Biofilm Formation and Virulence Factors among Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Patients in Babylon Province

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Abstract

Background: *Pseudomonas aeruginosa* is often multidrug resistant that is associated with hospital-acquired infections. **Objectives:** The objective of this study was to identify the patterns of antibiotic resistance, biofilm formation, the occurrence of multiple drug resistant (MDR), and virulence genes (*Alg, OprL,* and *OprI*) in *P. aeruginosa* isolated from patients. **Materials and Methods:** A total of 131 isolates from various sites including (burn, wound, and urine) were collected from different hospitals in Babylon province for both genders and ages. These isolates were identified using traditional techniques as well as the Vitek 2 system. *Pseudomonas aeruginosa* isolates were subjected to disc diffusion antimicrobial susceptibility testing. *Alg, oprL,* and *oprI*-specific primers were used in the polymerase chain reaction technique for the molecular identification of virulence factors genes. **Results:** *Pseudomonas aeruginosa* isolates that were one hundred thirty-one (100%) had *oprL* gene. On the other hand, *Alg* and *oprI* each have 129 (98.47%). **Conclusion:** Alarming factors for public health include the high rate of MDR among *P. aeruginosa* isolates and its capacity to form biofilm.

Keywords: Antibiotic resistance, biofilm formation, *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a toxigenic infectant that can cause nosocomial infection. To begin infection and invasion, they must suppress immune system defenses, then begin pathogenesis, survive, and escape the immune system.[1] There are two potential sources of opportunistic infection: nosocomial infections and infected wounds.[2,3] *Pseudomonas aeruginosa* infection in burn patients is prevalent and ranks among the most serious life-threatening illnesses in burn units because burn wounds generally serve as a good site for bacterial multiplication due to immune system suppression.[4] The use of antimicrobial agents is of great importance in the treatment of bacterial infections.[5] Compared to other Gram-negative bacilli, *P. aeruginosa* is typically less resistant to numerous antibiotics by nature. Strains of *P. aeruginosa* that are multiple drug resistant (MDR) are difficult to treat clinically.[6] MDR *P. aeruginosa* has become resistant to numerous medication classes, including carbapenems, fluoroquinolones, cephalosporins, and aminoglycosides, as a result of the emergence and dissemination of antimicrobial-resistant strains.[7] Despite the fact that they are often acquired and distributed through chromosomal changes. These comprise many mobile DNA segment types, including plasmids, transposons, and integrons.[8] It is believed that a key element contributing to persistent infections is *P. aeruginosa* capacity to generate biofilm. A complex of microbial cells known as a biofilm protects bacteria by enclosing them in an extracellular matrix. Biofilms are very difficult and expensive to cure with antimicrobial substances.[9] Alginate, an exopolysaccharide that *P. aeruginosa* produces, helps the bacteria adhere to solid surfaces and protects them from changing environmental

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Table 1: Primers for PCR amplification of biofilm and virulence factors genes

Gene	Sequence	Product size (bp)	Reference
Alg: F	5'- TTCCCTCGCAGAGAAAACATC -3'	520	[17]
Alg: R	5'- CCTGGTTGATCAGGTCGATCT -3'		
oprL: F	5'- ATGGAAATGCTGAAATTCGGC -3'	504	
oprL: R	5'- CTTCTTCAGCTCGACGCGACG -3'		
oprI: F	5'- ATGAACAACGTTCTGAAATTCTCTGCT -3'	249	
oprI: R	5'- CTTGCGGCTGGCTTTTTCCAG -3		

conditions.[10] The *algD* gene controls the transcription of the Alg proteins and alginate biosynthesis. It also produces the precursor GDP-mannuronic acid.[11] *Pseudomonas aeruginosa* has many virulence factors, including exotoxin A, exoenzyme S, and elastase, all of which are tightly regulated by cell-to-cell signaling systems.[12] The purpose of this research is to detect *P. aeruginosa* biofilm-forming and virulence factor potential, as well as its relationship to antibiotic resistance.

Materials and Methods

Study subjects

A total of 385 clinical samples from various clinical sources (burn, wound, and urine) were obtained from various hospitals in Babylon Province/Iraq in 2022. Regular biochemical assays and the Vitek2 System were used to identify the 131 *P. aeruginosa* isolates that were recovered from the research.

