmicin	0	8 (17%)	3 (6.4%)	1 (2.1%)	0	12 (25.5%)
Tobramycin	2 (4.3%)	6 (12.8%)	3 (6.4%)	2 (4.3%)	0	13 (27.7%)
Cefepime	9 (19.1%)	17 (36.2%)	7 (14.9%)	6 (12.8%)	0	39 (83%)
Ceftazidime	9 (19.1%)	17 (36.2%)	7 (14.9%)	5 (10.7%)	0	38 (80.9%)
Pepracillin	9 (19.1%)	17 (36.2%)	10 (21.2%)	5 (10.7%)	0	41 (87.2%)
Pep/Tazobactum	9 (19.1%)	17 (36.2%)	8 (17%)	6 (12.8%)	0	40 (85.1%)
Imipinem	6 (12.8%)	14 (29.7%)	7 (14.9%)	2 (4.3%)	0	29 (61.7%)
Meropenem	2 (4.3%)	8 (17%)	4 (8.5%)	3 (6.4%)	0	17 (36.2%)
Tigycycline	6 (12.8%)	19 (40.4%)	8 (17%)	5 (10.7%)	0	38 (80.9%)
Tri/sulfamthoxazole	8 (17%)	15 (31.9%)	8 (17%)	6 (12.8%)	0	37 (78.2%)
Ciprofloxacin	1 (2.1 %)	8 (17%)	3 (6.4%)	3 (6.4%)	0	15 (31.9%)
Levofloxacin	1 (2.1%)	8 (17%)	4 (8.5%)	4 (8.5%)	0	17 (36.1%)

Table 3. Prevalence of beta-lactamase production, biofilm formation and virulence genes in various clinical samples

	Urine n=9 (19.1%)	Sputum n=19 (40.4%)	Pus and Wound n= 11 (23.4%)	CSF n=6 (12.8%)	Blood n= 2 (4.3%)	Total n= 47
MBL n (%)	8 (17%)	10 (21.3%)	8 (17%)	4 (8.5%)	1 (2.1%)	31 (65.9%)
ESBL n (%)	5 (10.7%)	12 (25.5%)	10 (21.3%)	2 (4.2%)	1 (2.1%)	30 (63.8%)
Biofilm n (%)	7 (14.9%)	17 (36.1%)	10 (21.3%)	3 (6.4%)	2 (4.2%)	39 (82.9%)
tox A	9 (19.1%)	19 (40.4%)	11 (23.4%)	6 (12.8%)	2 (4.3%)	47 (100%)
alg D	6 (12.8%)	0	1 (2.1%)	0	2 (4.2%)	9 (19.1%)
ndvB	4 (8.6%)	1 (2.1%)	2 (4.2%)	1 (2.1%)	0	8 (17%)
tssC1	0	1 (2.1%)	0	2 (4.2%)	0	3 (6.3%)

isolates. One of the crucial gene identified in all of the isolates (100%) was the *toxA* gene, which encodes exotoxin A. The high prevalence of *toxA* among our isolates is comparable with others (28). Khattab et al. detected that the majority of their pulmonary isolates had *toxA* (29). On the other hand, these outcomes are incompatible with those obtained earlier, that found a smaller number of isolates harboring this gene (8, 30). Another virulence factor that influences *P. aeruginosa* pathogenicity is *algD* which is engaged in alginate production, which is an important component of biofilms because it is the principal extracellular polymer material (31). The increased expression of alginates not only shields *P. aeruginosa* from antimicrobial treatments, but also suppresses the immune response by suppressing complement activation, decreasing polymorphonuclear chemotaxis, and decreasing the process of phagocytosis (32). The GDP-mannose dehydrogenase enzyme, which contributes to adhesion and is encoded by the *algD* gene, was not found in high abundance in our isolates. Interestingly, this result is consistent with another study, which found its presence in patients with otitis (33). While the result findings are contradicted by Elmouaden et al. (34), they exposed a very high prevalence of *algD* in their respiratory isolates, due to the fact that this polysaccharide is associated with biofilm. Conversely, other studies have also displayed variation in the illuminating rate of the *algD* gene, which is conceivably related to the primer specificity and/or the source of the bacterial isolate. Biofilm-specific antibiotic resistance is influenced by numerous factors (35).

