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Relationship between Biofilm Regulating Operons and Various β-Lactamase Enzymes: Analysis of the Clinical Features of Infections caused by Non-Fermentative Gram-Negative Bacilli (NFGNB) from Iran

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Abstract

Bacteria are capable of evolving high doses of the drug in various infections by forming biofilms. Perhaps, biofilm regulator genes have different frequencies in β-lactam producing non-fermentative Gram-negative Bacilli (NFGNB). In this study, we investigated the role of biofilm operons of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* on the prevalence of different β-lactamase enzymes. One-hundred twenty (120) nosocomial NFGNB isolates were collected from different clinical samples of patients. PCR method was used for the amplification of resistance genes. Isolates were collected, including 50 isolates (41.66%) of *P. aeruginosa* and 70 isolates (58.33%) of *A. baumannii*. The distribution of ESBL, AmpC, KPC, and MBL β-lactamase enzymes in *P. aeruginosa* and *A. baumannii* isolates were 64%, 58%, 38%,44%, and 57.14%, 60%, 32.85%, 34.28%, respectively. The frequency of *csuABC*, *pgaABC* operon in *A. baumannii* were as follows: *pgaA* (45.71%), *pgaB* (32.85%), *pgaC* (42.85%), *csuA* (34.28%), *csuB* (32.85%), *csuC* (41.42%), and *ompA* (38.57%). Further, the prevalence of *pslABC* and *pelABC* operons in *P. aeruginosa* isolates were as follows: *pslA* (58%), *pslB* (58%), *pslD* (60%), *pelA* (64%), *pelB* (38%), *pelC* (44%), and *algD* (68%). This study revealed that the abundance of biofilm regulator genes in NFGNB strains is affected by different β-lactamase enzymes.

Keywords: β-lactamase enzymes, Biofilm formation, Antibiotic resistance, Virulence factors, non-fermentative Gram-negative Bacilli

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Abbreviation: CLSI: Clinical and Laboratory Standards Institute, NFGNB: Non-fermentative Gram-negative Bacilli, MDR: Multidrug resistant, ESBLs: Extended-spectrum β-lactamases, XDR: Extensively Drug Resistant, KPC: *Klebsiella pneumoniae* Carbapenemase, SHV: Sulfydryl variable, TEM: Temoneira, CDT: Combined disk method, MBL: Metallo beta-lactamase, MR/VP: Methyl Red Voges Proskauer.

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INTRODUCTION

Non-fermenting Gram-negative bacilli (NFGNB) pose a particular difficulty for the healthcare community. They are resistant to three or more drugs, and essential members of this group are *Pseudomonas aeruginosa* and *Acinetobacter baumannii*[1]. The recently extended spectrum of β-lactamases and carbapenemase resistance is reported in nosocomial infections[2-4]. Multidrug-resistant (MDR) strains carrying extended-spectrum β-lactamases (ESBLs) have become a growing problem worldwide[5].

The major challenge of biofilm removal is addressing their increased resistance to disinfection[6]. Induction of antimicrobial-degrading enzymes and biofilm operons have been found in biofilm-grown *P. aeruginosa*. In NFGNBs, there are many genes involved in controlling and producing biofilms, then each of these genes is specific to the genus and bacterial species[7,8]. The gene clusters of *P. aeruginosa* involved in biofilm formation have been studied, and two biofilm-forming operons, pel, and the psl have been identified in *P. aeruginosa*[5]. The pel operon affects biofilm maturation, and the psl operon affects biofilm initiation[8]. The expression of biofilms by *A. baumannii* clinical isolates, accompanied with changes in genetic expression, has been reported by several studies[5]. Nevertheless, several genes have been associated with biofilm formation in *A. baumannii*. *CsuABC*, *bap*, *OmpA*, and *pgaABCD* are some of these[6].

Some studies have reported that there is a relationship between biofilm formation and antibiotic resistance. However, some environmental factors may indicate the activity of biofilm formation and β-lactamase enzymes[9,10]. In other words, the presence of the ESBL, AmpC, MBL, and KPC enzymes may alter the biological activity of the organism and increase the pathogenicity and spread of the infection[11].

Therefore, the aim of this study was to investigate the frequency of biofilm regulating genes in different strains of *A. baumannii* and *P. aeruginosa*. We also intend to determine the relationship between different beta-lactamase enzymes and the abundance of biofilm-forming genes.

MATERIAL AND METHODS

Study design

A cross sectional study design utilizing a systematic random sampling technique was adopted. Admitted patients who had stayed for over 48 hours with catheters; tubing's, surgical wounds, burn wounds and whose consent wassought and given were eligible. Those patients whodid not meet these criteria were excluded from this study. One-Hundred twenty (120) isolates of nosocomial gram-negative were obtained from the Hamadan's Hospitals between Jun 2018 and Oct 2019.

Isolation, and identification of gram-negative bacteria

All collected samples were inoculated onto Nutrient Agar (Merck, Germany) and MacConkey Agar (Merck, Germany) and incubated aerobically at 37°C for 24 hr. Also, biochemical reactions (such as indole production, MR/VP test, Urea hydrolysis, Aesculin hydrolysis) were done. Finally, the *16sRNA* gene was used for molecular confirmation of *P. aeruginosa* and *A. baumannii* isolates. The Ethical Council approved (Ethical NO:17130507962001) the present study of Research of the Faculty of Basic Sciences, Hamadan Branch, Islamic Azad University, Hamadan, Iran.

Determination of Antibiotic Resistance Pattern

The isolates were subjected to antibiotic susceptibility testing by employing Kirby Bauer disc diffusion techniques according to Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines[12]. In the present study, susceptibility was tested against piperacillin(30µg), ticarcillin(75µg), cefepime(30µg), ceftazidime(30µg), meropenem(10µg), imipenem(10µg), amikacin(30µg), gentamicin(30µg), tetracycline(30µg), ciprofloxacin(5µg), and trimethoprim/sulfamethoxazole (1.25/23.75µg) (STX) antibiotics procured commercially from Himedia laboratories Ltd, Mumbai. The diameter of the zone was measured and interpreted according to the guidelines of CLSI. For quality control, *Klebsiella pneumoniae* ATCC 70063 and *Enterobacter Creole* NCBT 13406 were used in the study.

Screening and confirmation of ESBLs and AmpC producer strains

ESBL and AmpC producing strains were identified using the MAST AmpC + ESBL detection

set (UK, MAST, code: D68C) based on manufacturer instruction. *Klebsiella pneumoniae* ATCC 70063, *Escherichia coli* ATCC 25922, and *Enterobacter Creole* NCBT 13406 were used as a positive control.