Susceptibility testing for antimicrobials

Relative to (CLSI, 2022),[13] antibiotic susceptibility was tested by the disk diffusion test. As a control positive, *P. aeruginosa* ATCC 27853(ATCC/USA) was also examined as a reference strain for antibiotic resistance. However, isolates that were multiple-drug resistant were distinct as being resistant to at least three different antibiotic groups.[14]

Biofilm formation

Quantitative biofilm assay for detection the bacterial ability for biofilm formation by spectrophotometric method was performed as described.[15,16]

Extraction of genomic DNA

Genomic DNA was extracted from *P. aeruginosa* isolates. The genomic DNA extraction procedure was provided by (Favorgen DNA Extraction KIT, Korea) and stored at -20°C. DNA concentration and purity of the samples were assessed using Nano-drop (Analytik jena, Germany).

PCR assay

The presence of *Alg, oprL,* and *OprI* were examined in all isolates. For each gene, specific primers were constructed [Table 1]. A final volume of 25 µL was used for the PCR.

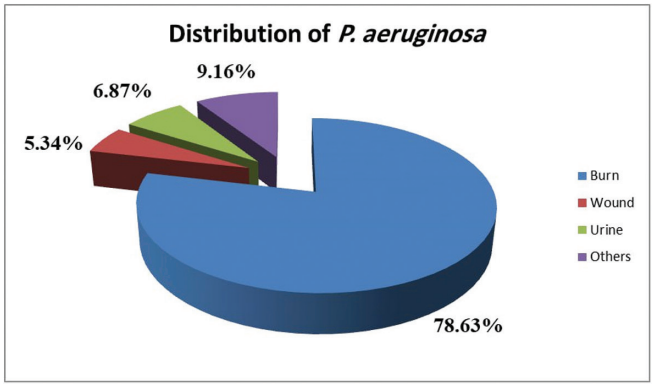

Figure 1: Sources of *P. aeruginosa*

Under the following conditions, PCR amplification for the identification of *Alg, oprL,* and *oprI* were performed as reported,[17] PCR products were separated on 1.5 % agarose gel.[18]

Statistical analysis

Chi-square at a probability of 0.05 was applied to assess the differences of tested variables by (SPSS).

Ethical approval

This research was performed abiding by the ethical principles set by the university of Babylon according to document number B220101 (including the number and the date in 17/01/2022) to get this approval.

Results

Isolates

From diverse clinical samples taken from several hospitals in the Babylon province of Iraq, 131 verified *P. aeruginosa* isolates were found. Total *P. aeruginosa* isolates were 51.9 % (68/131) from male and 48.1% (63/131) from female hospitalized patients. The mean (+SD) age of the group was 34.1 + 20.6 years. From 385 diverse clinical samples taken from several hospitals in the Babylon province of Iraq, 131 isolates were obtained [Figure 1].

Resistances patterns

All 131 *P. aeruginosa* isolates were tested for susceptibility to 22 antibiotics (Bioanalyse/ Turkey and Liofilchem/

Italy). The findings suggested that different isolates' rates of susceptibility to various antibiotics may be observed. Figure 2 shows that all *P. aeruginosa* isolates were resistant to Ampicillin, Cefixime, Cefotaxime in (100%). Whereas Ceftizoxime (99.23%), Cefepime (93.90%), Tetracycline (91.60%), Gentamicin (83.97%), Doxycycline (90.84%), Cefoperazone (84.73%), Tobramycin and Meropenem (81.68%) respectively, Ticarcillin clavulanic (80.91%), Amikacin (79.39 %) Piperacillin (77.86%), Imipenem (74.80%), Levofloxacin (75.57%), Aztreonam (74.05%), Ciprofloxacin (73.28%), Norfloxacin (71.75%), Piperacillin-tazobactam PIT (64.12%), and Doripenem (54.20%). Azithromycin demonstrated the highest level of sensitivity (40.46%).

All isolates (100%) displayed MDR resistance to at least three groups of antibiotics, with some isolates displaying resistance to nearly all classes [Figure 3].

Biofilm production

According to our research, 85.49% of *P. aeruginosa* isolates produced biofilm. In the present study, (16.03, 30.53, and 38.93) % of the isolates produced biofilms weakly, strongly, and moderately, respectively. While 14.50% of the isolates were regarded as non-biofilm producers [Figure 4].

