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Evalution of the Potential Antibiofilm Activity of Synthetic Peptide Idr-1018 Against Highly Virulent Clinical *Pseudomonas Aeruginosa* Isolates and Molecular Screening for the Prevalence of Toxinand Antitoxin Type Ii Genes

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Abstract : Multi-drug resistance *Pseudomonas aeruginosa* considered a significant threat to human health according to the WHO. The antimicrobial synthetic peptides have taken a significant attention as options of antibiotic for dealing with resistant bacteria. A total of 200 samples were collected from different clinical sources including both sex with an age range between (5months - 58year) in the period between July to end of November 2024. Samples collected from five main hospitals. Out of 122 isolates, 60 isolates (49.18%) as *P. aeruginosa* depending on conventional and molecular methods and the highest frequent isolates were burn sources. All isolates were subjected to the sensitivity test against twelve antibiotics. The results revealed that most *P. aeruginosa* isolates were resistant to Ticarcillin-Clavulanate (93.3%) while the lowest resistance was towards Ceftazidime (41.7%). The Biofilm formation test was characterized into three categories: 23.3% strong adherent, 35% moderately adherent, 30% weakly adherent and 11.7% non-producing biofilm. Range of IDR-1018 concentrations (7.8-1000µg/ml) were examined against eight selected MDR *P. aeruginosa* isolates to determine the minimum inhibitory concentrations (MICs) of IDR-1018 peptide. The affected concentration of *P. aeruginosa* varies from 62.5µg/ml up to 1000µg/ml as MIC. All isolates were subjected to molecular screening to determine the prevalence of virulence factors encoded for toxin-antitoxin system-type II, the results highlighted that *higBA*, *relBE*, *parDE* genes were present in 100% among of isolates. In conclusion, the antimicrobial peptides IDR-1018 are characterized as promising agent for use as antimicrobial products.

Keywords: *Pseudomonas aeruginosa*, toxin and antitoxin genes, antibiofilm, IDR-1018 peptide.

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Introduction

The most hospital-problem and community-acquired pathogen is the multi-drug resistant (MDR) *Pseudomonas aeruginosa* (opportunistic pathogen) (1). This bacterium can adapt as well as inheritance resistance, *P. aeruginosa* quickly acquired drug resistance (2 and 3). The bacteria possess Mobile genetic elements (MGEs) that give the bacteria the property of resistance to carbapenems, and this requires extra attention (4). There are a number of mechanisms for *P. aeruginosa* to increase pathogenicity

and antibiotic-resistant, such as: the production of biofilm and Toxin-Antitoxin system (5 and 6).

Biofilm is a type of life as extracellular polymeric substance matrix; that support bacterial survival and antibiotic resistance. The growth rate of cell is reduced and the up- and down-regulation of specific genes (7). The emergency importance issues are the rise and dissemination of multidrug resistant *P. aeruginosa* which is highly association with decrease therapeutic choice and become a major significant public health concern (8). Moreover, biofilms may be associated to lethal and serious conditions include inflammation in cystic fibrosis, endocarditis, and infections associated with chronic indwelling devices, for instance, heart valves and joint prostheses (9) Due to its association with certain prevalent pathogenic processes, including burn wound, cystic fibrosis, chronic wound and chronic obstructive pulmonary disorder (COPD) (10).

Toxin-antitoxin systems (TAs) were initially identified in 1983 on a conjugative plasmid, by post-segregation killing (PSK) serve as plasmid maintenance systems in bacteria and eventually, discovered to be locate on chromosomes (11). It considered as a novel target for antimicrobial treatment. TA are small genetic unit extensively distributed in the bacterial genome as stress response factors and closely associated with important physiological processes, such as: the formation of biofilm, defense against phages, control of growth, virulence enhancement (12). Toxins have many properties to become as a new method as antibacterial method. Firstly, encoded of toxin by single gene instead of large gene clusters. Secondly, considered suitable for genetic engineering when the structure of toxin is simple to manipulate and predict (13). Third, TA was restricted to specific bacterial strains. Finally, the most known toxins were cytosolic, with the exception of some types there are III and VII types of toxins (14).

Toxin-antitoxin system made up of two parts: a stable toxin (kills host cells by delivering toxicity) and a labile cognate antitoxin (degradation-prone antitoxin) (a DNA-binding protein conferring immunity to the toxin protein/RNA) (12). The TA system type II toxins and antitoxins are proteins. Toxin's toxicity was blocked by the antitoxin by following strategies: Direct interactions of protein–protein (type II) (15), TA systems are widely distributed throughout chromosomes and mobile genomic elements (plasmids, prophages, transposons, conjugate and integrate factors) in bacteria and archaea, where they play various functions in physiological processes and disease transmission (16).

