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ORIGINAL ARTICLE

The molecular characterization of colistin-resistant isolates of *Acinetobacter baumannii* from patients at intensive care units

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ABSTRACT

Background and Objectives: The objective of this study was to determine molecular characterization and genetic diversity of colistin-resistant *A. baumannii* clinical isolates in Intensive Care Unit hospitalized patients.
Materials and Methods: A total of 127 *A. baumannii* clinical isolates were evaluated for antimicrobial susceptibility. PCR reaction and sequencing were performed for the detection of mutations in *pmrAB* and *lpx ACD* genes.
Results: Based on antimicrobial susceptibility testing, 40.94% and 33.85% of the isolates were MDR and XDR respectively whereas 3.93% of them were found to be PDR. Results of agar dilution MIC and E-test indicated that 76% of the isolates were sensitive to colistin. All of the isolates were positive for $bla_{\text{OXA-51}}$ and 50% of them were positive for both $bla_{\text{OXA-23}}$-like and $bla_{\text{OXA-143}}$-like genes while only 25% of the isolates were positive for $bla_{\text{OXA-72}}$. None of them were positive for the $bla_{\text{OXA-58}}$-like gene. There is no mutation in *pmrA*. The V162A substitution for *pmrB* gene was repeated in two isolates, and E_{394} D and Y_{292}H substitutions in *lpxA* were observed in two isolates; also, C_{120}R and F_{165}L substitutions in *lpxC* gene was repeated in two isolates. Analysis of phylogenetic tree based on alterations in *lpxACD* and *pmrB* genes indicated the appearance of new isolates compared to the reference strain ATCC17978 *A. baumannii* isolates.
Conclusion: The present study indicated the prevalence of MDR and XDR *A. baumannii* isolates and the emergence of PDR isolates in the northwest portion of Iran. The appearance of colistin-resistant isolates with new mutations in *pmrB, lpxACD* genes indicates new resistance mechanisms.

Keywords: *Acinetobacter baumannii*; Beta-lactamase OXA-23; Beta-lactamase OXA-143; OXA-72 carbapenemase; Beta-lactamase OXA-58; Colistin

INTRODUCTION

Acinetobacter baumannii is the leading cause of a wide range of infections, including meningitis, pneumonia, wound, bloodstream, and urinary tract infections, especially in intensive care units (1, 2).

The escalating prevalence of antibiotic resistance due to inappropriate antibiotic use and the appear-

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ance of multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pan drug-resistant (PDR) strain is now a serious threat in clinical settings (3, 4). Based on reports, there is a direct association between the prevalence of MDR and XDR and the high risk of mortality and morbidity in *A. baumannii* infected patients (5, 6).

MDR *A. baumannii* isolates are identified based on resistance to β-lactams, aminoglycosides, and tetracyclines antibiotic classes (7). In this regard, carbapenems are known as the best therapeutic agent for MDR *A. baumannii* (8). For a while, colistin is used as a last resort antibiotic against carbapenem-resistant *A. baumannii* strains; however, based on recent reports, colistin-resistant *A. baumannii* strains have increased in clinical settings (9, 10).

Colistin is a cationic peptide that acts through disruption of the outer membrane (OM) of Gram-negative bacteria. Resistance to colistin is resulting from post-translational modification or loss of the lipopolysaccharide (LPS) molecules forming the outer leaflet of the OM (11). It occurs via alterations in the *pmrAB* two-component regulatory systems, *lpxACD*, or efflux pump mechanisms (7, 12, 13). According to studies, mutations within *pmrA* are not often related to resistance to colistin (14) while several mutations including non-synonymous SNPs, frameshifts, and deletions have been detected in *pmrB* gene (15). The *LpxACD* genes are involved in the first three stages of lipid A synthesis. Based on reports inactivation of *lpxA* or *lpxC* genes through mutation leads to colistin resistance (16).

This study was conducted to survey the molecular characterization of colistin-resistant *A.baumannii* clinical isolates in Tabriz, North-west Iran.