Association of genes of virulence factors with formation of biofilm

Prevalence of genes; *Alg*, *oprL*, and *OprI* were 129 (98.47%), 131 (100%), and 129 (98.47%), respectively. As demonstrated in Table 2, no association was found between *Alg, oprL,* and *oprI* with biofilm formation capability ($P < 0.05$).

Molecular profiles of virulence factors genes

The PCR result showed (98.47% and 100%) isolates tested positive for the *Alg* and *oprL* with amplification products of 520 bp and 504 bp, respectively, and that the presence of the gene encodes *oprI* was detected in 129 (98.47%) with amplification product of 249 bp [Figures 5-8].

Whereas the presence of the gene encodes *oprI* was detected in 129 (98.47%) of clinical isolate of *P.aeruginosa* with amplification product of 249 bp, as shown in Figure 7.

Table 3 illustrates the antimicrobial resistance trend of *P. aeruginosa* isolates in both biofilm formers and non-formers. Antimicrobial resistance was found to be significantly higher in biofilm-producing *P. aeruginosa*

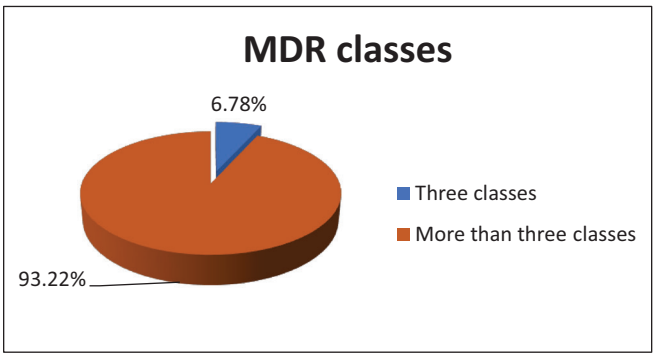

Figure 3: Resistance patterns of *P. aeruginosa* isolates

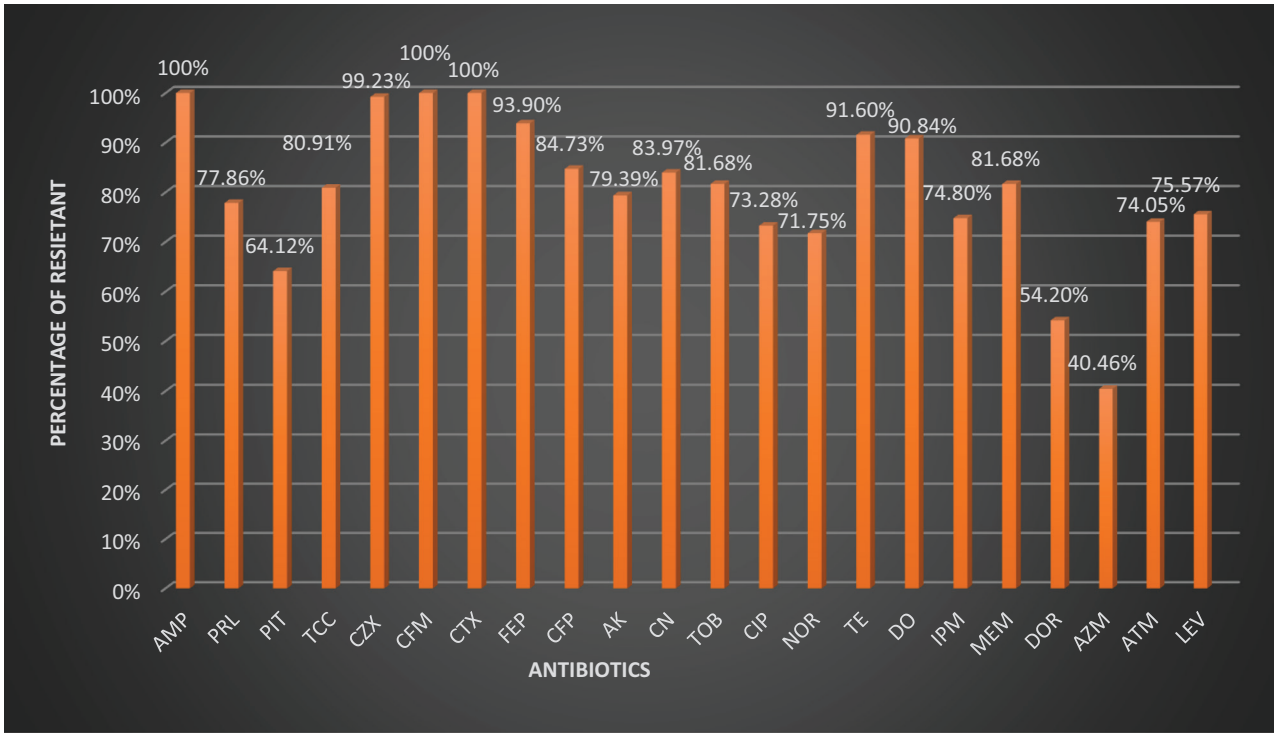

Figure 2: Antibiotics susceptibility of *P. aeruginosa* isolates

isolates than in non-biofilm-producing *P. aeruginosa* isolates ($P < 0.05$). Antibiotics from different classes, including Piperacillin-tazobactam PIT, Ticarcillin clavulanic TTC, and Levofloxacin LEV, were found to have a statistically significant ($P < 0.05$) correlation between biofilm production and antimicrobial resistance. However, the correlation was not found to be significant in other classes of antibiotics.

Discussion

Pseudomonas aeruginosa: MDR isolates have emerged and spread around the world, raising serious concerns that could make it more difficult to choose empirical agents. It is a hugely significant pathogen producing a variety of diseases in hospitals and healthcare situations.[19] The current study indicated that all isolates were MDR (100%). According to Corehtash *et al.*,[20] 93.1% of isolates were MDR. Multi-drug resistance in *P. aeruginosa* strains causes antibiotics intended to treat infections brought on by *P. aeruginosa* to lose their therapeutic effectiveness.