Toxin can be inactivated by action of labile antitoxin (either protein or RNA) as recognized in TA system type II genes in *P. aeruginosa*: *relBE* (*relB* toxin and *relE* anti-toxin), *parDE* (*parD* toxin and *parE* anti-toxin), *ccdAB* (*ccdB* toxin and *ccdA* anti-toxin), *higBA* (*higB* toxin and *higA* anti-toxin), *mazFE* (*mazF* toxin and *mazE* antitoxin) and *mqsRA* (*mqsR* toxin and *mqsA* anti-toxin) (17). So, TA system type II can possibily used as a new approach against MDR bacteria (18).

The synthetic peptide-1018 (IDR-1018) is considered as regulator innate defense consist of 12-amino acid (VRLIVAVRIWRR; abbreviated here as 1018), derivative of bactenecin, a bovine HDP (19). The function and structure of synthetic peptide IDR-1018 was butter understand in the model membrane; the peptide's structure consists of lipids with neutral head

groups. For example, zwitterionic dodecyl phosphocholine (DPC) micelles are similar to this issue. Sodium dodecyl sulfate is another example. Lipids with a combination of neutral and negatively charged head groups (there are phosphatidylcholine and phosphatidylglycerol, which is a model for bacterial cells) via solution state NMR and CD (16). This study determined the MIC of peptide IDR-1018 required to counter *P. aeruginosa.*

Materials and Methods

Isolation and Identification of *P. aeruginosa*

This research included samples collected from patients at five hospitals in Baghdad/Iraq there are: Burn, Gazi, Children welfare, AL-Kindi teaching and Ibn-Albaladi hospitals. A total of 200 clinical samples were aseptically acquired from several clinical sources in transport media and transferred to the laboratory for bacterial isolation. In this study samples were obtained between July 2024 to November 2024. A total of 122 isolates, sixty isolates (49.18%) were identified as *P. aeruginosa* depending on Cetramide agar (Liofilchem/Italy), McConkey agar (Liofilchem/Italy) and Blood agar (Liofilchem/Italy). After that, the culture plates were incubated at 37C˚ for 24hrs. Then, oxidase test was performed to more confirmation the identification. The *Pseudomonas* colonies were isolated under sterile conditions and maintained in pure form for further characterization. A variety of phenotypic, physiological and biochemical assays were accomplished to identify the microbial The Pseudomonas colonies were isolated under sterile conditions and maintained in pure form for further characterization. A variety of phenotypic, physiological, and biochemical assays were conducted to identify the microbial strains. Finally, the isolates were verified by using VITEK 2 GN ID cards by using VITEK 2 system (bioMerieux, France) as claimed by Zedan *et al.* (2013).

Antibiotic Susceptibility Test

The susceptibility to antibiotics was assessed via the disc diffusion technique. Briefly, sixty *P. aeruginosa* was prepared on Brain heart infusion broth (24hrs., 37C˚) Subsequently, resuspended in normal saline. The turbidity of the suspension was calibrated to 0.5 McFarland, and This suspension of bacteria was utilized for inoculating on Mueller-Hinton agar (Liofilchem/Italy) plates. This study employed twelve antibiotic discs from seven families, as follows:

Aminoglycoside family (Gentamycin (CN) (10Mg), Tobramycin (TOB) (10Mg), Amikacin (AK) (30Mg)), Cephems family (Ceftazidime (CAZ) (30Mg), Cefepime (FEP) (30Mg)), Carbapenems family (Imipenem (IMI) (10Mg), Meropenem (MRP) (10Mg)), Fluoroquinolones family (Ciprofloxacin (CIP) (5Mg), Levofloxacin (LEV) (5Mg)), Monobactams family (Aztreonam (ATM) (30Mg)), B-lactam combination agents' family (Ticarcillin-Clavulanate (TCC) (75/10Mg)), Penicillin family (Piperacillin (PRL) (100Mg)). Then, MH agar plates incubate overnight at 37C˚, after that, the inhibition zone was assessed in order to ascertain the percentages of those who were resistant, susceptible, and intermediate depend on CLSI guideline 2024 (21).

Biofilm Formation Assay

According to Badmasti *et al.* (2015), biofilms formation assay was demonstrated in 60 of *P. aeruginosa* strains by using a microtiter plate. The formation of biofilm achieved in triplicate. The abilities of adherence the test isolates were classified into four

categories; weak, moderate, strong and non-biofilm forming. Three standard deviations (SDs) above the mean OD (at 630nm) of the negative control (broth only) were considered as the cut-off optical density (ODc). Isolates were classified as follows: if OD ≤ ODc, the bacteria were non-adherent; if ODc < OD ≤ 2 × ODc, the bacteria were weakly adherent; if 2 × ODc < OD ≤ 4 × ODc, the bacteria were moderately adherent; if 4 × ODc < OD, the bacteria were strongly adherent (8).