MATERIALS AND METHODS

Samples collection and bacterial identification. From January 2016 to October 2020, a total of 127 non-duplicate *A. baumannii* isolates were recovered from patients hospitalized in the Intensive Care Unit (ICU) wards of Imam Reza and Sina hospitals in Tabriz, Iran. Several clinical samples were collected from hospitalized patients including trachea, urine, blood, wound swab, fluid (pleural, cerebrospinal), bronchial secretions, catheter, and throat. *A. baumannii* isolates were identified by standard microbiological (colony morphology, Gram-staining) and biochemical tests (catalase test, glucose oxidation, oxidase test, citrate utilization, and Oxidation-Fermentation of various sugars test (4, 17). Hospitalized patients in intensive care settings (ICU) were included in this study. Outpatient and hospitalized patients in non-intensive care settings and patients which were transferred from other hospitals were also excluded from the study.

Antibiotic susceptibility testing. The antibiotic susceptibility test of the isolates was carried out by the disk diffusion method based on the Clinical Laboratory Standards Institute (CLSI) guideline (18). The following antimicrobial disks (MAST, UK) were utilized for antibiotic susceptibility testing; imipenem (10 µg), meropenem (10 µg), ceftazidime (30 µg), amikacin (30 µg), amoxicillin-sulbactam (10 µg), cefepime (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), and tetracycline (30 µg). A bacterial suspension was prepared with the 0.5 McFarland turbidity from *A. baumannii* isolates and inoculated on the Muller-Hinton agar plats. The antibiotic disks were incubated with the inoculated plates at 37°C overnight. *A. baumannii* ATCC 19606 was used as positive control and ultrapure water as a negative control (19). MDR isolates were defined based on resistance to at least one agent from three classes of drug including cephalosporins, fluoroquinolones, and aminoglycoside. Extensively drug-resistant (XDR) isolates were detected from MDR isolates resistant to carbapenems. Pan-drug resistant (PDR) isolates were found from XDR isolates resistant to polymyxins (20, 21).

Colistin susceptibility testing. Colistin susceptibility test was carried out by agar dilution MIC and E- test methods. E- test was performed based on the manufacturer's guidelines (bioMérieux, Madrid, Spain). For this, Mueller Hinton agar plates were inoculated with a 0.5 McFarland suspension from each isolate, and E-test strips were placed on the pre-inoculated agar plate surface. Plates were incubated for 16-24 hours at 35°C. The MIC results were read where the inhibition zone intersects the E-test strip and interpreted according to the Clinical and CLSI guidelines. The possible range of MIC for the E-test (colistin) was 0.016 to 256 mg/liter.

The agar dilution method was performed according to the procedure detailed in the previous reports (22). Colistin powder (Sigma Aldrich, USA) was used in

this method. A serial dilution for colistin was provided, ranging from 0.03 to 64 µg/mL in Mueller-Hinton agar plates. Plates were inoculated with a 0.5 McFarland suspension from each isolate and incubated at 37°C overnight. The MIC values were read based on the lowest antibiotic concentration that can inhibit visible bacterial growth.

DNA extraction and PCR amplification. The DNA extraction was performed by the boiling procedure. The suspension of individual colonies in 300 µL of TE buffer (10 mM Tris, 1 mM EDTA) was boiled at 95ºC for 10 minutes and then centrifuged (3). The quantity and quality of the extracted DNA were evaluated by spectroscopy at 260 nm and 260/280, respectively. The PCR reaction was conducted to amplify $bla_{\text{OXA-like}}$, *lpxA*, *lpxC*, *lpxD*, *pmrA*, and *pmrB* genes using specific primers (Table 1). PCR reaction began with an initial denaturation at 94°C for 4 minutes, 40 cycles of (94°C for 1 minute, 49°C for 1 minute, and 72°C for 1 minute). The final extension was carried out at 72°C for 5 minutes (21). PCR products were analyzed by gel electrophoresis and sequenced on both DNA strands and compared with the reference strain ATCC17978 (GenBank Accession Number CP000521).

Phylogenetic analysis. We used MEGA7 (version 7.0.2) for point mutations analysis in five colistin-resistant isolates compared to the drug-sensitive strain ATCC 17978 and constructed the maximum likelihood phylogenetic tree by the bootstrap algorithm.

