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Survey on Genetic Diversity, Biofilm Formation, and Detection of Colistin Resistance Genes in Clinical Isolates of *Acinetobacter baumannii*

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Saeed Khoshnood[1,2]
Mohammad Savari[1,2]
Effat Abbasi Montazeri[1,2]
Ahmad Farajzadeh Sheikh[1,2]

[1]Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; [2]Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Introduction: *Acinetobacter baumannii* is an opportunistic pathogen responsible for nosocomial infections. The emergence of colistin-resistant *A. baumannii* is a significant threat to public health. The aim of this study was to investigate the molecular characterization and genotyping of clinical *A. baumannii* isolates in Southwestern Iran.

Methods: A total of 70 *A. baumannii* isolates were collected from patients admitted to Imam Khomeini Hospital in Ahvaz, Southwestern Iran. Minimum inhibitory concentration test was conducted by using Vitek 2 system. The presence of biofilm-forming genes and colistin resistance-related genes were evaluated by PCR. The isolates were also examined for their biofilm formation ability and the expression of *pmrA* and *pmrB* genes. Finally, multilocus sequence typing (MLST) and PCR-based sequence group were used to determine the genetic relationships of the isolates.

Results: Overall, 61 (87.1%) and 9 (12.8%) isolates were multidrug-resistant (MDR) and extensively drug-resistant (XDR), respectively. Colistin and tigecycline with 2 (2.8%) and 32 (45.7%) resistance rates had the highest effect. Among all the isolates, 55 (78.5%), 7 (10%), and 3 (4.3%) were strong, moderate, and weak biofilm producers, respectively. The frequency rates of biofilm-related genes were 64 (91.4%), 70 (100%), 56 (80%), and 22 (31.42%) for *bap, ompA, csuE*, and bla_{PER1}, respectively. Overexpression of *pmrA* and *pmrB* genes was observed in two colistin-resistance isolates, but the expression of these genes did not change in colistin-sensitive isolates. Additionally, 37 (52.8%) and 8 (11.4%) isolates belonged to groups 1 (ICII) and 2 (IC I), respectively. MLST analysis revealed a total of nine different sequence types that six isolates belonged to clonal complex 92 (corresponding to ST801, ST118, ST138, ST 421, and ST735). Other isolates were belonging to ST133 and ST216, and two colistin-resistant (Ab4 and Ab41) isolates were belonging to ST387 and ST1812.

Conclusion: The present study revealed the presence of MDR and XDR *A. baumannii* isolates harboring biofilm genes and emergence of colistin-resistant isolates in Southwestern Iran. These isolates had high diversity, which was affirmed by typing techniques. The control measures and regular surveillance are urgently needed to preclude the spread of these isolates.

Keywords: *Acinetobacter baumannii*, drug resistance, colistin, MLST, clonal complex

Correspondence: Ahmad Farajzadeh Sheikh
Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Khuzestan 61357-15794, Iran
Tel +98 9161133491
Fax +98 61 3333 2036
Email farajzadehah@gmail.com

Introduction

Acinetobacter baumannii is known as an opportunistic nosocomial pathogen in hospitals and is associated with a wide range of infections in healthcare facilities.[1] Antibacterial resistance in multidrug-resistant (MDR) *A. baumannii* isolates is an important issue, and molecular studies are considered serious strategies for controlling the outbreak of these isolates.[2] *A. baumannii* resistance to several antimicrobial

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drugs has increasingly been elevated in recent decades. This pattern of antibiotic resistance often varies with time and from one area to another or even within the same area.[3,4] Nowadays, in developing countries, including Iran, physicians face serious challenges to the treatment of patients infected with MDR *A. baumannii*, presenting severe healthcare problems owing to treatment failure.[5,6]

Carbapenem antibiotics, such as imipenem and meropenem, are the most effective therapeutic options for *A. baumannii* infections. Overuse of broad-spectrum carbapenems and/or cephalosporins is an important risk factor for the development of colonization or infection with carbapenemase-producing *A. baumannii* strains. The rapid spread of carbapenem-resistant MDR *A. baumannii* has led to the use of polymyxins, particularly polymyxin E (colistin), which its excessive use has recently given rise to the emergent resistance to this antibiotic.[7] Colistin, a cationic polypeptide antibiotic, targets the bacterial outer membrane via an initial charge-based interaction between the positively charged colistin and the negatively charged on the lipid A component of lipopolysaccharide (LPS). Various mechanisms, including mutations in the *pmrA* and *pmrB* genes of the PmrAB two-component regulatory system, plasmid-mediated *mcr-1* gene, mutations in any of lipid A biosynthesis genes (*lpxA, lpxC*, and *lpxD*), and the presence of insertion sequence IS*Aba11* in either *lpxC* or *lpxA* are involved in the emergence of resistance to this antibiotic.[8,9] Another leading factor involved in bacterial resistance to varied antibiotics, survival in hospital environments, and chronic infections is the biofilm formation capability of bacteria. Previous studies have shown a positive relationship between the antibiotic resistance and biofilm formation in *A. baumannii* strains. There are also a variety of virulence factors, including the biofilm-associated protein (BAP), outer membrane protein A (OmpA), chaperon-usher pilus (Csu), and quorum-sensing system, that are engaged in the biofilm formation of *A. baumannii*. The BAP encoded by the *bap* gene has a critical function in the biofilm establishment and intercellular adhesion.[10,11] The OmpA has negligible contribution to the development of the robust biofilm on the plastic surface. The biofilm-forming ability of *A. baumannii* is also largely dependent on pili. Therefore, the *csuE* gene plays a major role in *A. baumannii* biofilm formation.[12] The prevalence of *A. baumannii* in hospitals has increased dramatically worldwide; therefore, using molecular typing methods is essential for infection control and epidemiological studies. Based on the epidemiological studies and population genetic investigations of *A. baumannii*, there are several developed typing methods, including multiple-locus variable number tandem repeats (VNTRs) analysis (MLVA), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and PCR-based sequence group (SG) profiling.[13,14]