Screening and confirmation of MBL and KPC producer strains

For the detection of MBL producing strains, EDTA-imipenem microbiological (EIM) was used. For the detection of carbapenemase-producing strains, the Modified Hodge test (MHT) was used[13].

Screening of biofilm producer strains

Biofilm production was assessed using a crystal violet microtiter plate assay according to the method of Ghadaksaz et al study[11]. The OD of each well was measured at 550nm and 595nm using the microplate reader (Omega Fluostar, Germany). Bacterial biofilms were classified based on an OD cut-off ODc as described. In this case, *P. aeruginosa* PAO1 and *A. baumannii* ATCC 19606 was used as the positive control, and the culture medium used as negative controls.

DNA Extraction

DNA was prepared for PCR according to the method described previously with some modifications[13]. Briefly, the organisms were grown overnight, and from that young culture of *P. aeruginosa* were taken in a 2 ml microcentrifuge tube and centrifuged it at 3000 rpm for 10 minutes. Then the supernatant was discarded, and 100µl of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1mMEDTA [pH 8]) was added to the pellet. The

Table 1. Oligonucleotide sequences used in this study

Target	Gene	Sequence of Primers	Product size(bp)	Ref
csuABC operon	*csuA*	F: TGGTACAGCAGTAGCTTGGC		
		R: GACGGTGGTGAACGTACAGA	156	6
	csuB	F: GGCAAACTTTCCGTACAACGA		
		R: ATGCAGCAGATCCTCAGCTC	187	6
	csuC	F: GAAGCATCTTGCTCGTTGCC		
		R: TTCGCTTAACCAAAAGCGCC	109	6
pgaABC operon	*pgaA*	F: CCCGCTTCAAAATGCTGCTT		
		R: AAGGCTATTCGACGCACCTC	186	6
	pgaB	F: ATGCATCTGGGCTTGCATCT		
		R: GTGACAGAGCAGGCAAAAGC	190	6
	pgaC	F: ACGAATCGTTTGTCGGACCT		
		R: ATGGTAGGAGGCCTCTGGTT	132	6
Adhesion	*ompA*	F: ATTTACCAGGATGGGCCGTG		
		R: GCGCCACAACCAAGCAATTA	182	6
Acinetobacter baumannii	*16srRNA*	F: TTTAAGCGAGGAGGAGG		
		R: ATTCTACCATCCTCTCCC	240	3
pelABC operon	*pelA*	F: CCTTCAGCCATCCGTTCTTCT		
		R: TCGCGTACGAAGTCGACCTT	118	5
	pelB	F: CGGCTACGTGCAGCGTTAT		
		R: CACTGCATGCGTTCCTTGAC	150	1
	pelC	F: TGCTCCAGCTTCACCAG		
		R: CAGTTGCAGGTCGCCTT	192	8
pslABD operon	*pslA*	F: TGGGTCTTCAAGTTCCGCTC		
		R: ATGCTGGTCTTGCGGATGAA	119	5
	pslB	F: ACACCAACGAATCCACCTTCA		
		R: CGCTCTGTACCTCGATCATCAC	93	8
	pslD	F: CTCATGAAACGCACCCTCCT		
		R: TGCGACCGATGAACGGATAG	295	5
alginate	*algD*	F: ACGAAGTGGTGGCGAGTTC		
		R: TGGTGTGCGGCATGAAGC	126	31
Pseudomonas aeruginosa	*16srRNA*	F: TGGAGCATGTGGTTTAATTCGA		
		R: TGCGGGACTTAACCCAACA	105	4

microcentrifuge tubes were placed in a water bath at 100°C for 10 minutes and immediately cooled on Ice. Following centrifugation, the supernatant was used as a template for PCR.

Biofilm operons Genes Detection

Primer sets used were obtained from Macrogen, Korea (Table 1). Template DNA in a volume of 2µL was added to the 12.5µL master mix (Ready Mix TMTaq PCR Reaction Mix, Sigma) with 0.4µM of each primer for a final volume of 25µL in each PCR. DNA templates were subjected to one regime of amplification. After initial denaturation at 95°C for 5min. The PCR cycling consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1min annealing at 55 to 61°C (according to Table 1), 72°C for 90 sec and a final extension at 72°C for 5 min. The PCR products (10µL) were analyzed by electrophoresis on 1%agarose gel. Finally, the amplified bands in the gel were visualized by a trans-illuminator (UV light) to confirm the PCR products.

Statistical analysis

The WHONET software version 2018 (World Health Organization, Geneva, Switzerland) was used for entry and preliminary analysis of microbiology data. Also, the data were analyzed using SPSS software version 16 (SPSS, Com, Chicago, IL), and descriptive statistics such as means, standard deviations, frequencies, and percentages were generated; biofilm production was compared using the Mann–Whitney U test. Also, different criteria were used to analyze data in this research. These tests were Mann-Whitney U-Test (Two-tailed), Fisher exact test, and Chi-square ($\chi 2$) test. $P < 0.05$ values were considered statistically significant.