RESULTS

Antimicrobial susceptibility. According to disk diffusion results, all of the isolates were resistant to meropenem (Fig. 1). Based on the classification method of MDR, XDR, and PDR isolates (20), 41.73% (53/127) of cases were MDR, while the frequency of XDR and PDR strains was 33.85% (43/127) and 3.93% (4/127) respectively. XDR isolates were detected based on resistance to only one or two antibiotic classes, while PDRs were resistant to all antibiotic categories (23). The highest rate of MDR and XDR was related to tracheal samples ($p<0.05$) (Fig. 2). PDRs were mainly isolated from wound samples ($p<0.05$) (Fig. 2). The highest MDR and XDR rate was related to inpatients up to 50 years ($p<0.05$) (Table 2). The prevalence of PDR was dependent on gender, and all PDR isolates were isolated from males (Table 2). All of the colistin-resistant isolates were also resistant to

Table 1. Sequences of primers used to determine the presence of *pmrAB* and *lpxACD* genes

Genes		Sequence (5´-3´)	Size (bp)
$bla_{\text{OXA-51}}$	Forward	TAA TGC TTT GAT CGG CCT TG	353
	Reverse	TGG ATT GCA CTT CAT CTT GG	
OXA-58	Forward	G TAT TGG GGC TTG TGC TG	599
	Reverse	CCC CTC TGC GCT CTA CAT	
OXA-72	Forward	GGT TAG TTG GCC CCC TTA A	249
	Reverse	AGT TGA GCG AAA AGG GGA T	
OXA-23	Forward	TGT TGA ATG CCC TGA TCG	504
	Reverse	ATT TCT GAC CGC ATT TCC AT	
OXA-143	Forward	TGGCACTTTCAGCAGTTC	152
	Reverse	GTG TAA TCT TGA GGG GGC	
Pmr A	Forward	GGTGGAATGGGTCAATAAC	595
	Reverse	TTATGATTGCCCCAAACG	
Pmr B	Forward	GAAAGAACAGCTGAGCAC	1296
	Reverse	AACTTATGGACAGGCTGG	
Lpx A	Forward	CCATTCTACCGCCATTATTGA	746
	Reverse	CACAATTCCACGCTCTGA	
LpxC	Forward	CGTACTCTCAATCGTGTG	870
	Reverse	CGTATGGAATTGGACAGTC	
LpxD	Forward	AAGGTGAGCTAATTGGTGAAG	959
	Reverse	AGTGATTTGGGTCAATGGC	