MLVA and MLST schemes provide a genotype in the form of a code that can easily be shared. Both approaches allow for the investigation of the population structure and identification of clonal lineages.[15] The aim of this study was to investigate the presence of biofilm-forming genes and colistin resistance-related genes in *A. baumannii* isolates, which were examined for their ability to form biofilms and to express *pmrA* and *pmrB* genes. PCR-based sequence group and MLST were also applied to determine the genetic relationships of the isolates.

Materials and Methods

Bacterial Isolates and Identification

This cross-sectional study was conducted between October 2018 and July 2019 and confirmed by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No. IR.AJUMS.REC.1397.793). Before initiating this research work, informed consent forms were obtained from all the patients. The study was conducted in accordance with the Declaration of Helsinki. In total, 70 non-repetitive *A. baumannii* isolates were collected from different clinical samples from patients admitted to Imam Khomeini Hospital in Ahvaz, Southwestern Iran. Patients with concomitant infections not properly treated, human immunodeficiency virus (HIV) infection, and antibiotic treatment less than two days were excluded. All the included isolates were identified based on conventional microbiological tests,[16] and final identification was conducted by the PCR of $bla_{\text{OXA-51}}$-like gene and multiplex of *gyrB*.[17] The *A. baumannii* ATCC 19606 was used as a positive control.

Susceptibility Testing

The minimum inhibitory concentration (MICs) of each antibiotic was determined using the described Vitek 2 system (bioMérieux, Marcy l'Etoile, France). The MICs of all the selected antibiotics were interpreted by the aforesaid system as per CLSI guidelines.[18] For tigecycline, no MIC interpretive breakpoint was recommended by the EUCAST and

CLSI. A MIC of ≥8 μg/mL was regarded as the resistant breakpoint on the basis of the criteria suggested by Jones et al.[19] In addition, a MIC ≥ 4 μg/mL was proposed as the breakpoint of resistance for colistin and a MIC ≥ 8 μg/mL for both imipenem and meropenem.[18] MDR was defined as resistance to at least one agent in three or more categories of antibiotics. Isolates of *A. baumannii* with resistance to at least one agent in all but two or fewer antimicrobial categories were considered as XDR.[20] *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as the quality control.

Molecular Detection of Resistance Genes

Biofilm-related genes (*bap, ompA, csuE*, and bla_{PER1}) and colistin resistance-related genes (*mcr-1, mcr-2, pmrA*, and *pmrB*) were detected using PCR.[21–23] Bacterial DNA extraction was performed in accordance with the boiling method.[24] The PCR assay was carried out in a final volume of 25 μL containing Taq DNA polymerase (1 U; CinnaGen, Iran), dNTPs (100 μM), Taq buffer (5×), DNA template (50 ng), and forward and reverse primers (25 pM). PCR mixtures were subjected to the following thermal cycling: 5 min at 94°C, followed by 35 cycles with denaturation at 94°C for 50 s, annealing at 55–57°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Primer sequences used for the detection of the above-mentioned genes are presented in Table 1.

Expression of *pmrA* and *pmrB*

Quantitative real-time PCR assay was performed for isolates with colistin MICs ≥ 1 μg/mL. RNA extraction was conducted using a High Pure RNA Isolation Kit (Roche, Germany). The integrity and quality of the total RNA assessed with a NanoDrop spectrophotometer (Thermo

Table 1 Primers Used for the Amplification of Genes

Target Genes	Sequence (5′–3′)	Amplicon Size (Base Pairs)	References
bap	F:ATGCCTGAGATACAAATTAT R:GTCAATCGTAAAGGTAACG	1449	21
ompA	F:GTTAAAGGCGACGTAGACG R:CCAGTGTTATCTGTGTGACC	578	21
csuE	F:CATCTTCTATTTCGGTCCC R:CGGTCTGAGCATTGGTAA	168	21
bla_{PER-1}	F:ATGAATGTCATTATAAAAGC R:AATTTGGGCTTAGGGCAGAA	925	21
mcr-1	F:AGTCCGTTTGTTCTTGTGGC R:AGATCCTTGGTCTCGGCTTG	320	22
mcr-2	F:CAAGTGTGTTGGTCGCAGTT R:TCTAGCCCGACAAGCATACC	715	22
pmrA	F:ACTGGACATGTTGCACTCTTGT R:ATGCACTTTTATGAAGTCCCGA	757	23
pmrB	F:TCGGGACTTCATAAAAGTGCAT R:CAGTCACAGGTGTTCGTAATT	722	23
16S_rRNA	F:CAGCTCGTGTCGTGAGATGT R:CGTAAGGGCCATGATGACTT	150	23
Group1ompAF306	GATGGCGTAAATCGTGGTA	355	27
Group1and2ompAR660	CAACTTTAGCGATTTCTGG		
Group1csuEF	CTTTAGCAAACATGACCTACC	702	27
Group1csuER	TACACCCGGGTTAATCGT		
Gp1OXA66F89	GCGCTTCAAAATCTGATGTA	559	27
Gp1OXA66R647	GCGTATATTTTGTTTCCATTC		
Group2ompAF378	GACCTTTCTTATCACAACGA	343	27
Group1and2ompAR660	GGCGAACATGACCTATTT		
Group2csuEF	GGCGAACATGACCTATTT	580	27
Group2csuER	CTTCATGGCTCGTTGGTT		
Gp2OXA69F169	CATCAAGGTCAAACTCAA	162	27
Gp2OXA69R330	TAGCCTTTTTTCCCCATC		