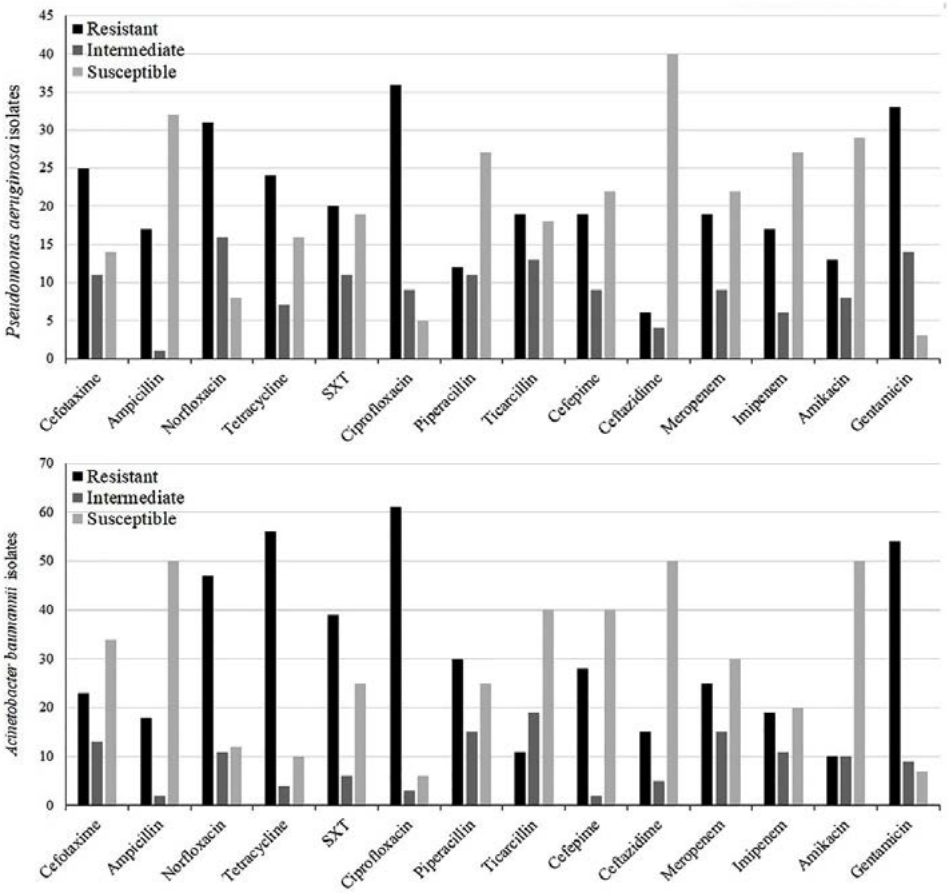

Fig. 1. Antibiotic resistance pattern in clinical isolates of *P. aeruginosa* and *A. baumannii*

Table 2. Phenotypic characteristics of biofilm formation and antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Phenotypic test	Number (%) of clinical isolates of *Pseudomonas aeruginosa*					p^a	Number (%) of clinical isolates of *Acinetobacter baumannii*					p
	Total	wounds	blood	urine	catheters		Total	wounds	blood	urine	catheters	
	n=50	n=19	n=10	n=9	n=12		n=70	n=23	n=17	n=19	n=11	
Biofilm production b												
Low	3 (6)	3 (1oo)	0 (0)	0 (0)	0 (0)	0.685	10 (14.2)	6 (60)	0 (0)	4 (40)	0 (0)	0.072
Moderate	14 (28)	5 (35.7)	0 (0)	1 (7.1)	8 (57.1)	0.012	19 (27.4)	7 (36.9)	1 (5.2)	1 (5.2)	10 (52.6)	<0.001
Strong	16 (32)	9 (56.2)	1 (6.2)	2 (12.5)	4 (25)	<0.001	20 (28.5)	11 (55)	1 (5)	3 (15)	5 (25)	<0.001
Antibiotic Resistance												
Gentamicin	33 (66)	7 (21.1)	10 (30)	9 (27.7)	7 (21.1)	<0.001	54 (19.3)	22 (40.7)	16 (29.6)	13 (24)	3 (5.5)	<0.001
Amikacin	13 (26)	2 (15.3)	7 (53.8)	3 (23)	1 (7.6)	0.085	10 (19.3)	3 (30)	5 (50)	2 (20)	0 (0)	<0.001
Imipenem	17 (34)	9 (52.9)	5 (29.4)	1 (5.8)	2 (15.3)	0.039	19 (19.3)	11 (57.8)	7 (36.8)	1 (5.2)	0 (0)	0.043
Meropenem	19 (38)	4 (21)	9 (47.3)	5 (26.3)	1 (5.2)	<0.001	25 (19.3)	9 (36)	5 (20)	4 (16)	1 (4)	0.051
Ceftazidime	6 (12)	1 (16.6)	5 (83.3)	0 (0)	0 (0)	0.016	15 (19.3)	4 (26.6)	7 (46.6)	4 (26.6)	0 (0)	0.033
Cefepime	19 (38)	4 (21)	7 (36.8)	5 (26.3)	3 (15.7)	<0.001	28 (19.3)	9 (32.1)	11 (39.2)	7 (19.3)	2 (19.3)	<0.001
Ticarcillin	19 (38)	9 (47.3)	4 (21)	5 (26.3)	2 (10.5)	0.119	11 (19.3)	2 (19.3)	1 (19.3)	7 (25)	1 (4)	0.053
Piperacillin	12 (24)	3 (25)	5 (41.6)	2 (16.6)	2 (16.6)	0.093	30 (19.3)	11 (36.6)	6 (20)	10 (33.3)	3 (1)	0.013
Ciprofloxacin	36 (72)	11 (30.5)	6 (20)	9 (30)	10 (33.3)	0.004	61 (19.3)	23 (37.7)	11 (18)	18 (29.5)	9 (14.7)	<0.001
Trimethoprim/ sulfamethoxazole	20 (40)	7 (35)	9 (40)	4 (20)	0 (0)	0.072	39 (19.3)	11 (28.2)	17 (43.5)	9 (23)	1 (2.5)	<0.001
Tetracycline	29 (58)	13 (44.8)	8 (27.5)	8 (27.5)	1 (3.4)	0.112	49 (19.3)	15 (30.6)	10 (20.4)	17 (34.6)	7 (14.2)	0.109
Norfloxacin	31 (62)	11 (35.4)	7 (22.5)	8 (25.8)	5 (16.1)	<0.001	47 (19.3)	12 (25.5)	11 (23.4)	18 (36.7)	6 (12.7)	<0.001
Ampicillin	17 (34)	9 (52.9)	2 (11.7)	3 (17.6)	2(11.7)	<0.001	18 (19.3)	6 (33.3)	6 (33.3)	5 (27.7)	1 (5.5)	<0.001
Cefotaxime	25 (50)	10 (40)	6 (24)	7 (28)	2 (8)	0.097	23 (19.3)	9 (39.1)	3 (13)	11 (47.8)	0 (0)	0.025
MDR	11 (22)	6 (54.5)	2 (18.1)	2 (18.1)	1 (9)	<0.001	22 (31.4)	11 (50)	5 (27.7)	4 (18.8)	2 (9)	<0.001
XDR	7 (14)	4 (57.1)	2 (28.5)	1 (14.2)	0 (0)	0.024	11 (15.7)	7 (63.6)	1 (9)	3 (27.7)	0 (0)	0.041
β-lactamase producer isolates												
AmpC enzymes	29 (58)	16 (55.1)	4 (13.79)	7 (24.1)	2 (6.8)	<0.001	42 (60)	19 (45.2)	10 (23.8)	8 (19)	5 (11.9)	<0.001
ESBL enzymes	32 (64)	10 (31.2)	6 (18.7)	9 (28.1)	7 (21.8)	<0.001	40 (57.1)	17 (42.5)	7 (17.5)	11 (27.5)	5 (12.5)	<0.001
KPC enzymes	19 (38)	4 (21)	6 (31.5)	7 (36.8)	2 (10.5)	<0.001	23 (32.8)	20 (86.9)	1 (4.3)	2 (8.6)	0 (0)	0.011
MBL enzymes	22 (44)	13 (59)	5 (22.7)	2 (9)	2 (9)	<0.001	24 (34.2)	19 (79.1)	1 (4.6)	3 (12.5)	0 (0)	0.049

a: χ^2 test.
b: Mann–Whitney U test.