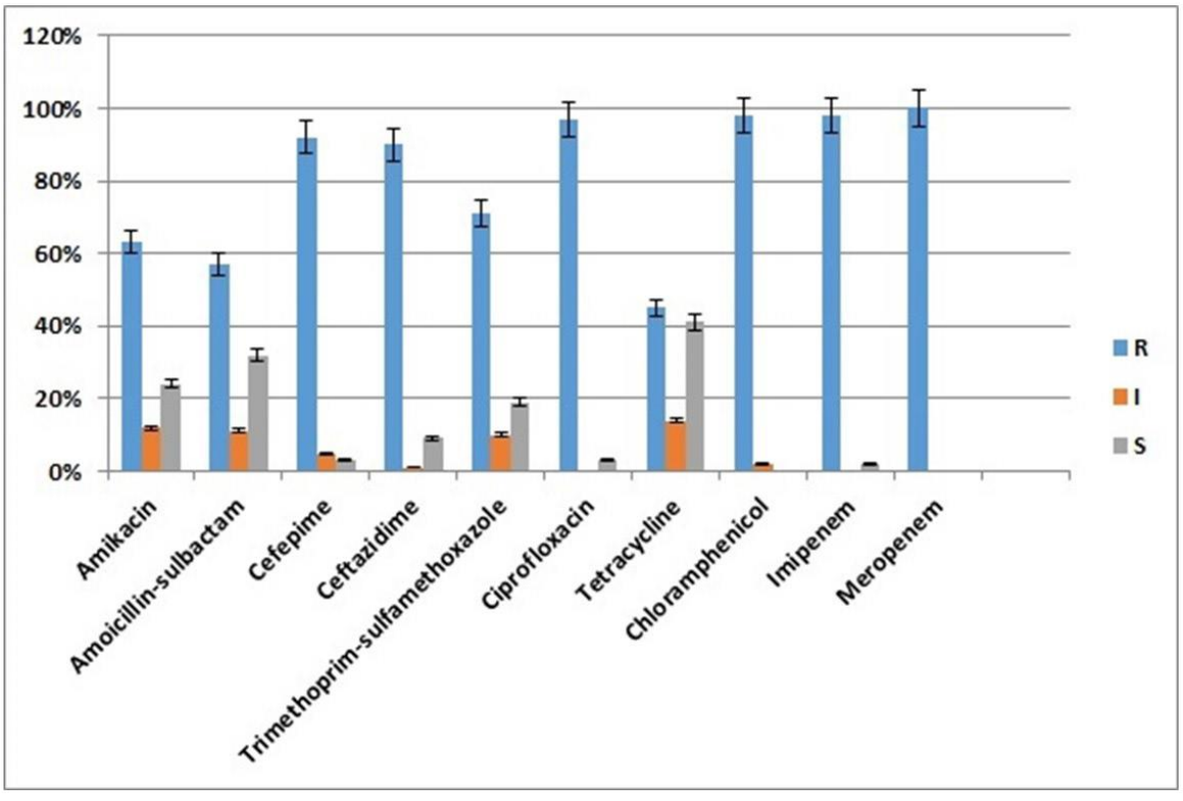

Fig. 1. Results of antibiotic susceptibility testing of *A. baumannii* clinical isolates obtained from Imam Reza and Sina hospitals in Tabriz, Iran.

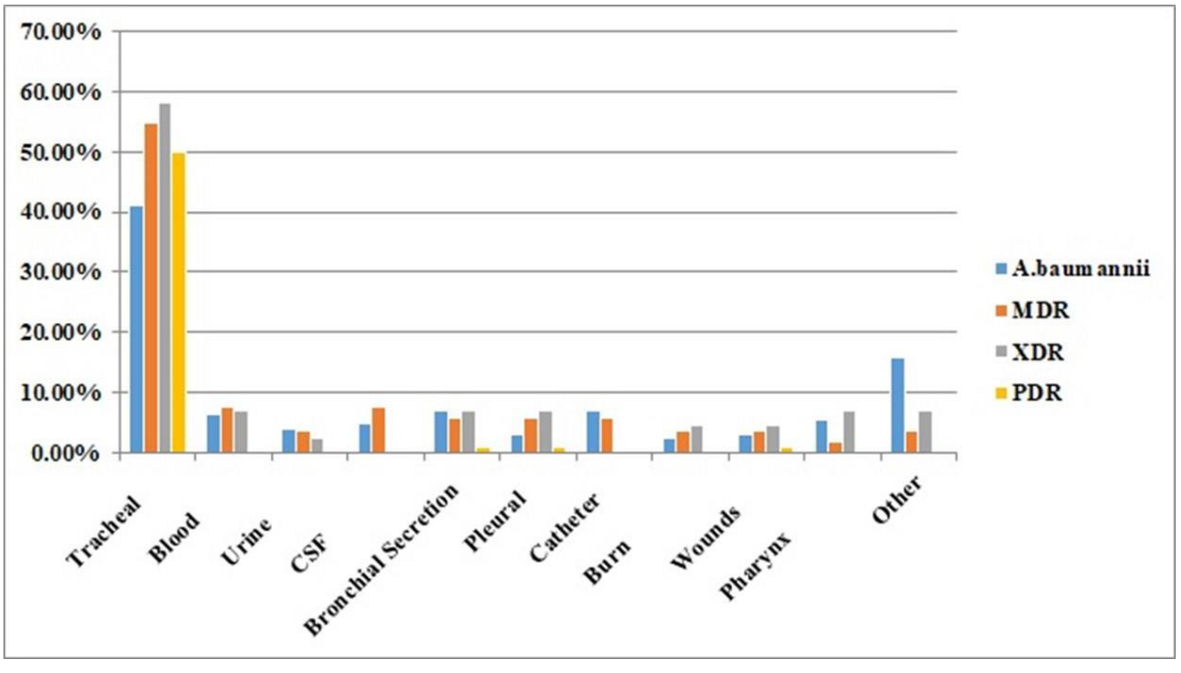

Fig. 2. Frequency of MDR, XDR, and PDR *A. baumannii* isolates in ICU ward of Imam Reza and Sina hospitals of Tabriz, Iran.