[21] This study found that Azithromycin had the highest antibiotic susceptibility rate in the current study, with a susceptibility rate of 59.54%, while the majority of *P. aeruginosa* isolates displayed significant antibacterial agent resistance rates. The results of the present study, patients are becoming more resistant (100%) to several categories of antibiotics like ampicillin, cefixime, and cefotaxime. Mutations in class A-lactamases are the primary source of the resistance to third-generation cephalosporins.[22] In the middle of the 1990s, the fourth-generation Cephalosporin Cefepime was released into clinical use.[23] Furthermore, 83.97% and 79.39% of our isolates were resistant to aminoglycoside groups; gentamicin and amikacin, respectively. Resistance of *P. aeruginosa* isolates to aminoglycoside antibiotics in this study was parallel to the study of Poonsuk *et al.*[24] In gram-negative bacilli, carbapenem resistance is becoming more commonplace. The most significant mechanism driving carbapenem resistance is thought to be the acquisition of genes encoding MBL.[25] The antibiotic most successful in treating MDR isolates is carbapenem. However, a current global challenge is the rise in carbapenem-resistant *P. aeruginosa*.[26] Ugwuanyi *et al.*,[27] All *Pseudomonas* isolates (100%) in their investigation conducted in Nigeria were revealed to be resistant to CZX, CZM, and AMP-clavulanate. Additionally, a study conducted in southwestern Nigeria by Odumosu *et al.*,[28] reported that *Pseudomonas* isolates were 100% resistant to amoxicillin-clavulanate, and 87.1% resistant to ceftriaxone. The extracellular and cell-mediated virulence factors of *P. aeruginosa*, including *toxA, exoY, oprL, oprI, lasA, lasB, and oprD*, are also associated with the pathogenesis of the organism. These factors have the ability to invade and propagate throughout the host body and destroy host tissue.[29] The main components of *P. aeruginosa* outer membrane lipoproteins, which are also utilized as indicators for the detection of *P. aeruginosa*-associated infections, include virulence genes such as *oprL, oprI,* and *oprD*.[30,31]

Production of biofilms has been identified as a key pathogenicity factor in *P. aeruginosa* infections.[32]