Fisher Scientific, Waltham, MA, USA) were electrophoresed on 1% agarose gel. The RNA was reverse transcribed to cDNA using Prime Script™ 1st strand cDNA Synthesis Kit (Takara Biotechnology Co., LTD., China) according to the instructions provided by the manufacturer. To carry out real-time PCR, we used the specific primers of 16S rRNA (as an internal control), *pmrA,* and *pmrB* genes. *A. baumannii* ATCC 19606 was selected as the reference strain. Real-time PCR amplification reaction was prepared in a 20-µL final volume, with 0.5 µL of each forward and reverse primer (10 nM each), 10 µL of RealQ Plus Master Mix Green (Ampliqon, Denmark), 400 ng of cDNA, and DNase- and RNase-free water up to a 20-µL final volume. This reaction was accomplished in the ABI Thermocycler System (Thermo Fisher Scientific). The reaction conditions were initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The relative expression fold changes of mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method.[21]

In Vitro Bacterial Biofilm Assay

The biofilm formation capacity of *A. baumannii* isolates on 96-well polystyrene microtiter plates (MTP) was evaluated using the crystal violet staining (CVS) method, as described elsewhere.[25] The results of this assay were interpreted according to the criteria explained before by Zhang et al.[26] The isolates were no biofilm producers when $A \leq Ac$, weak biofilm producers when $Ac < A \leq 2Ac$, moderate biofilm producers when $2Ac < A \leq 4Ac$, and strong biofilm producers when $A > 4Ac$; A represents optical absorbance and Ac displays OD value. Also, *A. baumannii* ATCC 19606 and Mueller Hinton Broth were used as positive and negative controls for the evaluation of the biofilm formation, respectively. All experiments were performed in triplicate.

Molecular Typing Methods

International clonal (IC) types were determined based on the absence or presence of the alleles of *csuE, ompA*, and the intrinsic carbapenemase ($bla_{OXA\text{-}51\text{-}like}$)-encoding genes in two multiplex PCRs, as previously described.[27] Identification of a strain as a member of Group 1 or Group 2 required the amplification of all fragments in the corresponding multiplex PCR and an absence of any amplification by the other multiplex PCR. Group 3 isolates were defined by the amplification of only the *ompA* fragment in the Group 2 PCR, and the amplification of only the *csuE* and $bla_{OXA\text{-}51}$-like fragments in the Group 1 PCR. Isolates pertained to the novel variant of the PCR-based group, according to the new combination of amplified products. The amplification reaction was carried out by using a thermal cycler (Mastercycler Eppendorf, Germany) with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min. Besides, a final extension step was conducted at 72°C for 5 min. The MLST was also performed the previously described method by Bartual.[28] Primer sequences, sequence types (STs), allele sequences, clonal complexes (CCs), and other details are available in the MLST website at http://pubmlst.org. This typing method was carried out for isolates with colistin MICs ≥ 1 µg/mL.

Statistical Analysis

Descriptive data were analyzed using Microsoft Excel and SPSS version 22 statistics software (IBM Corporation, Armonk, NY, USA). Fisher's exact test was employed to analyze significance. In addition, P-value < 0.05 was considered as significance level. The results are presented as descriptive statistics in terms of relative frequency.

Results

Study Population, Sampling, and Antibiotic Susceptibility

Seventy non-duplicate isolates of *A. baumannii* were obtained from the studied hospital and included 42 (60%) from male patients and 28 (40%) from female patients with the mean age of 42.5 ±10 years (ranged 5–80 years). All the 70 isolates originated from the following wards: 31 (44.2%) from the ICU, as well as 5 (7.1%) from pediatric, 8 (11.5%) from urology, 10 (14.3%) from surgery, 6 (8.6%) from infectious diseases, 7 (10%) from general and 3 (4.3%) from neurology units. These isolates were recovered from different clinical specimens, including CSF (5, 7.1%), pleural fluid (7, 10%), urine (8, 11.4%), blood (9, 12.8%), catheter (15, 21.4%), and tracheal aspirates (26, 37.1%). Based on antibiotic susceptibility testing, among 70 *A. baumannii* isolates screened, 61 (87.1%) isolates were MDR and 9 (12.8%) isolates were extensively drug-resistant (XDR).