Table 2. Demographic data and frequency of MDR, XDR, and PDR in *A. baumannii* clinical isolates obtained from inpatients in Imam Reza and Sina hospitals of Tabriz, Iran.

Gender	*A. baumannii* N=127(%)	MDR (n=53)	P. value	XDR (n=43)	P. value (p=0.86)	PDR (n=4)	P. value
Male	81 (63.78%)	39 (73.58%)	(p=0.05)	27 (62.80%)	(p=0.86)	4 (100%)	-
Female	46 (36.22%)	20 (37.74%)	(p= 0.05)	16 (37.20%)	(p=0)	0	-
Age							
<17	4 (3.14%)	2 (3.77%)	(p=0.73)	0	(p=0.95)	0	-
18-30	18 (14.17%)	2 (3.77%)	(p=0 .004) P<.05	6 (13.95%)	(p=0.18)	1 (20%)	P=0 .52
31-40	12 (9.44%)	4 (7.54%)	(p=0.53)	2 (4.65%)	(p=0.15)	0	-
41-50	20 (15.74%)	5 (9.43%)	(p=0.09)	4 (9.30%)	(p=0.017)	0	-
>51	73 (57.48%)	40 (75.47%)	(p= .0005) p<.05	31 (72.09%)	P<.05	3 (80%)	P=0.47

chloramphenicol, imipenem, imipenem, ciprofloxacin, and cefepime. Most of the isolates were resistant to ceftazidime and cefepime (Table 3).

MIC test. Five *A. baumannii* carbapenem-resistant isolates were identified based on the E- test and agar dilution results. MICs of colistin-resistant isolates were ranging from 16 to 64 mg/L. Based on results 76% of the isolates were sensitive to colistin while 19% of them indicated an intermediate resistance to colistin.

Frequency of carbapenemase encoding genes. Based on PCR results, a high proportion of MDR (41/77.36%) and XDR (36/85.71%) isolates were positive for the $bla_{\text{OXA-23}}$ gene while only 2 (50%) of PDR strains was carried the $bla_{\text{OXA-23}}$ gene. Also, 29 (54.72%) of MDR isolates were positive for the $bla_{\text{OXA-143}}$ gene whereas 13(30.95%) of XDR and 2 (50%) of PDR isolates were carried the $bla_{\text{OXA-143}}$ gene. The frequency of the $bla_{\text{OXA-72}}$ gene was almost identical between MDR (13/24.53%) and XDR (9/21.42%) isolates and only 1(25%) of PDR strains was positive for the $bla_{\text{OXA-72}}$ gene.

Resistance mechanisms to colistin. The colistin-resistant isolates were screened for the incidence of mutations in *lpxACD* and *pmrAB* genes. For this, after successful amplification of *lpxACD* and *pmrAB* genes, the potential mutations were revealed by sequencing (Table 4). The results were compared to the reference strain ATCC17978 (GenBank Accession Number CP000521) to detect the presence of alterations related to resistance to colistin. Based on Clustal W results, $V_{299}G$ substitution was also detected in all colistin-resistant and colistin susceptible isolates, compared to the sequences of the *A. baumannii* ATCC 17978.

The $V_{162}A$ substitution in *pmrB* gene was repeated in two isolates, also $E_{394}D$, and $Y_{292}H$ substitutions in *lpxA*, were found in two isolates and $C_{120}R$ substitution

Table 3. The profile of antibiotic susceptibility of colistin-resistant *A. baumannii* isolates

Colistin resistant isolates	CAZ	C	MEM	AN	GM	TE	SXT	IPM	SAM	CIP	FEP
A158	S	R	R	S	S	R	S	R	S	R	S
A307	R	R	R	S	R	R	R	R	R	R	R
A384	R	R	R	S	S	S	R	R	R	R	R
A529	R	R	R	R	R	S	S	R	S	R	R
A115	R	R	R	R	S	R	R	R	R	R	R

Caz: ceftazidim; C: coloramfenicol; Mem: meropenem; Ipm: imipenem; AN: Amikacin; GM: Gentamicin; TE: Tetracyclin; SXT: Trimetoprim-sulfametoxazol; SAM: Ampicilin – sulbactam; CIP: Ciprofloxacin; FEP: cefepim

Table 4. Details of point mutations in *pmrAB* and *Lpx ACD* genes involved in resistance to colistin.