Table 3. Molecular and phenotypic characteristics of biofilm formation regulatory genes and antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Phenotypic test	Number (%) of biofilm formation regulatory genes in *Pseudomonas aeruginosa* isolates								Number (%) of biofilm formation regulatory genes in *Acinetobacter baumannii* isolates							
	Total	*pslA*	*pslB*	*pslD*	*pelA*	*pelB*	*pelC*	*algD*	*Total*	*pgaA*	*pgaB*	*pgaC*	*csuA*	*csuB*	*csuC*	*ompA*
	n=50	n=29	n=29	n=30	n=33	n=19	n=22	n=34	n=70	n=32	n=23	n=30	n=24	n=20	n=29	n=27
Biofilm production																
Low	3 (6)	0 (0)	3 (100)	1 (30.3)	3 (100)	3 (100)	0 (0)	2 (66.6)	10 (14.2)	3 (30)	7 70)	3 (30)	1 (10)	0 (0)	1 (10)	4 (40)
Moderate	14 (28)	14 (100)	10 (71.4)	13 (63.3)	14 (92.8)	6 (42.8)	9 (64.2)	14 (100)	19 (27.4)	12 (63.5)	11 (57.8)	11 (57.8)	9 (47.3)	3 (15.7)	11 (57.8)	7 (36.8)
Strong	16 (32)	15 (93.7)	16 (100)	16 (100)	16 (100)	10 (62.5)	13 (81.2)	16 (100)	20 (28.5)	17 (85)	5 (25)	16 (80)	14 (70)	17 (85)	17 (85)	16 (80)
Antibiotic Resistance																
Gentamicin	33 (66)	19 (57.7)	22 (66.6)	26 (78.8)	20 (60.6)	14 (42.4)	19 (57.7)	29 (87.7)	54 (19.3)	32 (32.2)	19 (42.2)	26 (48.1)	13 (24.1)	13 (24.1)	27 (50)	24(44.4)
Amikacin	13 (26)	5 (38.4)	7 (53.8)	11 (84.6)	9 (69.2)	7 (53.8)	11 (84.6)	11 (84.6)	10 (19.3)	6 (60)	4 (40)	9 (90)	7 (70)	7 (70)	10 (100)	10 (100)
Imipenem	17 (34)	9 (52.9)	11 (64.7)	11 (64.7)	14 (84.3)	7 (41.1)	13 (76.4)	16 (94.1)	19 (19.3)	11 (57.8)	11 (57.8)	17 (19.3)	10 (89.4)	13 (76.4)	14 (73.6)	18(94.7)
Meropenem	19 (38)	12 (63.1)	12 (63.1)	9 (47.3)	15 (78.9)	17 (89.4)	17 (89.4)	14 (73.6)	25 (19.3)	19 (76)	21 (84)	15 (60)	19 (76)	11 (44)	16 (64)	22 (88)
Ceftazidime	6 (12)	1 (16.6)	5 (83.3)	4 (66.6)	1 (16.6)	4 (66.6)	5 (83.3)	5 (83.3)	15 (19.3)	9 (60)	11 (73.3)	7 (46.6)	11 (73.3)	9 (60)	14 (93.3)	14(93.3)
Cefepime	19 (38)	17 (89.4)	11 (57.8)	11 (57.8)	14 (83.4)	16 (84.2)	10 (52.6)	18 (94.7)	28 (19.3)	19 (67.8)	24 (85.7)	17 (69.7)	20 (71.4)	12 (42.8)	10 (35.7)	25(89.2)
Ticarcillin	19 (38)	13 (68.4)	17 (89.4)	14 (83.4)	11 (57.8)	16 (84.2)	12 (63.1)	7 (36.8)	11 (19.3)	6 (54.5)	2 (18.1)	7 (63.6)	7 (63.6)	9 (81.8)	6 (54.5)	10 (90)
Piperacillin	12 (24)	9 (75)	11 (91.6)	9 (75)	7 (58.3)	3 (25)	9 (75)	11 (91.6)	30 (19.3)	23 (76.6)	21 (70)	26 (86.6)	20 (66.6)	13 (43.3)	24 (80)	26(86.6)
Ciprofloxacin	36 (72)	27 (75)	29 (80.5)	29 (8.05)	31 (86.1)	11 (30.5)	20 (55.5)	33 (91.6)	61 (19.3)	23 (37.7)	20 (32.7)	29 (47.5)	23 (37.7)	19 (31.1)	24 (39.3)	26(47.5)
Trimethoprim/ sulfamethoxazole	20 (40)	11 (55)	19 (95)	14 (70)	19 (75)	15 (75)	11 (55)	18 (90)	39 (19.3)	17 (43.5)	19 (48.7)	26 (66.6)	16 (41)	11 (28.2)	27 (69.2)	24(61.5)
Tetracycline	29 (58)	23 (79.3)	28 (96.5)	25 (86.2)	12 (41.3)	14 (48.2)	19 (65.5)	29 (100)	49 (19.3)	12 (24.4)	19(38.7)	10 (20.4)	17 (34.6)	17 (34.6)	19 (38.7)	13(26.5)
Norfloxacin	31 (62)	22 (70.9)	27 (87)	28 (90.3)	29 (93.5)	19 (61.2)	31 (100)	29 (93.5)	47 (19.3)	10 (21.2)	6 (14.2)	11 (23.4)	18 (38.2)	16 (34)	14 (34)	11(23.4)
Ampicillin	17 (34)	13 (76.4)	12 (70.5)	11 (19.3)	9(52.9)	9 (52.9)	15 (88.2)	15 (88.2)	18 (19.3)	6 (19.3)	6 (19.3)	11 (19.3)	9 (19.3)	7 (19.3)	14 (19.3)	16(19.3)
Cefotaxime	25 (50)	19 (76)	21 (84)	20 (80)	19 (76)	17 (68)	19 (76)	23 (92)	23 (19.3)	16 (69.5)	20 (86.9)	20 (86.9)	19 (82.6)	13 (56.5)	15 (65.2)	17(73.9)
MDR	11 (22)	4 (36.3)	9 (81.1)	3 (27.7)	9 (81.1)	9 (81.1)	3 (27.7)	11 (100)	22 (31.4)	19 (47.8)	17 (47.8)	20 (29.4)	17 (21)	19 (18.1)	21 (19.3)	22(19.3)
XDR	7 (14)	6 (85.7)	6 (85.7)	3 (42.8)	6 (85.7)	5 (71.4)	4 (57.1)	6 (85.1)	11 (15.7)	9 (81.1)	11 (100)	10 (90)	6 (54.5)	6(54.5)	11 (100)	11 (100)
β-lactamase producer isolates																
AmpC enzymes	29 (58)	27 (93.1)	4 (13.7)	7 (24.1)	2 (6.8)	14 (48.2)	14 (48.2)	24 (82.7)	42 (60)	29 (69)	20 (47.6)	29 (69)	24 (57.1)	12 (28.5)	27 (64.2)	25(59.5)
ESBL enzymes	32 (64)	29 (90.6)	16 (50)	19 (59.3)	7 (21.8)	17 (54.8)	21 (65.6)	29 (90.6)	40 (57.1)	27 (67.5)	17 (42.5)	27 (67.5)	21 (52.5)	15 (37.5)	24 (60)	23(57.5)
KPC enzymes	19 (38)	15 (78.9)	16 (84.2)	17 (89.4)	10 (52.6)	13 (68.4)	19 (100)	18 (94.7)	23 (32.8)	20 (86.9)	20 (86.9)	21 (91.3)	12 (52.1)	19 (82.6)	23 (100)	17(73.9)
MBL enzymes	22 (44)	20 (90)	15 (68.1)	12 (54.5)	12 (54.5)	19 (86.3)	20 (90)	22 (100)	24 (34.2)	18 (75)	21 (87.5)	21 (87.5)	23 (95.8)	21 (87.5)	24 (100)	24 (100)