The results showed that colistin and tigecycline with the resistance rates of 2.8% and 45.7%, respectively, were the most active agents, while cephalosporins with 100% resistance had no effect on *A. baumannii* isolates. Also, resistance to other antibiotics was ≥50% (Table 2). Among 70 *A. baumannii* isolates, 52 isolates (74.2%) were carbapenem-resistant *A.baumannii* (CRAB). Resistance to colistin was

found in 2 isolates (Ab 4 and Ab 41) with MICs 32 and 8 µg/mL, XDR phenotype, and international clones 1 and 2 (Table 2).

These isolates were strong biofilm producers and had biofilm-related genes and *pmrA, pmrB* genes. Among the meropenem-resistant and -sensitive isolates, 52 (74.2%) and 18 (25.7%) isolates had the MICs range from ≥8 to 256 µg/mL and from 0.25 to ≤2, respectively. The MICs for imipenem ranged from 0.25 to ≤2 µg/mL in 17 (24.2) isolates, while 50 (71.4%) isolates had MICs ≥ 8–256 µg/mL for this antibiotic.

Distribution of Resistance Genes and Expression of *pmrA* and *pmrB*

PCR results showed the presence of the $bla_{\text{OXA-51-like}}$ gene in all the isolates. The frequency rates of biofilm-related genes were 64 (91.4%), 70 (100%), 56 (80%), and 22 (31.42%) for *bap, ompA, csuE*, and bla_{PER1}, respectively (Table 3). Also, 15 (21.42%) isolates had these genes simultaneously, and all the isolates included at least one biofilm-related gene. The relationship between the biofilm formation and related genes in *A. baumannii* strains with MDR and XDR phenotypes is presented in Table 4. Coexistence genes, *bap* and *ompA*, were observed in all the strong and moderate biofilm producers, and more than 55% did not have bla_{PER1} gene.

This study found a strong association between the biofilm intensity and presence of *bap* gene in *A. baumannii* isolates (P=0.001). Two colistin-resistant isolates contained all three genes (*bap, ompA*, and bla_{PER1}) involved in the biofilm formation. Besides, all the isolates susceptible to meropenem had *bap* and *ompA* genes. *PmrA* and *pmrB* genes were detected in all the isolates, but *mcr1* and *mcr2* were not identified in any of the isolates. In the end, we determined the expression of the *pmrA* and *pmrB* genes in two colistin-resistant (Ab 4 and Ab 41) and eight colistin-susceptible isolates (Ab 49, Ab 61, Ab 69, Ab 22, Ab 26, Ab 7, Ab 32, and Ab 37). Overexpression means that the isolates have fourfold increase in the expression level of genes compared with the control strain *A. baumannii* ATCC 19606. The *pmrA* and *pmrB* genes were overexpressed in two colistin-resistant isolates, but their expression did not change in colistin-sensitive isolates (Figure 1). In colistin-resistant isolates, the *pmrB* gene had higher expression than the *pmrA* gene.

Biofilm Formation Assay

Assessment of biofilm formation was carried out using the MTP method. Among all the isolates, 55 (78.5%), 7 (10%), and 3 (4.3%) were strong, moderate, and weak biofilm producers, respectively, while no biofilm was observed in 5 (7.1%) isolates. The OD_{570} values for negative and positive controls were 0.044 ± 0.005 and 0.411 ± 0.041 (strong biofilm producer), respectively. Also, of 55 strong biofilm producers, 47 (77%) isolates were MDR, and 8 (88.9%) isolates were XDR. The relationship between the biofilm intensity and antibiotic susceptibility is shown in Table 4. All the weak and moderate biofilm producers were MDR phenotype and meropenem resistant. In addition, the *bap* and *ompA* genes were detected in all the strong and moderate biofilm producers.

Molecular Typing

IC type analysis showed six different PCR-based groups (G1, G2, G4, G7, G10, and G15) among *A. baumannii* isolates. Eight (11.4%) and 37 (52.8%) isolates belonged to group 2 (IC I) and group 1 (IC II), respectively. In addition, 3 (4.2%), 5

Table 2 Antibiotic Susceptibility Pattern of *A. baumannii* Isolates

Antibiotic		LEV	CTZ	CFP	PIP	TGC	AMP/S	COL	CTX	SXT	AMK	CIP	IMI	MER
Susceptibility pattern	R	65	70	70	68	32	55	2	70	67	59	65	50	52
	N (%)	(92.8)	(100)	(100)	(97.1)	(45.7)	(78.6)	(2.8)	(100)	(95.7)	(84.2)	(92.8)	(71.4)	(74.2)
	I	0	0	0	0	0	0	0	0	0	2	0	3	0
	N (%)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(2.8)	(0.0)	(4.3)	0.0)
	S	5	0	0	2	38	15	68	0	3	9	5	17	18
	N (%)	(7.1)	(0.0)	(0.0)	(2.8)	(54.3)	(21.4)	(97.1)	(0.0)	(4.3)	(12.8)	(7.1)	(24.2)	(25.7)
Interpretive categories and MIC breakpoints (µg/mL)	R	≥8	≥32	≥32	≥128	≥8	≥32/16	≥4	≥64	≥4/76	64	≥4	≥8	≥8
	I	4	16	16	32–64	–	16/8	–	16–32	–	32	2	4	4
	S	≤2	≤8	≤8	≤16	<8	≤8/4	≤2	≤8	≤ 2/38	16	≤1	≤2	≤2

Abbreviations: LEV, levofloxacin; CTZ, ceftazidime; CFP, cefepime; PIP, piperacillin; TGC, tigecycline; Amp/S, ampicillin/sulbactam; COL, colistin; CTX, cefotaxime; SXT, trimethoprim-sulphamethoxazole; AMK, amikacin; CIP, ciprofloxacin; IMI, imipenem; MEM, meropenem; R, resistant; I, intermediate; S, susceptible.