Antimicrobial peptide preparation

IDR-1018 (VRLIVAVRIWRR-NH2) has been used Novopro company/China. The peptide was immediately used in MIC after dissolved in sterile water to obtain concentration 4000µg/ml (23).

Minimum inhibitory concentration (MIC) of IDR-1018 peptide

To calculate the MIC of the IDR-1018 peptide, the broth microdilution method of Muller Hinton broth was used in 96-wells microtiter plate (23), the peptide was prepared in range of half-dilution concentrations between (1000 to 7.8µg/ml). The working solution was made in sterile D.W. at a concentration of peptide 4000µg/ml. Eight *P. aeruginosa* (A5, A6, A7, A18, A28, A32, A33 and A36) selected according to sources (table 1). These isolates were prepared on Brain heart infusion broth (24hrs., 37C˚) (Liofilchem/Italy) followed by resuspension in Muller Hinton broth. The turbidity of suspension was calibrated to 0.5 McFarland. The MICs determines as the lowest achievable concentrations of the peptide at which no change in color of the resazurin broth experiment from blue to pink, which were evaluated visually in broth micro dilutions (24 and 25).

Table (1): Characterization of isolates using in MIC with peptide

No.	Isolate ID	source	Gender	Age
1	A5	Sputum	Male	1Year
2	A28	Sputum	Female	25 Years
3	A6	Burn	Male	15 Years
4	A32	Burn	Female	47 Years
5	A7	Urine	Female	20 Years
6	A18	Urine	Male	45 Years
7	A33	ETT	Male	9 Months
8	A36	Wound	Female	5 Years

Molecular detection of *P. aeruginosa*

Molecular detection of *P. aeruginosa* strains was conducted using bacterial specific primer (table 2) to amplify *16S rRNA* gene upon DNA extraction genomic DNA extraction kit (Geneaid/Taiwan). Then, the DNA concentration was measured using Quantus fluorometer (Promega/USA). Moreover, table (3) shows the reaction mixture for amplification. While conditions of reaction mentioned as follows in table (4) as described previously (26).

Detection of Toxin-antitoxin type II system genes

The prevalence of TA system (*higBA*, *relBE* and *parDE)* genes were screened among *P. aeruginosa* isolates A5, A6, A7, A18, A28, A32, A33 and A36. The PCR primers, reaction mixture, and the PCR conditions for genes amplification were illustrated in table (2), table (3) and table (5), respectively. The visualization of DNA bands by UV gel documentation and photographed (28).

Table (2): The sequence of primers used in this study.

Primers	Sequence (5'-3')	Product size (bp)	Ref.
16S rRNA	F- GGGGGATCTTCGGACCTCA	956	26
	R- TCCTTAGAGTGCCCACCCG		
higBA	F-GGCCAACATAGCATCAGGATC	305	27
	R-GGACGTATCAAAGTAACGCCC		
relBE	F-CGCAGTACCTGGAAAGGCAGC	349	27
	R-GCCTTTAACCCGAAACGGG		
parDE	F-GCGGCTGACCTGGATTTATC	556	27
	R-CCAAGCAGTAGCGGATCAATTG		

Table (3): PCR reaction components for *16S rRNA*, *higBA*, *relBE* and *parDE* genes

PCR reaction components	Volume (µl)
master mix (2x) ($MgCl_2$, 1.5mM, Taq polymerase 1U, each dNTPs 200µM)	12.5
DNA template	2
Forward primer (10pmol)	1
reverse primer (10pmol)	1
nucleases free water (Promega/USA)	8.5
Final volume	**25µl**

Table (4): Program of PCR thermocycling conditions for *16S rRNA* gene

No.	Steps	Temp. (C°)	Time	No. of cycle
1	Initial denaturation	92	3min.	1 cycle
2	Denaturation	92	30sec	35 cycles
3	Annealing	58	30sec	
4	Extension	72	1min.	
5	Final extension	72	3min.	1 cycle

Table (5): Program of PCR thermocycling conditions for *higBA*, *relBE* and *parDE* genes

No.	Steps	Genes	Temp. (C°)	Time	No. of cycle
1	Initial denaturation		95	2min.	1 cycle
2	Denaturation		95	30sec	35 cycles
3	Annealing	*higBA*	50.8	45sec	
		relBE	58	45sec	
		parDE	58	1min	
4	Extension		72	1min.	
5	Final extension		72	10min.	1 cycle

Results and Discussion

Bacterial Isolates

Sixty MDR *P. aeruginosa* isolates were collected from different clinical sources, including burn, sputum, urine, ETT (Endotracheal tube), blood, wound, stool, skin, ear, CSF (Cerebrospinal fluid) and B.W (Bronchial Wash) in many hospitals, Baghdad, Iraq. *P. aeruginosa* isolates were gained from several sources, there are: burn 40%, sputum 18.3%, urine 11.7%, ETT 10%, wound 5% blood, B.W and ear swab 3.3%, while, stool, skin and CSF were 1.7%. The bacteria appeared on McConkey media as non-lactose fermenters, while on blood agar as Beta-hemolysis, while on selective cetrimide agar was green. The oxidase test was positive (blue color).