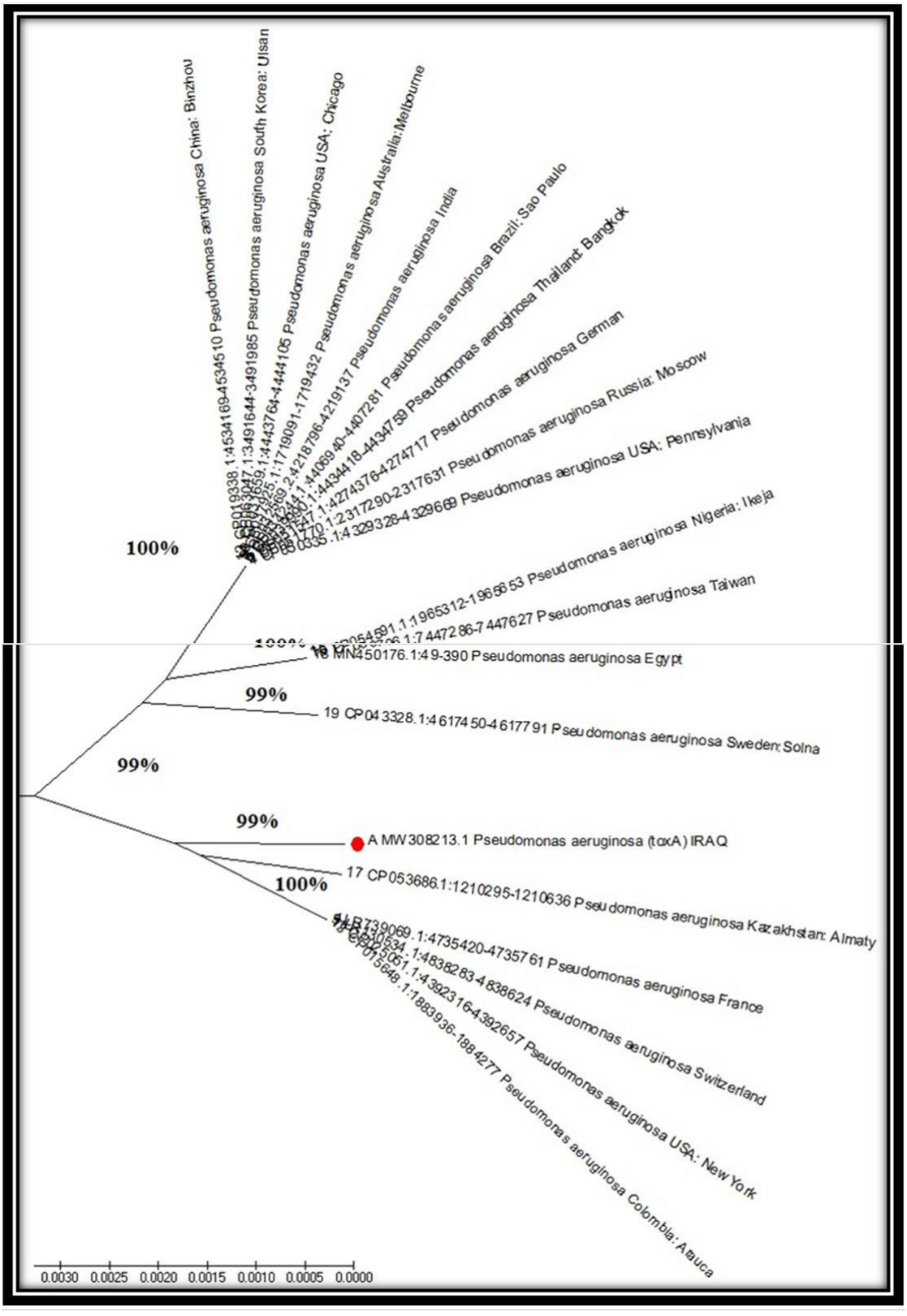

Fig. 1. Neighbor-joining tree demonstrates the phylogenetic relationships between *P. aeruginosa toxA* sequences and other *toxA* sequences. International nucleotide databases assign accession numbers to identify them. Phylogenetic tree was made via MEGA 7