Table 3 Characteristics of *A. baumannii* Strains

Strain ID	Biofilm	Phe	MIC			Biofilm Genes	IC	MLST	
			MER	TGC	COL				
Ab 1	S	XDR	16	64	0.5	bap, ompA, csuE	G1	–	–
Ab 2	S	MDR	0.25	16	0.5	bap, ompA, csuE, blaPER-1	G1	–	–
Ab 3	S	XDR	64	2	0.5	bap, ompA, csuE	G2	–	–
Ab 4	S	XDR	128	8	32	bap, ompA, csuE, blaPER-1	G2	387	–
Ab 5	W	MDR	16	16	0.25	bap,ompA	G1	–	–
Ab 6	S	MDR	0.5	4	0.25	bap, ompA, csuE, blaPER-1	G2		
Ab 7	S	MDR	128	0.25	1	bap, ompA, csuE	G15	138	92
Ab 8	S	MDR	16	32	0.5	bap, ompA, csuE	G1	–	–
Ab 9	S	MDR	8	1	0.5	bap, ompA, csuE, blaPER-1	G1	–	–
Ab 10	N	XDR	32	0.5	0.25	ompA, csuE, blaPER-1	G10	–	–
Ab 11	M	MDR	0.5	64	0.125	bap, ompA, csuE	G1	–	–
Ab 12	S	MDR	128	0.25	0.25	bap, ompA, csuE, blaPER-1	G1	–	–
Ab 13	S	MDR	0.25	2	0.5	bap, ompA, csuE	G1	–	–
Ab 14	S	MDR	16	32	0.5	bap, ompA	G4	–	–
Ab 15	S	MDR	256	0.25	0.25	bap, ompA	G10	–	–
Ab 16	N	MDR	256	1	0.25	ompA, csuE, blaPER-1	G1		
Ab 17	S	MDR	32	0.5	0.25	bap, ompA, csuE	G2	–	–
Ab 18	S	MDR	0.5	32	0.125	bap, ompA, csuE	G1	–	–
Ab 19	M	MDR	0.5	0.25	0.25	bap, ompA, csuE	G10	–	–
Ab 20	S	MDR	32	16	0.5	bap, ompA, csuE	G1	–	–
Ab 21	S	MDR	16	1	0.25	bap, ompA	G1	–	–
Ab 22	S	MDR	128	0.5	1	bap, ompA, csuE, blaPER-1	G1	118	92
Ab 23	S	MDR	256	16	0.25	bap, ompA, csuE, blaPER-1	G15	–	–
Ab 24	N	MDR	16	2	0.25	ompA, csuE	G 1	–	–
Ab 25	S	MDR	2	8	0.125	bap, ompA, csuE	G 4	–	–
Ab 26	S	MDR	16	4	2	bap, ompA	G 1	421	92
Ab 27	S	XDR	16	32	0.25	bap, ompA, csuE, blaPER-1	G 10	–	–
Ab 28	W	MDR	64	0.5	0.25	ompA, csuE	G 1	–	–
Ab 29	S	MDR	0.25	8	0.25	bap, ompA, csuE, blaPER-1	G 1	–	–
Ab 30	S	MDR	0.5	8	0.5	bap, ompA, csuE	G 2	–	–
Ab 31	S	MDR	8	16	0.25	bap, ompA	G 1	–	–
Ab 32	S	MDR	128	1	1	bap, ompA, csuE	G 4	216	405
Ab 33	S	MDR	0.5	0.25	0.125	bap, ompA, csuE	G 7	–	–
Ab 34	M	MDR	32	32	0.25	bap,ompA, csuE, blaPER-1	G 10	–	–
Ab 35	S	MDR	0.5	1	0.25	bap, ompA	G 1	–	–
Ab 36	M	MDR	16	0.5	0.5	bap,ompA, csuE	G 15	–	–
Ab 37	N	MDR	256	8	2	ompA, csuE, blaPER-1	G 1	735	92
Ab 38	M	MDR	0.5	32	0.5	bap, ompA, csuE, blaPER-1	G 2	–	–
Ab 39	S	MDR	0.25	4	0.25	bap, ompA, csuE	G 10	–	–
Ab 40	S	MDR	16	1	0.25	bap, ompA, csuE	G 1	–	–
Ab 41	S	XDR	64	16	8	bap, ompA, blaPER-1	G 1	1812	–
Ab 42	S	MDR	16	0.5	0.25	bap, ompA, csuE	G 1	–	–
Ab 43	S	MDR	2	4	0.25	bap, ompA, csuE	G10	–	–
Ab 44	S	MDR	16	4	0.5	bap, ompA, csuE	G 1	–	–
Ab 45	M	MDR	128	16	0.25	bap,ompA, blaPER-1	G15	–	–
Ab 46	S	MDR	16	0.5	0.5	bap, ompA, csuE	G4	–	–
Ab 47	S	MDR	2	32	0.25	bap, ompA, csuE	G 1	–	–
Ab 48	S	MDR	16	1	0.5	bap, ompA, csuE, blaPER-1	G1	–	–
Ab 49	S	MDR	16	8	1	bap, ompA	G2	133	225

(*Continued*)

Table 3 (Continued).