Sexes and Ages

The results revealed that the gender of patient were 28 and 32 for males and female, respectively. The relation between ages and sex shows in figure(1).

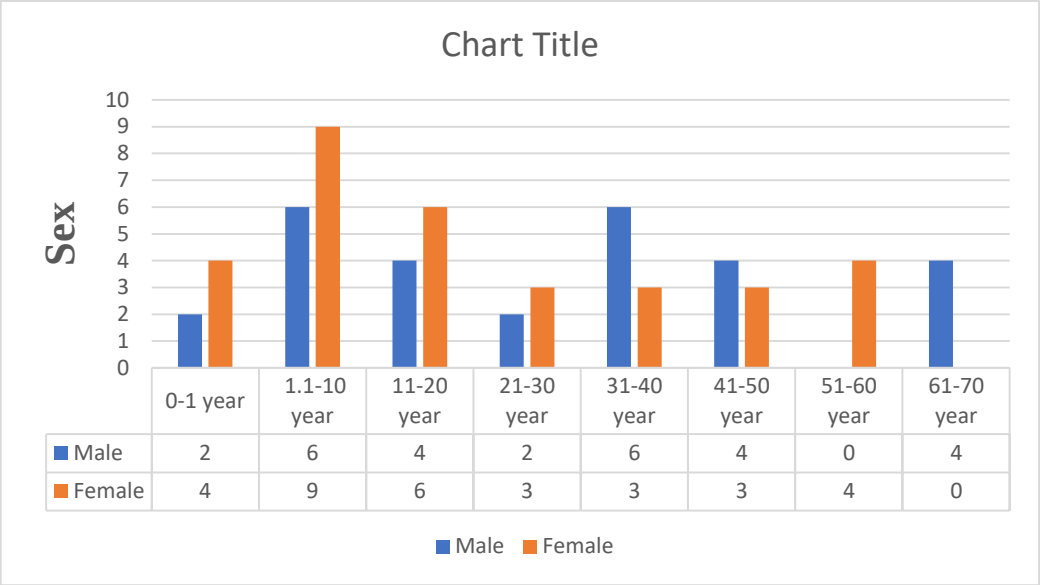

Figure (1): The distribution of P. *aeruginosa* among patients according to sex and age.

Antimicrobial Susceptibility

Twelve antibiotics (seven families) were obtained for all isolates of *P. aeruginosa*. The results showed that most *P. aeruginosa* isolates were resistant to Ticarcillin-Clavulanate (75/10Mg) 93.3%, Imipenem (10Mg) 85%, Tobramycin (10Mg) 58.3%, Cefepime (30Mg), Piperacillin (100Mg) and Aztreonam (30Mg) were 53.3%, Ciprofloxacin (5Mg) and Levofloxacin (5Mg) 50%, Gentamycin (10Mg) 48.3% and Meropenem (10Mg) 46.6%, Amikacin (30Mg) 45% and Ceftazidime (30Mg) 41.7%. The majority of isolates were multi-drug resistant (Figure 2).