Table 4. Relationship between biofilm formation regulatory genes and antimicrobial resistance

Antibiotics	virulence factors of *Acinetobacter baumannii*[a]								virulence factors of *Pseudomonas aeruginosa*[a]							
	pgaA	*pgaB*	*pgaC*	*csuA*	*csuB*	*csuC*	*ompA*	Biofilm[b]	*pslA*	*pslB*	*pslD*	*pelA*	*pelB*	*pelC*	*algC*	Biofilm[b]
Ampicillin	0.047	0.o52	0.025	0.029	0.097	0.097	0.001	0.009	0.059	0.014	0.029	0.068	0.050	0.067	0.024	0.019
Gentamicin	0.041	0.065	0.003	0.001	0.004	0.033	0.006	0.595	0.017	0.048	0.033	0.091	0.170	0.023	0.079	0.074
Amikacin	0.003	0.080	0.033	0.033	0.029	0.074	0.039	0.174	0.039	0.004	0.019	0.036	0.041	0.052	0.035	0.035
Imipenem	0.069	0.071	0.040	0.032	0.021	0.097	0.045	0.597	0.004	0.017	0.051	0.039	0.047	0.023	0.088	0.050
Meropenem	0.046	0.007	0.020	0.065	0.001	0.064	0.011	0.009	0.031	0.011	0.055	0.071	0.018	0.033	0.049	0.040
Ceftazidime	0.017	0.005	0.083	0.095	0.047	0.001	0.019	0.004	0.064	0.059	0.044	0.083	0.074	0.049	0.038	0.054
Cefepime	0.046	0.097	0.020	0.065	0.051	0.064	0.022	0.009	0.073	0.049	0.036	0.093	0.069	0.057	0.034	0.063
Ticarcillin	0.218	0.824	0.343	0.083	0.647	0.420	0.061	0.682	0.093	0.061	0.081	0.047	0.084	0.058	0.077	0.074
Piperacillin	0.072	0.599	0.042	0.062	0.024	0.272	0.074	0.260	0.037	0.029	0.063	0.058	0.099	0.011	0.084	0.070
Ciprofloxacin	0.622	0.416	0.106	0.574	0.705	0.261	0.560	0.902	0.366	0.564	0.156	0.857	0.706	0.101	0.101	0.101
Trimethoprim/ sulfametho-xazole	0.016	0.214	0.091	0.041	0.033	0.086	0.101	0.083	0.218	0.824	0.343	0.383	0.647	0.420	0.682	0.081
Tetracycline	0.758	0.429	0.004	0.055	0.572	0.190	0.091	0.088	0.068	0.488	0.073	0.066	0.782	0.309	0.060	0.087
Norfloxacin	0.627	0.801	0.080	0.603	0.079	0.900	0.075	0.858	0.080	0.062	0.021	0.097	0.065	0.597	0.040	0.072
Ampicillin	0.627	0.801	0.180	0.603	0.879	0.900	0.101	0.058	0.034	0.017	0.011	0.019	0.047	0.013	0.088	0.014
Cefotaxime	0.688	0.488	0.073	0.667	0.782	0.309	0.101	0.008	0.041	0.013	0.049	0.061	0.023	0.064	0.047	0.049
MDR	0.066	0.064	0.056	0.057	0.006	0.040	0.003	0.039	0.053	0.050	0.045	0.033	0.013	0.041	0.039	0.024
XDR	0.021	0.045	0.007	0.078	0.020	0.045	0.025	0.055	0.047	0.040	0.033	0.026	0.015	0.024	0.047	0.042
β-lactamase producer isolates																
AmpC enzymes	0.022	0.016	0.323	0.074	0.005	0.061	0.030	0.042	0.069	0.074	0.056	0.027	0.006	0.091	0.028	0.022
ESBL enzymes	0.047	0.019	0.051	0.011	0.027	0.056	0.041	0.066	0.051	0.024	0.038	0.091	0.071	0.038	0.082	0.016
KPC enzymes	0.077	0.059	0.024	0.049	0.085	0.028	0.056	0.097	0.041	0.035	0.013	0.021	0.034	0.053	0.050	0.015
MBL enzymes	0.013	0.028	0.041	0.017	0.044	0.050	0.069	0.079	0.023	0.038	0.051	0.033	0.019	0.034	0.059	0.044

a: χ^2 test.
b: Mann–Whitney U test.

RESULTS

Phenotypic characteristics

One-hundred twenty isolates were collected, including 50(41.66%) of *P. aeruginosa* isolates and 70(58.33%) of *A. baumannii* strains. Of these 50 isolates, 19 were isolated from wounds (38%), ten from the blood (20%), nine from urine (18%), and 12 from catheters (24%). Also, out of 70 isolates of *A. baumannii*, 23 isolates from wounds (32.85%), 17 isolates from blood (24.28%), 19 isolates from urine (27.14%) and 11 isolates from catheters (15.71) were isolated (Table 2).