Strain ID	Biofilm	Phe	MIC			Biofilm Genes	IC	MLST	
			MER	TGC	COL				
Ab 50	S	MDR	32	8	0.25	bap, ompA, csuE	G1	–	–
Ab 51	S	XDR	16	0.25	0.5	bap, ompA, csuE	G1	–	–
Ab 52	M	MDR	16	2	0.25	bap,ompA	G7	–	–
Ab 53	S	XDR	0.5	16	0.25	bap, ompA, csuE	G10	–	–
Ab 54	S	MDR	16	8	0.5	bap, ompA, csuE, blaPER-1	G1	–	–
Ab 55	S	MDR	64	0.5	0.5	bap, ompA, csuE	G 1	–	–
Ab 56	S	MDR	16	4	0.25	bap, ompA, csuE, blaPER-1	G 1	–	–
Ab 57	S	MDR	32	16	0.5	bap, ompA, csuE	G 4	–	–
Ab 58	S	MDR	16	8	0.25	bap, ompA, csuE	G1	–	–
Ab 59	S	MDR	0.25	0.5	0.5	bap, ompA, csuE, blaPER-1	G4	–	–
Ab 60	S	MDR	16	1	0.25	bap, ompA	G1	–	–
Ab 61	S	MDR	128	0.5	1	bap, ompA, csuE	G7	848	92
Ab62	S	MDR	16	8	0.25	bap, ompA, csuE	G15	–	–
Ab 63	S	MDR	32	1	0.5	bap, ompA, csuE	G10	–	–
Ab 64	N	MDR	16	2	0.25	ompA, csuE, blaPER-1	G2	–	–
Ab 65	S	MDR	125	32	0.25	bap, ompA, csuE	G1	–	–
Ab 66	S	XDR	16	32	0.5	bap, ompA	G1	–	–
Ab 67	S	MDR	16	64	0.25	bap, ompA, csuE	G10	–	–
Ab 68	S	MDR	0.25	0.5	0.25	bap, ompA, blaPER-1	G1	–	–
Ab 69	W	MDR	16	2	1	bap,ompA, csuE	G10	118	92
Ab 70	S	MDR	128	16	0.25	bap, ompA, csuE	G1	–	–

Abbreviations: Phe, phenotype; MDR, multidrug-resistant; XDR, extensively drug-resistant; M, medium; N, non-biofilm-forming; W, weak; S, strong; MIC, minimum inhibitory concentration; MER, meropenem; TGC, tigecycline; COL, colistin; IC, international clonal lineage.

(7.1%), 6 (8.5%), and 11 (15.7%) isolates were belonging to four IC variants PCR-based groups, ie, G7, G15, G10, and G4, respectively. All the isolates belonged to IC I were resistant to eight antibiotics, and more than 65% of the isolates belonged to IC II were resistant to all the antibiotics, except for tigecycline and colistin. Among strong biofilm-producing isolates, 6 (10.9%), 31 (56.36%), and 18 (32.72%) were belonging to IC I, IC II, and IC variants, respectively (Table 5).

MLST analysis for isolates with colistin MICs ≥ 1 µg/mL revealed a total of nine different STs. It also showed six isolates belonging to CC 92 (corresponding to ST801, ST118, ST138, ST421, and ST735). These isolates were meropenem resistant (MIC range: 16–256 µg/mL), MDR phenotype, and belonged to IC G1, G7, G10, and G15. Other isolates were, however, belonging to ST133 and ST216 and two colistin-resistant (Ab 4 and Ab 41) isolates to ST387 and ST1812.

Discussion

A. baumannii, due to its multidrug resistance characteristic, has been one of the axes of interest of the medical community in recent years.[29,30] Scattered reports of its resistance to the last-line treatment, colistin, have raised further concerns for the medical field.[7,8,23,24] *A. baumannii*

Table 4 The Relationship Between Biofilm Formation and Related Genes in *A. baumannii* Strains with MDR and XDR Phenotypes

Biofilm Intensity (No.)	bap No (%)		ompA No (%)		csuE No (%)		bla-PER1 No (%)	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Strong (55)	55 (100)	0 (0)	55(100)	0 (0)	44 (80)	11(20)	15(27.27)	40(72.73)
Moderate (7)	7 (100)	0 (0)	7 (100)	0 (0)	5(71.4)	2(28.57)	3 (42.85)	4 (57.14)
Weak (3)	2 (66.6)	1 (33.3)	3(100)	0 (0)	2 (66.6)	1 (33.3)	0 (0)	3 (100)
Non- Biofilm forming (5)	0 (0)	5 (100)	5(100)	0 (0)	5 (100)	0 (0)	4 (80)	1(20)
Total	64(91.4)	6 (8.6)	70(100)	0 (0)	56 (80)	14 (20)	22 (31.42)	48(68.58)
P value	<0.001		–		0.552			0.053