Colistin resistant isolates	*pmrA*	*pmrB*	*lpxA*	*lpxC*	*lpxD*
A115	no	227 frameshift mutation	299V—G	94E—G	7L—V
			292Y—H	115M—L	29I—V
			394E—D	151G—A	
				120C—R	
A158	no	162V—A	342P—R	120C—R	99I—N
			292Y—H	136E—R	174R—P
				137A—L	
				166A—P	
A307	no	371A—V	394E—D	109T—P	109I—L
				125T—P	112S—A
					140Q—P
					152G—E
A384	no	133V—D	405P—R	100V—G	62 I—V
		162V—A		165F—L	90L—P
					110H—R
					131N—I
					186F—L
A529	no	No	107K—Q	131I—N	127 frameshift mutation
			283I—N	165F—L	180V—G
			298, Frameshif mutation	182T—P	
				222V—G	

related to *lpxC* gene was also detected in two isolates. A phylogenetic tree was designed based on alterations in *lpxACD* and *pmrB* genes and results indicated the appearance of new isolates compared to the reference strain ATCC17978 *A. baumannii*. The examination of point mutations in the *lpxA* gene for five colistin-resistant isolates was indicating four phylogenetic groups that two isolates (A307 and A384) were in a group (Fig. 3). According to point mutations for *lpxD* gene, A307 and A529 isolates were classified in a group, and based on the analysis of variations for *lpxC* gene, A384, and A158 isolates were found in a single group.

DISCUSSION

The escalating prevalence of carbapenem-resistant *A. baumannii* isolates has led to the overuse of colistin as a last-line antibiotic. Currently, the appearance of colistin-resistant strains has limited the therapeutic options against MDR *A. baumannii* in clinical settings.

This study was conducted to characterize colistin-resistant *A. baumannii* isolates. In this study, all of the clinical isolates were resistant to carbapenem based on resistance to meropenem or imipenem. Also, 76% of the carbapenem-resistant isolates were sensitive to colistin consistent with results reported from Saudi Arabia (76% cases sensitive to colistin) (24). Colistin resistant rate, in our study, was almost similar to results obtained from Shiraz, Southwest of Iran, in which 6% of isolates were resistant to colistin (25).

The frequency of MDR and XDR isolates was 41.73% and 33.85%, respectively which was not consistent with the frequency of MDR and XDR reported from a burn center hospital in the northwest of Iran (74.75% MDR and 73.13% XDR) (26). This inconsistency can be associated with the hospitals under study. Also, MDR and XDR rates in our study were fewer than studies done in the west (84% MDR and 48% XDR) (26) and the North of Iran (91.4%MDR and 58.3% XDR) (27). The incidence of PDR in this study was equal to 3.93% which was less than the PDR rate reported from Shahid Motahari Burns Hospital, Tehran, Iran (14.5%) (28).

The highest proportion of MDR was isolated from tracheal samples not consistent with the recent re-

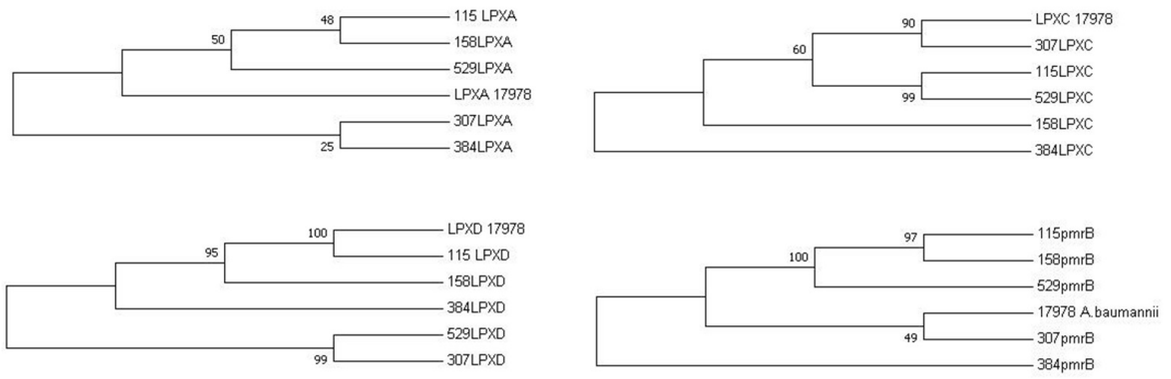

Fig. 3. Phylogenetic analysis of five colistin-resistant *A. baumannii* isolates based on point mutations in *pmrB* and *lpxACD* genes.

ports regarding the high rate of MDR in wound and blood samples (26, 29). It is indicating a lack of association between the sample type and MDR rate. The prevalence of MDR was dependent on age which was more detected in patients older than 51 years old similar to the previous study (30).