Antibiotic resistance pattern

The prevalence of antimicrobial susceptibility testing of *P. aeruginosa* and *A. baumannii* is shown in Fig. 1. Ciprofloxacin and gentamicin was found to be the least active antimicrobial agent as 72% (n=36) and 66% (n=33) of the *P. aeruginosa* isolates were resistant to it, respectively. Also, 11 MDR strains (22%) and 7 XDR strains (14%) were reported. Moreover, In *A. baumannii* isolates, resistance to gentamicin (77.14%, n=54) and ciprofloxacin (87.14%, n=61) was the most frequent; and 22 MDR isolates (31.42%) and 11 XDR isolates (15.71%) were detected.

Frequency of ESBL and AmpC β-lactamases strains

Results of ESBL and AmpC producing strains are shown in Fig. 2A and Table 2. Among 50 isolates of *P. aeruginosa*, 29 isolates (58%) were AmpC producers, and 32 isolates (64%) were ESBL producers. In 70 isolates of *A. baumannii*, 40 isolates (57.14%) were ESBL-producer, and 42 isolates (60%) were AmpC producer.

Frequency of MBL and KPC β-lactamases strains

Results of MBL and KPC-producing strains are shown in Fig. 2B, Fig. 2C, and Table 2. Out of 50 isolates of *P. aeruginosa*, 19 isolates (38%) were KPC producers, and 22 isolates (44%) were MBL producers. In 70 isolates of *A. baumannii,* 24 isolates (34.28%) were MBL producers, and 23 isolates (32.85%) were KPC producers.

Biofilm production

Overall, 33 strains (66%) of *P. aeruginosa* were detected as biofilm producers, and 17 (34%) isolates were a non-biofilm producer. Also, 49 strains (70%) of *A. baumannii* were identified as biofilm producers, and 21 (30%) strains were a non-biofilm producer. The details of the biofilm-forming strains are shown in Tables 2 and 3.

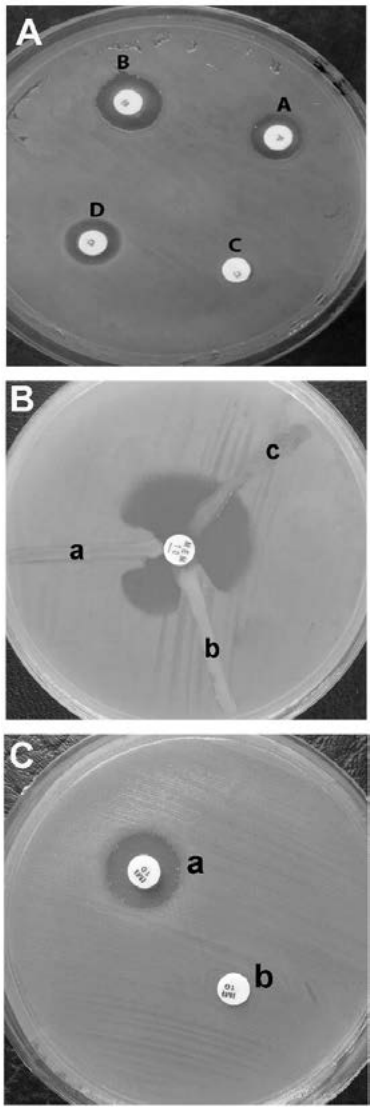

Fig. 2. The result of phenotypic detection of AmpC, ESBL (A) KPC (B) and MBL (C) strains of clinical isolates of *P. aeruginosa* and *A. baumannii*. A: For ESBL positive: B - A and D - C ≥5mm, D - B and C - A < 5mm. For AmpC positive: B - A and D - C <5mm, D - B and C - ZA ≥ 5mm. For AmpC and ESBL positive: D - C ≥ 5mm, ZB - ZA < 5mm. For AmpC and ESBL negative: Difference of the zones ≤ 2mm. B: The Modified Hodge test performed on a Muller Hinton Agar plate. a: MHT positive result; b: a clinical isolate; and c: negative result. A 10µg meropenem (MEM 10µg) or ertapenem susceptibility disk is placed in the center of the test area. C: Combined EDTA disk diffusion test: In the combined disc test, if the increase in inhibition zone with the Imipenem+EDTA disc (a) was ≥ 7 mm than the Imipenem disc (b) alone, it was considered as MBL positive.

Frequency of Biofilm Operon genes

The result of the amplification and frequency of biofilm regulatory genes in *P. aeruginosa* and *A. baumannii* is shown in Fig. 4 and Table 3. Out of the 50 *P. aeruginosa* isolates, 29 isolates (58%) were *pslA* gene, 29 strains (58%) were *pslB* gene, 30 isolates (60%) were *pslD* gene, 33 isolates (64%) were *pelA* gene, 19 isolates (38%) were *pelB* gene, 22 isolates (44%) were *pelC* gene, and 34 isolates (68%) were *algD* gene. Also, out of 70 *A. baumannii*, 24 isolates (34.28%) were *csuA* gene, 23 isolates (32.85%) were the *csuB* gene,