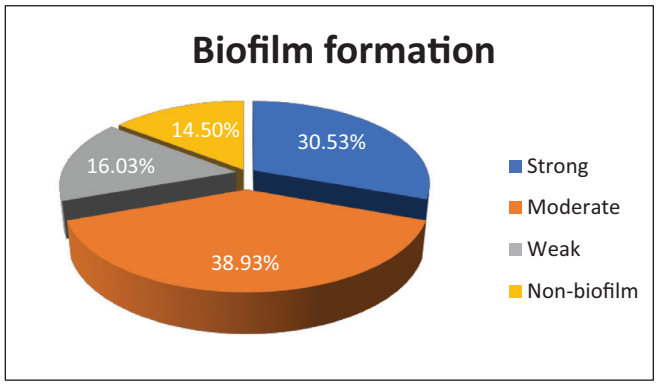

Figure 4: Biofilm formation of *P aeruginosa* isolates

Table 2: Biofilm formation and related genes, virulence factors genes among *P. aeruginosa*

Biofilm formation	*Alg* No. %	*oprL* No. %	*oprI* No. %
Strong	39(97.5%)	40(100%)	39(97.5%)
Moderate	51(100%)	51(100%)	50(98.03%)
Weak	21 (100%)	21(100%)	21(100%)
Non-biofilm	18(94.73%)	19(100%)	19(100%)
P. value	0.98	1.00	0.99

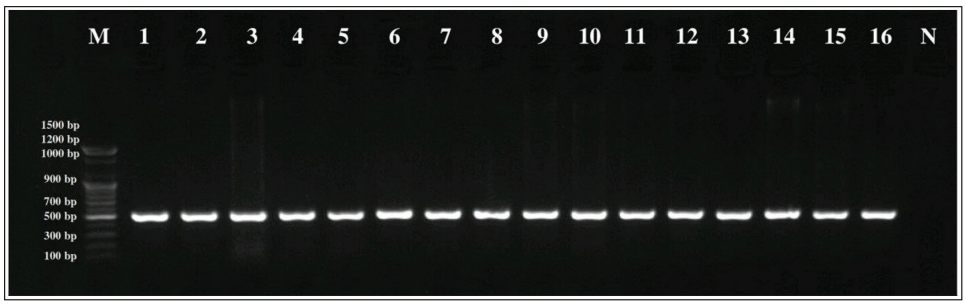

Figure 5: PCR amplification of *Alg* gene (520 bp) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. Lanes (1-16) were positive isolates and N (negative control)

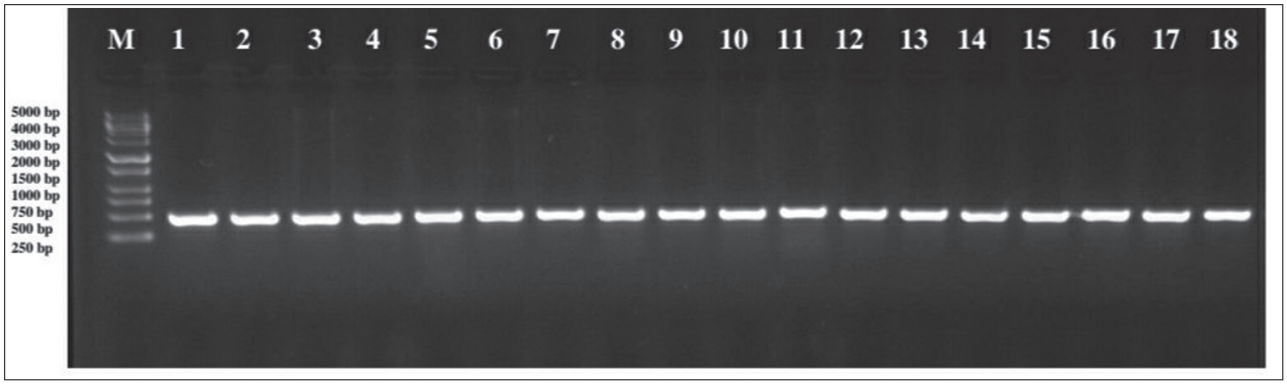

Figure 6: PCR amplification of *oprL* gene (504 bp) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. All isolates were positive isolates