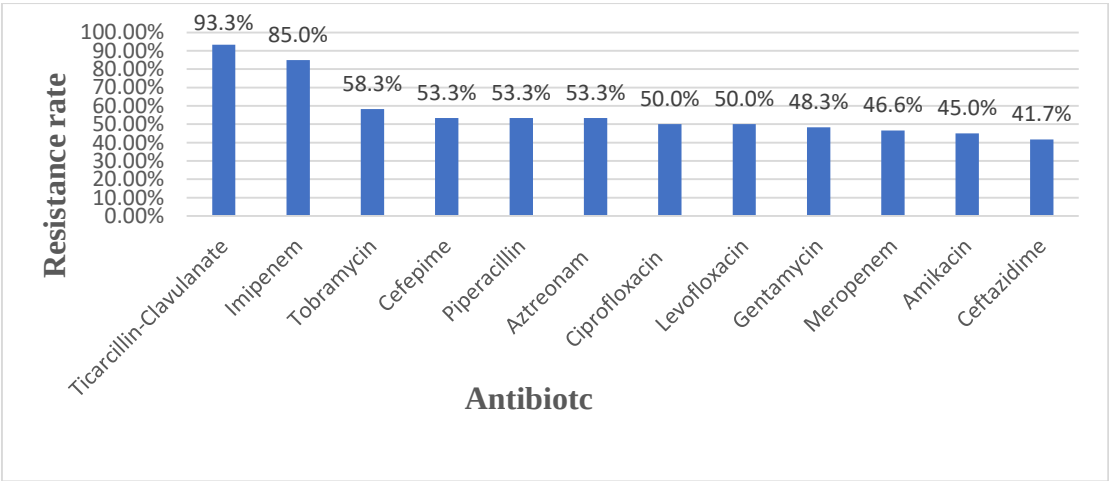

Figure (2): Percentages of antibiotic resistance for *P. aeruginosa* isolates against 12 antibiotics.

The emerging MDR *P. aeruginosa* isolates considered large concern in healthcare systems due to different factors such as mortality, cost and longer hospital stays (8). Our findings would agreement with data was found in local research achieved by Al-Shamary (2018), who revealed the rate of high resistance was 87.3% by *P. aeruginosa* strains toward cefotaxime, followed by amikacin was 61.9%, ciprofloxacin was 55.55% and gentamycin was 69.84%, however, our result report lower MDR rates of these antibiotics. The bacteria may be producing hydrolytic enzymes and

harboring various methods to resist such antibiotics, as indicated by their high resistance rate.

Results of Japoni *et al.* in (2014), confirmed our results and illustrated that *P. aeruginosa* isolates producing β-lactamase were 22% resistant to ampicillin, imipenem, aztreonam, meropenem and piperacillin/tazobactam completely, on the other hand, Tavajjohi and Moniri (2011), mentioned that the multidrug-resistant isolates were detected in the rate 30% in their study. For this reason, solving the MRPA's problem needed novel approaches. Some techniques have depended on creating a conjugate vaccine (22) or mixing herbal extracts or other antimicrobials with antibiotics (32). The explanations for these variations in the prevalence of MDR among several researches might be associated with variation in the antibiotic usage patterns, characteristics of the subjects, the source of the isolates and a variety of policies for infection control.

Biofilm production

Biofilm is considered quantitative technique for estimating the production of biofilm. The results of this study demonstrated that the clinical MDR *P. aeruginosa* isolates have a high capacity (87.5%) to create biofilm. Subsequently, the production of biofilm was divided into three groups based on the formula for biofilm data analysis depending on the formula, figures (3) and (4) illustrated that 30% weakly adherent, while moderately and strong adherent were 35% and 22.5%, respectively. Whereas 12.5% isolates were non-producer of biofilm.

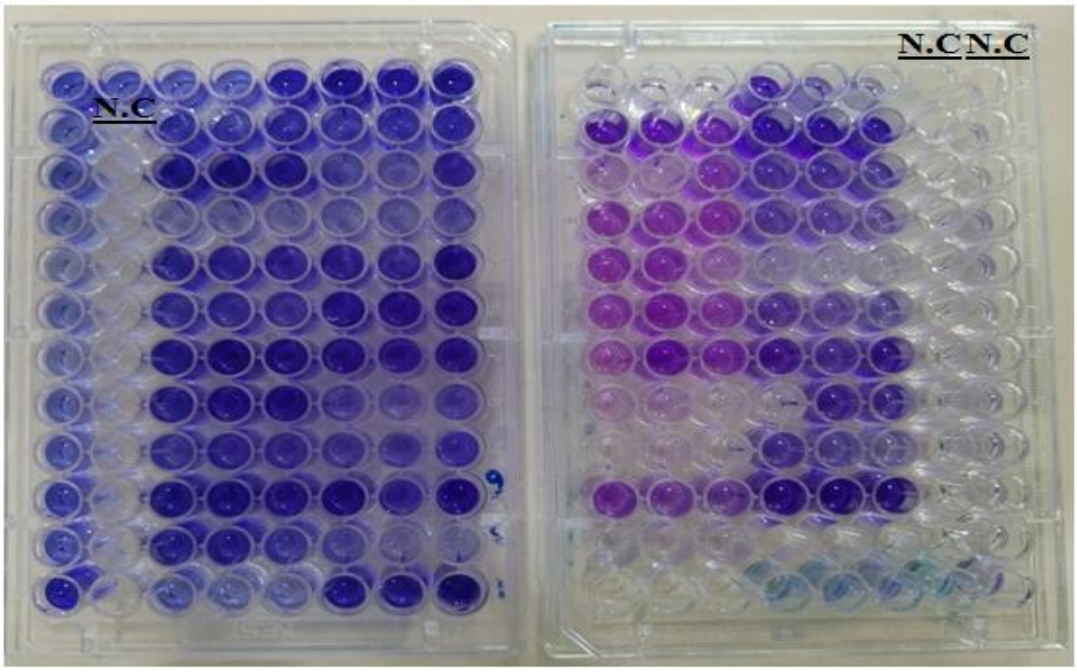

Figure (3): The Biofilm formation of sixty isolates of *P. aeruginosa*
N.C= Negative control