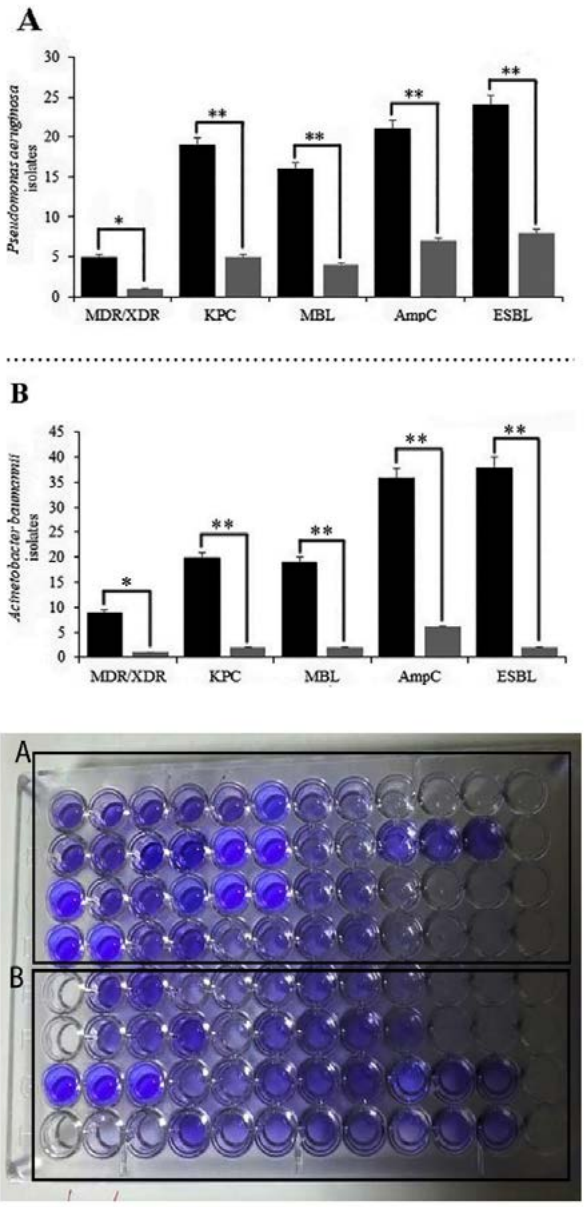

Fig. 3. Biofilm biomass stained with crystal violet and association of biofilm formation and β-lactamase enzymes in *P. aeruginosa* (A) and *A. baumannii* (B). Biofilm biomass is measured in absorbance at 570nm; Black well: Biofilm forming strains; Clear well: Strains without biofilm. Error bars indicate standard errors of the means from a representative triplicate time. Mann–Whitney U test and χ^2 test was performed for testing differences between groups. *: $p < 0.05$, **: $p < 0.001$, ***

29 isolates (41.42%) were *csuC* gene, 32 isolates (45.71%) were *pgaA* gene, 23 isolates (32.85%) were *pgaB* gene, 30 isolates (42.85%) were *pgaC* gene, and 27 isolates (38.57%) were *ompA* gene.

Statistical analysis

Based on Fig. 3A and 3B, there was a significant relationship between the biofilm formation and β-lactamase enzymes in *P. aeruginosa* and *A. baumannii* (p≤0.001). Besides, there was a significant association between biofilms regulatory genes and β-lactamase enzymes (p≤0.05). No statistical association was detected when the virulence factors were compared to some antibiotic. Moreover, we found no significant difference in antibiotic susceptibility between the fluoroquinolones, aminoglycosides (except for amikacin), and biofilm regulatory genes, a very similar distribution of disinfectant resistance genes than others (p≤0.05).

Besides, a high abundance of biofilm regulator genes was observed in strains resistant to carbapenems, monobactams, and amikacin (Table 2 and Table 4). However, in some isolates, harboring carbapenemase enzymes and biofilm operon genes was negatively associated with biofilm formation (p<0.05). In some MBL and AmpC producer isolates, harboring β-lactamase enzymes was negatively associated with biofilm production (p<0.05).

DISCUSSION

Alterations of chromosomal genes are still by far the most critical mechanisms of β-lactam resistance in NFGNBs, although transferable carbapenem resistance is becoming increasingly important[1].

As shown in Fig. 1, this paper reported that high resistance rate to ciprofloxacin (72% and 87.4%), gentamycin (66% and 77.1%), and trimethoprim/sulfadiazine (40% and 55.7%) in Iranian isolates of *P. aeruginosa* and *A. baumannii*, respectively. Some researcher demonstrated

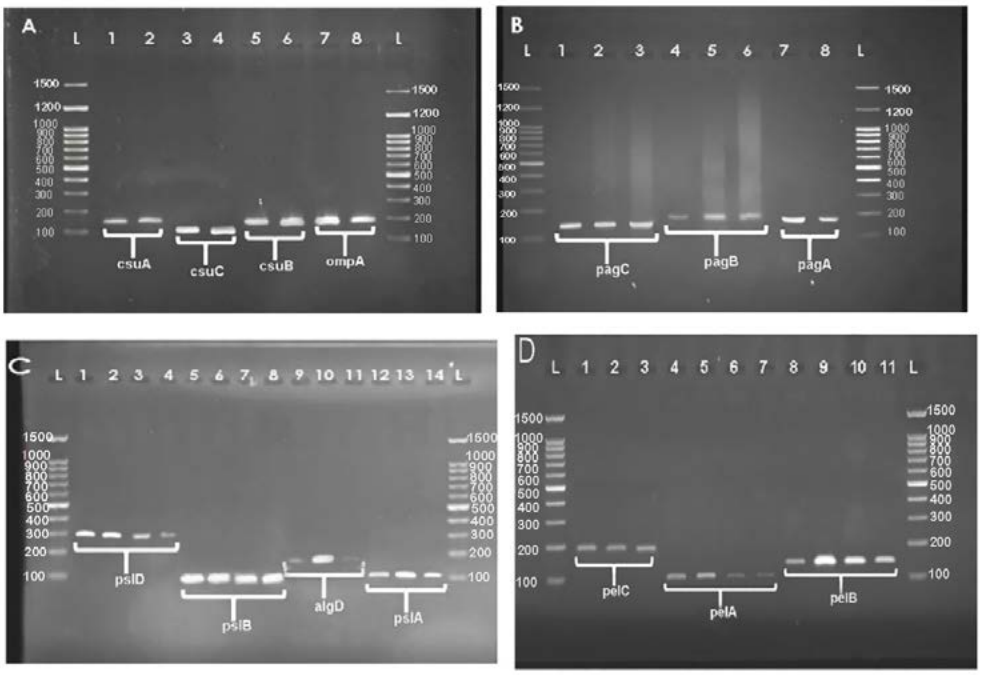

Fig. 4. The amplification and gel electrophoresis agarose 1.5% of biofilm operons of *A. baumannii* (A and B) and *P. aeruginosa* (C and D). A: *csuA* with 156bp (well 1 and 2), *csuB* with 187bp (well 5, 6), *csuC* with 109bp (well 3 and 4) and *ompA* with 182 (well 7 and 8) genes in clinical isolates of *A. baumannii,* B: *pgaA* with 186bp (well 7 and 8), *pgaB* with 190bp (well 4 to 6), and *pgaC* with 132bp (well 1 to 3) genes in *A. baumannii.* C: *pslD* with 295bp (well 1 to 4), *pslB* with 93bp (well 5 to 8), *algD* with 126bp and (well 9 to 11), and *pslA* with 119bp (well 12 to 14) genes in of *P. aeruginosa* isolates. D: *pelC* with 192bp (well 1 and 3), *pelA* with 113bp (well 4 to 7), and *pelB* with 150bp (well 8 to 11) genes.L: Ladder 100bp.

that ciprofloxacin and trimethoprim/sulfadiazine are the two most frequently co-transferred resistance phenotypes among *P. aeruginosa* and *A. baumannii* isolates[14]. The significantly high level of resistance to these antimicrobials was probably an indication of their extensive usage in the clinic for therapeutic and prophylactic purposes both for NFGNBs other infections.