The most percentage of MDR and XDR were related to patients up to 50 years ($p<0.05$). In our study, there were no mutations in *pmrA* consistent with the previous studies (31-33) indicating a lack of correlation between colistin resistance and incidence of mutation in *pmrA* gene. Remarkably, all colistin-resistant isolates were harbored at least one point mutation in the *pmrB* gene. In this study, three types of alterations in the *pmrB* gene (V$_{162}$A, A$_{371}$V, V$_{133}$D) were detected that V$_{162}$A substitution was repeated in two isolates and this mutation seems to be key in the occurrence of resistance to colistin. A mutation related to the change of alanine to valine reported in this study was previously detected in recent studies indicating a crucial role of this mutation in resistance to colistin (32, 34). Until now, a variety of mutations have been reported.

In a recent study performed on colistin-resistant isolates, five types of mutations (P$_{233}$S, A$_{142}$V, A$_{227}$V, and T$_{235}$I) were observed in the *pmrB* gene (34).

Also, in a study done by Park et al. six kinds of point mutations were identified in the *pmrB* (I$_{121}$F, T$_{192}$I, Q$_{228}$P, A$_{184}$V, P$_{190}$S, and A$_{183}$T) (31), and Beceiro et al. reported eight alterations in the *pmrB* gene (L$_{87}$F, M$_{145}$K, S$_{14}$L, P$_{233}$S, A$_{227}$V, S$_{403}$F, F$_{387}$Y, and N$_{353}$Y) (32).

In our study, several mutations were found in the *lpxA* gene (V$_{299}$G, P$_{342}$R, E$_{394}$D, Y$_{292}$H, P$_{405}$R, K$_{107}$Q, I$_{283}$N, and frameshift mutations) that E$_{394}$D and Y$_{292}$H substitutions were repeated in two isolates. Also, V$_{299}$G substitution was detected in all colistin-resistant and colistin susceptible isolates, compared to the sequences of the *A. baumannii* ATCC 17978. It was indicating a lack of correlation between V$_{299}$G substitution and resistance to colistin.

In *lpxC*, fourteen alterations were found in the five colistin-resistant isolates that C$_{120}$R and F$_{165}$L substitutions were repeated in two isolates. In *lpxD*, fifteen mutations were observed in the five colistin-resistant isolates. In a recent study, Nurtop et al. reported three mutations (V$_{63}$I, Q$_{4}$K, and E$_{117}$K) within the *lpxD* gene (35).

Until now, a variety of mutations have been identified in colistin-resistant isolates that may be related to resistance to colistin however it is obvious that repetition of some mutations in our study can be directly associated with resistance to colistin.

Analysis of the phylogenetic tree made based on alterations in *lpxACD* and *pmrB* genes indicated the appearance of new isolates in comparison with the reference strain ATCC17978 *A. baumannii*. Based on point mutations in the *lpxA* gene in five colistin-resistant isolates, four phylogenetic groups were classified which two isolates (A307 and A384) were in a group. Based on the diversity of point mutations in *lpxD* gene, A307 and A529 isolates were in a group and the analysis of variations in *lpxC* gene was indicating the classification of A384, and A158 isolates in a single group. Also, the A384 isolate was in a single group based on point mutations that occurred in *pmrB, lpxA, lpxC* genes.

In conclusion, the present study indicated the prev-

alence of MDR and XDR *A. baumannii* isolates and the emergence of PDR isolates in the northwest portion of Iran. The appearance of colistin-resistant isolates with new mutations in *pmrB, lpxACD* genes highlights the incidence of new resistance mechanisms in *A. baumannii* clinical isolates.

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