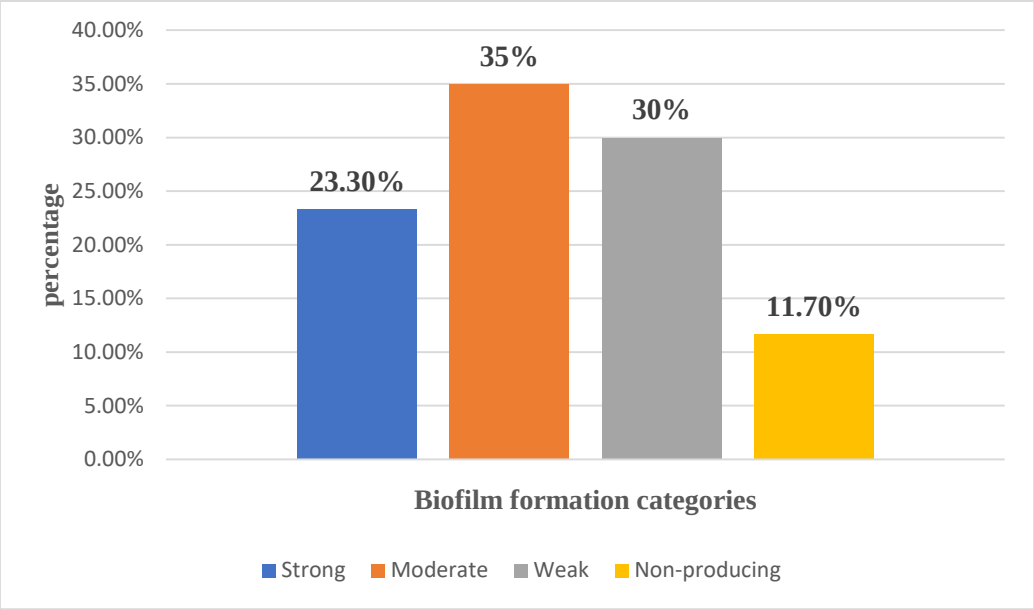

Figure (4): The categories of the Biofilm formation of sixty isolates of *P. aeruginosa* (triplicate for each isolate).

The opinion of von Rosenvinge *et al*. at (2013) that *P. aeruginosa* regularly causes chronic infections associated with biofilms. The antibiotic-stress eliminated the bacterial cells. The biofilm-embedded cells provide defense against the immune system, followed dispersal and cause infection by launching planktonic cells (34).

The limited efficiency of antibiotics against bacteria and the increasing of antibiotic resistance and tolerance of microbes that led to the need to new antimicrobial method. The maintenance of antimicrobial resistance is largely associated with TAs linked to antimicrobial genes (8).