Moreover, based on Table 1, MDR and XDR strains were detected in 22%, and 14% of *P. aeruginosa* and 31.4% and 15.7% of *A. baumannii* isolates, respectively. A similar pattern of results were obtained in many studies, who reported a high frequency of MDR and XDR strains in NFGNBs[15,16]. In contrast to our findings, some studies in Egypt[17] indicate the different prevalence of MDR and XDR strains in NFGNBs. In Table 2, we reported that the frequency of AmpC-producing, ESBL-producing, KPC-producing, and MBL-producing *P. aeruginosa* isolates were 56%, 64%, 48%, and 38%, respectively; which was in line with Tohamy et al.[17].

Furthermore, AmpC-producing, ESBL-producing, KPC-producing, and MBL-producing *A. baumannii* isolates were 57.1%, 60%, 34.2%, and 32.8%, respectively. This result ties well with Goel et al.[18] study and shows that the distribution of β-lactamase enzymes in *A. baumannii* is higher than *P. aeruginosa*. However, the rates of ESBL and AmpC producer strains were higher when compared to the rates reported from India[19] and Lebanon[20]. Our study found that more than 50% of the strains from the west of Iran were β-lactam-resistant is undoubtedly a cause for concern as β-lactam has been the drug of choice for *A. baumannii* and *P. aeruginosa* infections for over a decade.

Biofilm formation and regulatory genes have been investigated as controversial and critical issues in healthcare settings. However, in some studies, no apparent relationship between β-lactamase enzymes and biofilm formation has been detected[21,22]. Further, as shown in Fig. 3, the current study confirmed the strong association between biofilm formation and β-lactamase enzymes ($p< 0.001$), which was reported similar results in several studies[23,24]. Also, based on Table 2 and Table 4, our finding showed that resistance to meropenem, ceftazidime, and amikacin, was associated with a higher prevalence of the biofilm formation and regulatory genes. Nonetheless, many studies showed that antibiotic resistance was associated with the production of the biofilm phenotype, such as cellular appendages and adhesions[9].

In this study, a high prevalence of *pgaA*, *pgaC,* and *csuC* genes (45.7%, 42.8%, and 41.4%, respectively; $p< 0.05$) in resistant *A. baumannii* was observed. These results go beyond Liu et al.[25] reports how demonstrated the frequency of pathogenic genes (*ompA*) and biofilm regulators in the β-lactamase producer strains of *A. baumannii* was higher. Additionally, in Table 3 and Table 4, we confirmed that *algD*, *pelA,* and *pslD* (68%, 66%, and 60%, respectively; $p< 0.05$), which was also more prevalent in resistant strains. Another studies shown a significant relationship between virulence factors and antibiotic resistance in *P. aeruginosa*[13,26]. This evidence highlights that the possibility of acquisition of both resistance and virulence traits via horizontal gene transfer could be responsible for the appearance of strains simultaneously virulent and resistant[13].

In the current study, based on Table 3 and Table 4, statistically significant differences were observed for resistance to the various class of antibiotics and biofilm regulating genes in *A. baumannii* and *P. aeruginosa*. Table 4 also showed that a strong relationship between *pgaABC* and *csuABC* operons with β-lactamase enzymes (~50% in resistant vs. ~19% in susceptible, $p< 0.001$). By comparing the results of various studies we determine a significant relationship between β-lactamase enzymes and biofilm operons in *A. baumannii*[24,27]. However, some researcher showed that there is no significant relationship between biofilm formation and β-lactamase enzymes in *P. aeruginosa*[22]. This is in contrast to our results, which confirm that the biofilm regulatory gene in *P. aeruginosa* is most abundant in the β-lactamase producing strains (~35% in resistant vs. ~11% in susceptible, $p< 0.001$). This contrast in results may be due to differences in the number and type of clinical specimen. In other words, the statistical analysis with these explanations may not be the same in various studies. Therefore, Wang et al.[28] found that there was no significant relationship between antibiotic resistance and biofilm formation in *A. baumannii* bacteraemic pneumonia.

So far, the knowledge of the relationship between resistance and virulence traits in NFGNBs compared to other bacteria is limited. Environmental factors are one of the most critical factors that can clarify the relationship between biofilm formation (the activity of biofilm operons) and β-lactamase enzymes. Antimicrobial resistance could also be stress-dependent; for example, some studies found that antibiotic resistance increased when subjected to pH (5.0 and 4.0) and salt stresses, although antimicrobial susceptibility returned to previously tested levels after removing the stressors, bacterial sustained antimicrobial resistance[29,30]. This suggests that the pressures of stressors could permanently alter antimicrobial resistance in bacteria; Likewise, these variables can affect biofilm formation. By studying different variables on the activity of biofilm operons and increasing antibiotic resistance, we can determine the effect of environmental factors on the pathogenicity and antibiotic resistance of NFGNBs.

The limitations of the present studies include the academic budget deficit to evaluate the expression level of biofilm regulatory genes in β-lactamase producing strains. Because the expression of biofilm regulatory genes in MDR/XDR strains may be different with β-lactamase producing strains.

CONCLUSIONS

Our results demonstrated that biofilm operons play an essential role in the antibiotic resistance of *P. aeruginosa* and *A. baumannii*. In other words, our data show an association between biofilm operons and the abundance of β-lactamase producing strains. Some associations were detected that could help in predicting the degree of virulence of a certain isolate. Nevertheless,this association could be useful for clinicians in terms of adjusting treatment regimens based on the expected degree of virulence and the severity of the illness of the patient. Moreover, this association could be exploited by infection control specialists through the adaptation of eradication protocols to specific isolates. Although the interplay between resistance and biofilm operons seems to be a highly complex one, this observation could suggest that its lack of resistance could be attributing to its increased virulence. Performing a similar study on more sporadic isolates, and isolates from different origins could reveal further clinically important associations and help better understand the interaction between antimicrobial resistance and biofilm operons. Therefore, the identification of biofilm operons in different strains of *P. aeruginosa* and *A. baumannii* helps to control antibiotic resistance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

MHP and MP carried out the molecular genetic studies, participated in the primers sequence alignment, and drafted the manuscript. MHP carried out the sampling and culture method, participated in the design of the study, and performed the statistical analysis and writing the manuscript. All authors read and approved the final manuscript.

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ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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