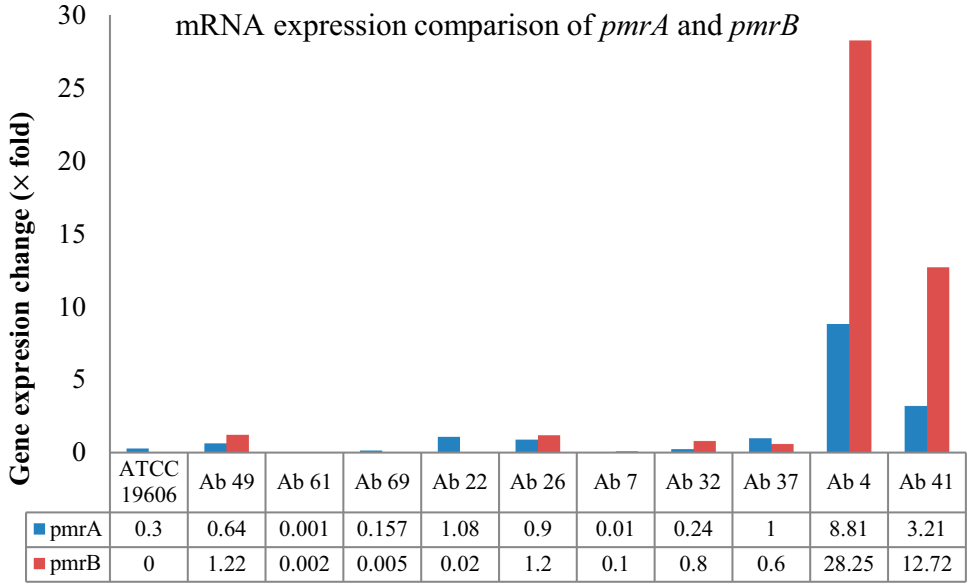

Figure 1 Gene expression comparison of *pmrA* and *pmrB* in colistin-resistant (Ab 4, Ab41) and susceptible isolates (Ab49, Ab61, Ab69, Ab22, Ab26, Ab7, Ab32, Ab37) and *A. baumannii* ATCC 19606.

has different mechanisms for resistance to colistin. It is also featured by having ability to produce biofilms, which are very effective in maintaining and retaining this bacterium in hospital environments.

In this study, we investigated the biofilm-producing properties and some colistin resistance mechanisms of clinical *A. baumannii* isolates collected from Ahvaz City, Southwestern Iran. Based on the results of antibiotic susceptibility test, the tigecycline and colistin with 54.3% and 97.1% sensitivity were the most effective antibiotics against *A. baumannii* isolates, which confirms previous reports from Iran by Hatami et al[2] and Noori et al.[31] Besides, 87.1% frequency of MDR *A. baumannii* isolates in our study was comparable with the results obtained in Iran by Salehi et al[32] and Khalilzadegan et al.[33] However, studies in Brazil[34] and Morocco[35] have disclosed the lower rates of MDR *A. baumannii* than our study. A possible reason for this high level of antibiotic resistance in Iran is non-compliance with antibiotics use standards, overuse and misuse of antibiotics, and easy access to over-the-counter drugs in third world countries.

The current study demonstrated the rate of 2.9% for colistin-resistant *A. baumannii* isolates, which was lower than the resistance rate (5.6%) reported from north of Iran by Ezadi et al.[36] In previous investigations in Pakistan[37] and Saudi Arabia,[38] no colistin-resistant *A. baumannii* isolates were found in clinical samples. These diverse resistance rates can be explained by differences in the epidemiology of the

Table 5 The Relation Between Biofilm Intensity with Antibiotic Susceptibility, Phenotype, and IC

Biofilm Intensity (No.)	Antibiotic N (%)						Phenotype N (%)		IC		
	Colistin		Meropenem		Tigecycline		MDR	XDR	I	II	IV
	R	S	R	S	R	S					
Strong (55)	2 (3.6)	53 (96.4)	40 (72.72)	15 (27.28)	26 (47.27)	29(52.73)	47 (85.45)	8(14.54)	6	31	18
Moderate(7)	0 (0)	7 (100)	4 (57.15)	3 (42.85)	4 (57.14)	3 (42.86)	7 (100)	0(0)	1	1	5
Weak(3)	0 (0)	3(100)	3 (100)	0 (0)	1(33.33)	2 (66.67)	3 (100)	0(0)	0	2	1
Non- Biofilm forming (5)	0 (0)	5 (100)	5(100)	0 (0)	1(20)	4(80)	4 (80)	1(20)	1	3	1
p-value	1		0.342		0.608		0.662				
Total	2 (2.8)	68 (97.1)	52	18	32	38	61(87.1)	9 (12.8)	8	37	25

regions, the patterns of administration, and use of antibiotics, as well as by dissimilarities in infection treatment regulatory policies.[39]

According to the information available so far, no evidence of *mcr* genes have been reported in clinical *A. baumannii* isolates before 2018.[40] In the present study, none of the isolates carried *mcr-1* and *mcr-2* resistance genes.