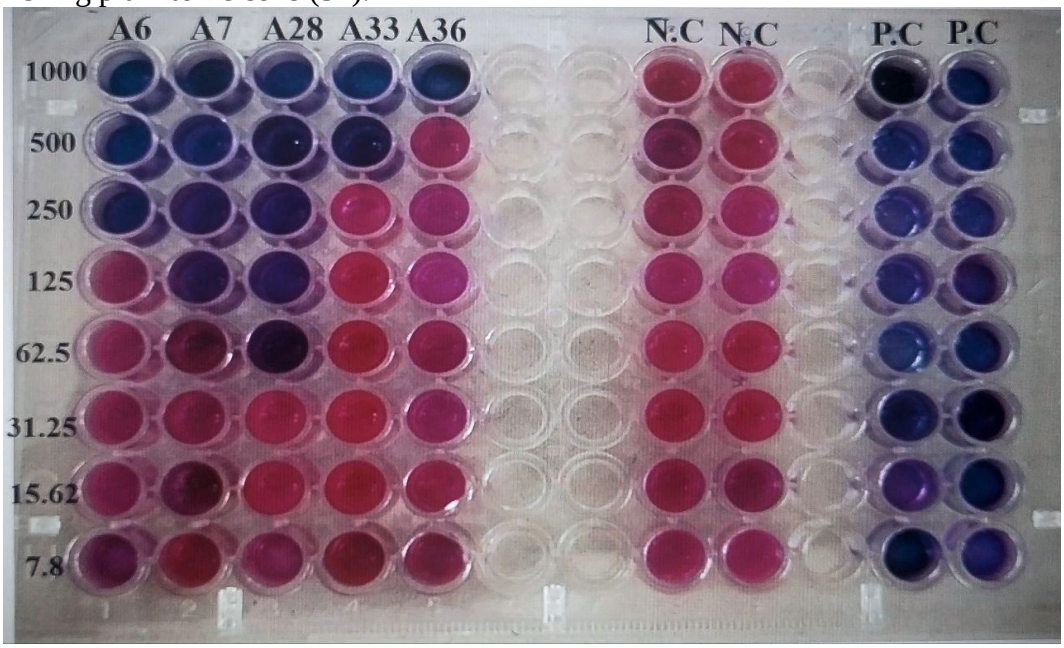

Figure (5): The minimum Inhibitory Concentrations (MICs) of IDR-1018 peptide at concentrations (7.8-1000µg/ml) against *P. aeruginosa*

N.C=Negative control P.C= Positive control

Eight isolates of *P. aeruginosa* was performed to MIC after mixed with IDR-1018 peptide to select the minimum concentration that allows the bacteria to survive wand to determine the appropriate concentration in the expression of toxin and antitoxin genes, (Figure 5). Three isolates from the *P. aeruginosa* isolates were producing strong biofilm (A6, A7 and A32) and two moderates (A5 and A18), while the last three were produce weak biofilm (A28, A33 and A36). These eight isolates were treated with peptide IDR-1018 and table (6) shows the values of MIC and sub-MIC.

Table (6): MIC and sub-MIC with peptide IDR-1018 of *P. aeruginosa*

No.	Isolate ID	MIC with IDR-1018 peptide	Sub-MIC with IDR-1018 peptide
1	A5	500	250
2	A28	62.5	31.25
3	A6	250	125
4	A32	62.5	31.25
5	A7	125	62.5
6	A18	1000	500
7	A33	500	250
8	A36	1000	500

The increasing MDR rate is a warning signal that more stringent antibiotic prescription is required. Moreover, the antibacterial resistance of *P. aeruginosa*. The results of Wu *et al.* (2022) revealed that MIC of IDR-1018 against *P. aeruginosa*, Many Gram-negative bacteria (*Pseudomonas*, *Escherichia*) and Gram-positive bacteria (*Staphylococcus*, *Enterococcus*) are affected by the antimicrobial peptide because of its ability to penetrate their membrane and interact with intracellular targets. Developing antibiotics that can target TA systems may be one of these strategies. In general, in cell survival antitoxin unit serve as positive regulator while toxin serve as negative regulator. Interactions between toxin and antitoxin molecule expression levels are essential for bacterial life under abnormal and stressful circumstances. Thus, they are being considered as possible targets for the creation of novel antimicrobial substances (8). When the bacterial species have difficult to identify by biochemical methods, so, molecular identification can be used to this purpose depend on amplification of *16S rRNA* gene which considered as flexible and precise method. Spilker *et al.* (2024) indicated that these results were validated by several of clinical strains, which had been previously identified through biochemical testing. The 16S rRNA gene was utilized in this conventional PCR, and the results indicated that all isolates produced a clear band measuring 956 bp in molecular size.

A new strategy required to address the crisis of MDR isolates involves the development of novel antimicrobials that specifically target TAs for the treatment of MDR-bacterial infections.

According to many researches, it was previously thought that respiratory viral infections were less common in cases of hospital-acquired infections. Some studies on TAs refer to that activating the toxin-antitoxin system may lead to the acquisition of new sensitive region in bacterium. The results of the investigation suggested that increasing level of gene expression of TAs genes can decrease antibiotic resistance. These

results confirmed the previous results obtained by Williams *et al.* (2011).

Toxin systems one of the most recent and most targeted antimicrobial systems because of their wide distribution in clinical bacterial strain and restricted structure of genetic material. So that, increased interest to develop new drugs against *P. aeruginosa*, such as MRPA. To reach the above goal, it is necessary to estimate the expression and functionality of TA systems in the life cycle of bacteria (32).

TAs system type II genes

The TAs type II genes used in this study including *higBA*, *relBE* and *parDE*. These genes were detected in 100% of all bacterial isolates (Figures 6, 7 and 8). In this study, all isolates of *P. aeruginosa* harboring these three genes.