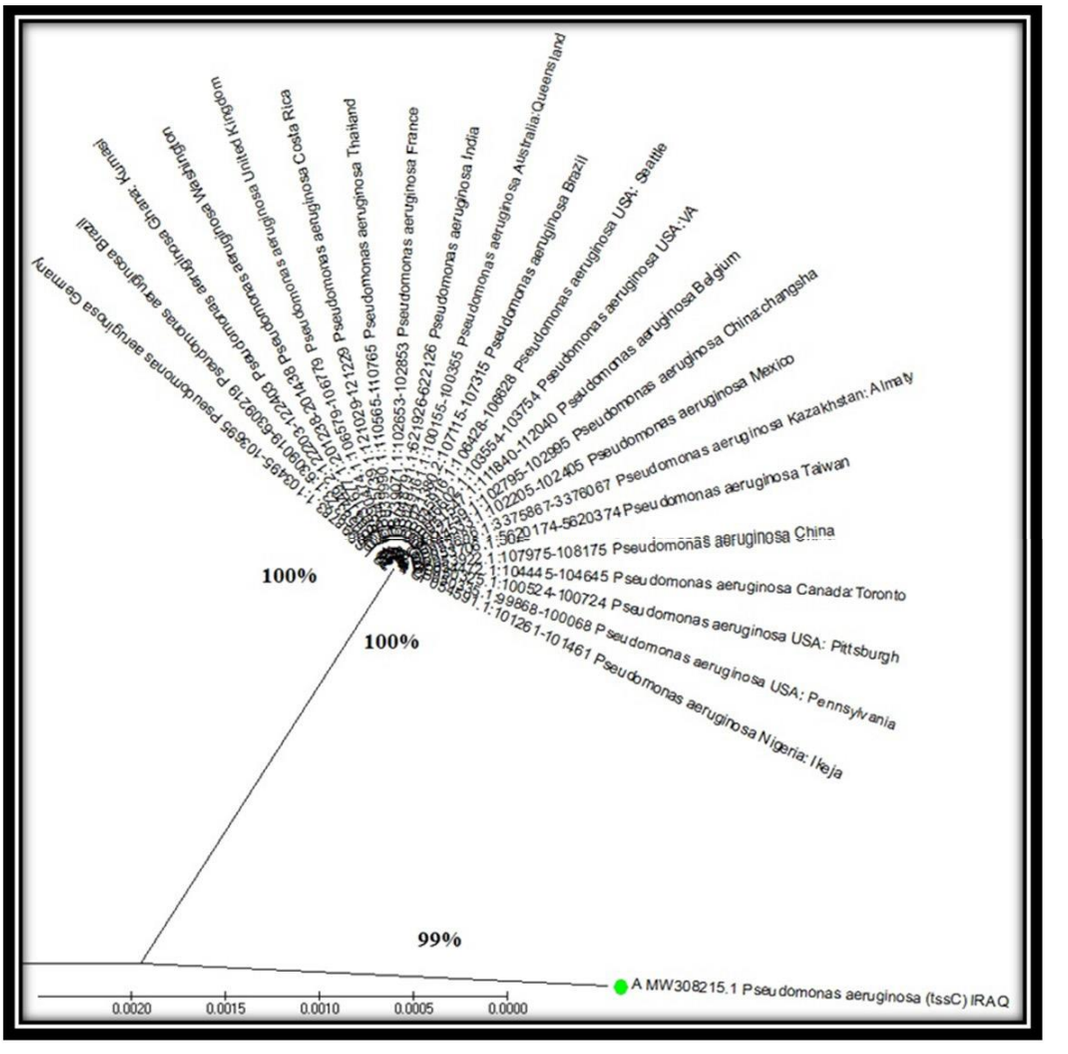

Fig. 2. *Tssc1* neighbor-joining unrooted phylogenetic analysis for *P. aeruginosa* clinical local isolates. The analysis was performed using MEGA7 sequence software.

Zhang, 2011 demonstrated that *P. aeruginosa tssC1* is essential for the resistance of biofilms to a subset of antimicrobial agents and established that *tssC1* is required for the secretion of T6S. Although we found a low frequency of the *tssC1* gene in our isolates. Many studies indicate that *tssC1* promotes antibiotic resistance in biofilms. Our results are incomparable to others (16) that a higher incidence of both *ndvB* and *tssC1* genes was reported to be displayed in 96.7% and 90.2% of the *P. aeruginosa* isolates respectively, and in a study obtained from Iraq, the expression of the *ndvB* gene in biofilms is reliant on the stationary-phase sigma factor RpoS (36). It is possible that the discrepancy is attributable to the variation in sample source, as in the earlier research, samples were collected from a variety of hospital sources. The phylogenetic tree was established by comparing the *toxA* and *tssc1* sequences from this study with GenBank database sequences at NCBI to determine the relationships among the groups investigated. Figs. 1 and 2 illustrate a neighbor-joining phylogenetic tree for *toxA* and *tssc1* sequences, which demonstrates the existence of one cluster that is new in the global gene bank. The availability of phylogenetic and epidemiological data, imperative to speculate about whether or not the pathogen is a recently emerging or has been prevalent in the population of the world for a longer term of time (33).

In conclusion, *P. aeruginosa* isolates obtained from different specimens possess an extensive array of virulence determinants. Furthermore, we found a high prevalence of the virulent genotype in our study

especially *toxA*, the presence of which was found to be of incredible significance due to the harmful composite and inadequate treatment options for these patients. Outcomes indicate one isolate, which is a new recorded gene bank isolated from patients in the Erbil city- Iraq.

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