Other surveys that have hitherto reported the presence of different *mcr* genes in the clinical isolates of the *A. baumannii* include the studies of Hameed et al[37] who reported a rate of 1.6% (1/62) for *mcr-1* in Pakistan; Al-Kadmy et al[41] who detected *mcr-1, mcr-2*, and *mcr-3* genes in 73.5% (89/121), 64.5% (78/121), and 67.8% (82/121) *A. baumannii* isolates in Iraq; Martins-Sorenson et al[42] who found *mcr-4.3* in an MDR *A. baumannii* clinical isolate from a meningitis case in Brazil; Bitar et al[43] who pinpointed *mcr-4.3* in an *A. baumannii* isolate from a clinical origin in Czech Republic; and Rahman et al[44] who reported 20% frequency rate of *mcr-1* in MDR *Acinetobacter* isolates from India.

In another study, identified *mcr* genes have mostly been related to *Enterobacteriaceae* family bacteria or the samples of animal origin.[41] In our investigation, the evaluation of *pmrA* and *pmrB* gene expression rate in colistin-resistant *A. baumannii* isolates showed the increased expression rate of both genes compared to colistin-sensitive strains. Park et al[45] have also achieved the same result in the PmrAB two-component system in colistin-resistant *A. baumannii* isolates. However, in an experiment in Iran by Sepahvand et al,[46] the expression rate of *pmrA* gene in colistin-resistant *A. baumannii* isolates was higher than colistin-susceptible strains, and the expression rate of *pmrB* gene had no significant change.

Indeed, the increased expression of the *pmrA* and *pmrB* genes reduces lipopolysaccharide, resulting in the impaired membrane permeability and colistin ineffectiveness on *A. baumannii* membrane.[46] Additionally, mutations within *pmrAB* genes contribute to colistin resistance in *A. baumannii* strains.[45,47]

One of the major aspects of *A. baumannii* causing persistent nosocomial infections and antibiotic resistance is the biofilm formation ability of this bacterium in a variety of environments.[10] In the current study, the results of MTP assay showed that 78.5% (55/70) of the isolates were strong biofilm producers, which was much higher than previous reports from Thailand with 23.6%[10] and China[25] with 27.3% frequency rates. The most frequent biofilm-related gene was *ompA* (100%), followed by *bap* (91.4%), *csuE* (80%), and bla_{PER1} (31.4%), which contradicts Zeighami et al's[48] result that the *csuE* (100%) was the most prevalent gene.

This investigation suggested a significant association between the biofilm production and presence of *bap* gene in *A. baumannii* isolates (P=0.001), which is in line with Monfared et al's[49] and Sung et al's[50] studies. A comparison of biofilm-positive and biofilm-negative *A. baumannii* isolates revealed that the presence of *ompA, csuE*, and bla_{PER-1} was not significantly associated with biofilm production. Existing evidence in this field has shown different results regarding the correlation of *bap, ompA,* bla_{PER-1}, and *csuE* with the biofilm formation in *A. baumannii* isolates.[49,51] For instance, insignificant relationship between the biofilm formation and bla_{PER-1} gene was detected by Bardbari et al,[51] while Monfared et al[49] observed a positive association between the presence of this gene and the biofilm production.

In this work, we carried out two typing methods, including three-locus dual assay multiplex PCR and MLST. The results of the first method reflected six diverse IC lineages (G1, G2, G4, G7, G10, and G15) among *A. baumannii* isolates, which G1 (IC II) with the rate of 52.8% was the most frequent group. The predominance of IC II was in good agreement with previous reports from Japan[52] and Brazil[53] that recognized the IC II as the most common clone in *A. baumannii* isolates. Meanwhile, the prevalence of IC I with 11.4% frequency rate was lower than the frequency rate (19%) stated by Bahador et al[54] in Tehran, Iran. Another difference between this and that study was the strong biofilm production by the majority of our CI I and CI II lineages, while in the Bahador's study,[54] they were moderate biofilm producers.

MLST is a highly informative, reproducible, and portable technique, which puts isolates in a global context and can directly assign them to their CC. Thus, it is regarded as the method of choice for long-term and phylogenetic studies.[55] MLST was performed only on isolates with colistin MICs ≥ 1 µg/mL, which suggested a total of nine different STs, including ST801, ST118, ST138, ST421, ST735, ST133, ST216, ST387, and ST812. All ST clones, except for ST387 and ST812, have formerly reported from Iran by Farshadzadeh et al.[56] Besides, the ST801, ST118, ST138, ST421, and ST735 clones were categorized as the CC92 lineages, a similar result with the findings of Farshadzadeh et al[56] and Hojabri et al[57] who identified CC92 as the most prevalent clone in Iran. All the isolates allotted to CC92 were MDR and meropenem resistant. The prevalence of CC92 clone in all around the world may be due to its high adaptation and survival ability in various environments and its resistance to antibiotic pressures, including resistance to carbapenems.[58]

Conclusion

The present study showed a high frequency of MDR and XDR *A. baumannii* isolates, with high prevalence of biofilm-forming genes, *ompA* and *bap*. Moreover, the emergence of colistin-resistant *A. baumannii* isolates could be ascribed to the elevated expression of *pmrAB* gene in our region in Southwestern Iran and also its high diversity, which was verified by typing techniques. Taken together, to better understand these relationships, further studies with a higher number of colistin-resistant isolates are suggested. Moreover, control measures and regular surveillance are urgently needed to preclude the spread of these isolates.

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Disclosure

The authors report no conflict of interest in this study.

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