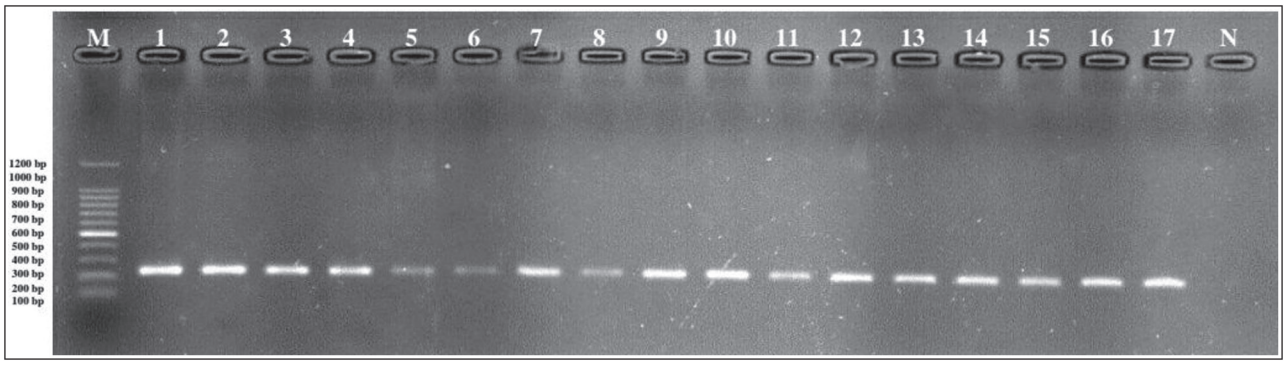

Figure 7: PCR amplification of *oprI* gene (249 bp) in *P. aeruginosa* isolates, on 1.5% agarose at 80 volt for 1 hr. All isolates were positive isolates

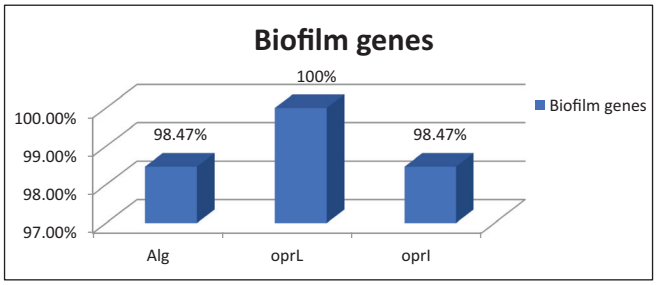

Figure 8: Prevalence of genes; *Alg*, *oprL*, and *OprI* of *P. aeruginosa* isolates

According to our findings, 85.49% of *P. aeruginosa* isolates produced biofilm. When *P. aeruginosa* forms biofilms, it loses its antibacterial resistance and must be treated with higher concentrations of antibiotics. The persistence of delayed infections is caused by two key virulence features of *P. aeruginosa*: biofilm development and antimicrobial resistance. According to research[33] on the relationship between the *AlgD* gene and biofilm production in *P. aeruginosa*, *AlgD* is highly expressed in all biofilm formers and is lacking in non-biofilm formers. Heidari *et al*.[34] reported 100% of *P. aeruginosa* biofilm producers carried the *AlgD* gene. A variety of methods, such as the creation of multidrug efflux systems, enzymes production, outer membrane protein (porin) loss, and target alterations, can be used by *P. aeruginosa* to mediate MDR.[35] The rise in MDR strains observed in the current investigation may be related to the recent, unrestricted usage of antibiotics in healthcare. The primary cause of MDR, which results in the selection and spread of bacteria resistant to antibiotics in clinical medicine, is the reckless use of antibiotics without antibiotic sensitivity testing.

Conclusion

The management of infections brought on by *P. aeruginosa* has significant public health consequences because of the organism's capacity for multidrug resistance, biofilm formation, and virulence factors.

Acknowledgements

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Financial support and sponsorship

This study was self-funded.

Conflict of interests

No conflict of interest was reported.

Table 3: Antimicrobial resistance pattern of *P. aeruginosa* among biofilm formation isolates