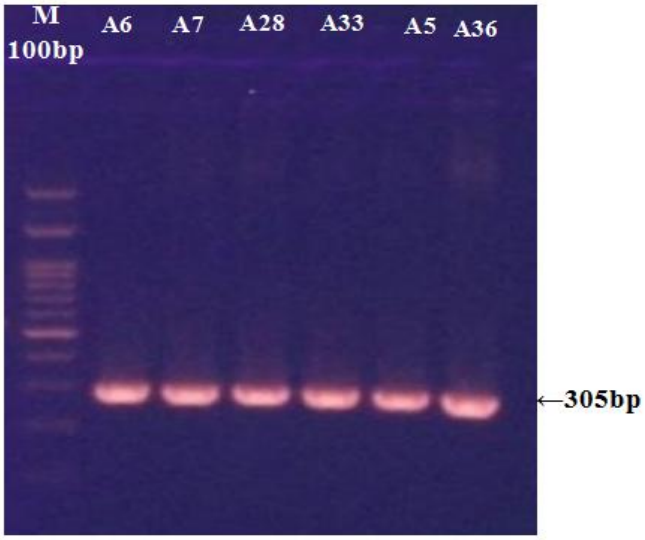

Figure (6): Gel electrophoresis for amplicons of *higBA* gene in *P. aeruginosa* (1% agarose gel; 150volts for 30min).

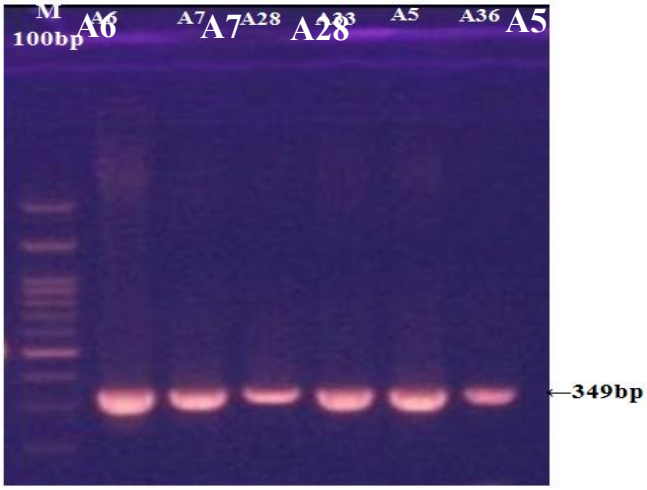

Figure (7): Gel electrophoresis for amplicons of *relBE* gene in *P. aeruginosa* (1% agarose gel; 150volts for 30min).

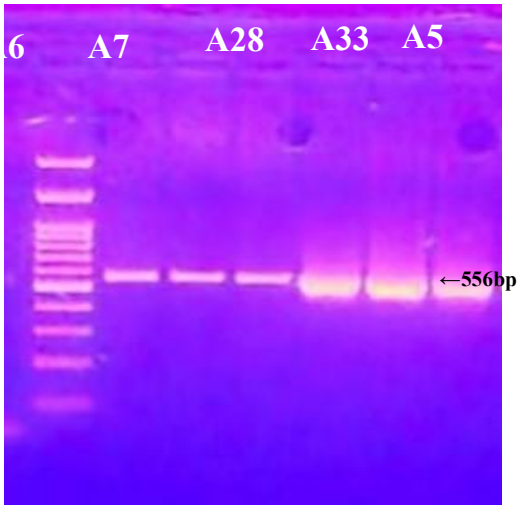

Figure (8): Gel electrophoresis for amplicons of *parDE* gene in *P. aeruginosa* (1% agarose gel; 150volts for 30min).

There have been few investigations examining clinical isolates of bacteria for the existence and operation of TA systems. Our results agreement with Williams *et al.* (2011), that revealed in the *P. aeruginosa* strains, *higBA* 100% and *relBE* 100% genes were universal but disagreement with *parDE* 30% gene were considered less prevalent. Comparison of the *P. aeruginosa* strains have different band patterns *higBA*, *relBE* and *parDE.* Our finding was similar to the report of the Perez *et al.* (2013) and Hemati (2014), they reported frequency *relBE* was 100% in *P. aeruginosa*.

Conclusion

The study found that clinical isolates from Baghdad had a high incidence of multidrug-resistant *P. aeruginosa*, with concerning resistance rates to important medicines including imipenem (85%) and ticarcillin-clavulanate (92.5%). The isolates' importance in chronic infections and treatment failure was highlighted by the noteworthy biofilm-forming potential of 87.5% of them, of which 35% showed moderate adherence and 22.5% strong adherence. The toxin-antitoxin (TA) system genes (*higBA*, *relBE*, and *parDE*) were present in all isolates, indicating their role in antibiotic resistance and bacterial survival under stress. Although resistance to ceftazidime (42.5%) and meropenem (5%) was relatively lower, the pervasive MDR patterns underscore the pressing need for better antimicrobial stewardship. The IDR-1018 peptide's inhibitory action on these isolates presents a possible substitute treatment approach. These results highlight the urgent need for innovative therapies that target TA systems and biofilm breakdown in order to fight rsistent *P. aeruginosa* infections in clinical settings.

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