Bi Biofilm	Strong (40)			Moderate (51)			Weak (21)			Non-biofilm (19)			*P* value
Antibiotics	**R no.(%)**	**S no.(%)**	**I no.(%)**	**R no.(%)**	**S no.(%)**	**I no.(%)**	**R no.(%)**	**S no.(%)**	**I no.(%)**	**R no.(%)**	**S no.(%)**	**I no.(%)**	
Ampicillin AMP	40(100)	-	-	51(100)	-	-	21(100)	-	-	19(100)	-	-	0.967
Piperacillin PRL	29(72.5)	6(15)	5(12.5)	40(78.43)	6(11.76)	5(9.8)	18(85.71)	2(9.52)	1(4.76)	15(78.95)	4(21.05)	-	0.844
Piperacillin-tazobactam PIT	23(57.5)	9(22.5)	8(20)	36(70.5)	7(13.72)	8(15.69	10(47.62)	4(19.05	7(33.33	15(78.25)	3(15.79)	1(5.26)	0.043*
Ticarcillin -clavulanic TTC	31(77.5)	5(12.5)	4(10)	45(88.23)	4(7.84)	2(3.92)	15(71.42)	3(14.29)	3(14.29)	15(78.96)	2(10.52)	2(10.52)	0.036*
Ceftizoxime CZX	40(100)	-	-	50(98.04)	1(1.96)	-	21(100)	-	-	19(100)	-	-	0.970
Cefixime CFM	40(100)	-	-	51(100)	-	-	21(100)	-	-	19(100)	-	-	0.967
Cefotaxime CTX	40(100)	-	-	51(100)	-	-	21(100)	-	-	19(100)	-	-	0.967
Cefepime FEP	36(90)	2(5)	2(5)	51(100)	-	-	19(90.48)	1(4.76)	1(4.76)	17(89.47)	1(5.26)	1(5.26)	0.938
Cefoperazone CFP	33(82.5)	6(15)	1(2.5)	45(88.24)	5(9.8)	1(1.96)	18(85.71)	2(9.52)	1(4.76)	15(78.95)	4(21.05)	-	0.869
Amikacin	31(77.5)	8(20)	1(2.5)	41(80.4)	9(17.64)	1(1.96)	18(85.71)	3(14.29)	-	14(73.68)	5(26.32)	-	0.946
Gentamicin CN	34(85)	5(12.5)	4(10)	45(88.24)	4(7.84)	2(3.92)	18(85.71)	3(14.29)	-	14(73.68)	4(21.05)	1(5.26)	0.702
Tobramycin TOB	32(80)	8(20)	-	42(82.35)	9(17.65)	-	18(85.71)	3(14.29)	-	15(78.95)	4(21.05)	-	0.980
CiprofloxacinCIP	28(70)	12(30)	-	40(78.43)	11(21.57)	-	14(66.66)	7(33.33)	-	15(78.95)	4(21.05)	-	0.880
Norfloxacin NOR	25(62.5)	15(37.5)	-	40(78.43)	10(19.6)	1(1.96)	13(61.9)	7(33.33)	1(4.76)	15(78.95)	4(21.05)	-	0.535
Tetracycline TE	36(90)	3(7.5)	1(2.5)	48(94.12)	1(1.96)	2(3.42)	20(95.24)	1(4.76)	-	16(84.21)	2(10.53)	1(5.26)	0.819
Doxycycline DO	37(92.5)	3(7.5)	-	44(86.27)	1(1.96)	6(11.76)	21(100)	-	-	17(89.47)	2(10.53)	-	0.451
Imipenem IPM	31(77.5)	6(15)	3(7.5)	38(74.5)	9(17.65)	4(7.84)	14(66.66)	5(23.81	2(9.52)	11(57.9)	4(21.05)	4(21.05)	0.632
Meropenem MEM	33(82.5)	7(17.5)	-	42(82.35)	8(15.68)	1(1.96)	18(85.71)	3(14.29)	-	13(68.42)	5(26.31)	1(5.26)	0.897
Doripenem DOR	23(57.5)	11(27.5)	6(15)	29(56.86)	15(29.41)	7(13.73)	12(57.14)	6(28.57)	3(14.29)	7(36.84)	5(26.32)	7(36.84)	0.421
AzithromycinAZM	18(45)	22(55)	-	16(31.37)	35(68.63)	-	7(33.33)	14(66.66)	-	13(68.42)	6(31.58)	-	0.684
Aztreonam ATM	26(65)	13(32.5)	1(2.5)	40(78.43)	7(13.73)	4(7.84)	17(80.95)	3(14.29)	1(4.76)	14(73.68)	5(26.32)	-	0.386
Levofloxacin LEV	28(70)	10(25)	2(5)	41(80.39)	9(17.65)	1(1.96)	15(71.43)	6(28.57)	-	15(78.95)	4(21.05)	-	0.029*

*Significance differences at ($P < 0.05$)

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