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MOLECULAR CHARACTERIZATION OF THE *pmrCAB* GENES ASSOCIATED WITH COLISTIN RESISTANCE IN CLINICAL ISOLATES OF *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumnnii (*A. baumannii*) represents a significant threat to public health, severely limiting the effectiveness of existing antimicrobial treatments. Currently, colistin is used as a last-resort treatment for infections caused by multidrug-resistant and extensively drug-resistant *A. baumannii*, due to the increasing rates of resistance to both first- and second-line antibiotics. Regrettably, reports of colistin resistance (CoR) in *A. baumannii* clinical isolates have been growing frequently. This study aims to investigate the incidence of mutations in the *pmrCAB* operon, characterize the alterations found within isolates, and examine the diversity and complexity of colistin resistance in *A. baumannii.* In this study, 46 unique isolates of *A. baumannii* from hospitalized patients are identified using the Phoenix BD Diagnostic System, which serves for bacterial identification and antimicrobial susceptibility profiling. Additionally, these isolates are confirmed through PCR analysis of the $bla_{\text{OXA-51}}$-like gene. Based on CLSI 2024 guidelines, a significant proportion of isolates were multidrug-resistant 25 (54.4%), extensively drug-resistant 18 (39.1%), or pandrug-resistant 6 (6.5%). High resistance rates were observed for meropenem (93.4%) and imipenem (91.3%). Colistin resistance was detected in 6.5% (3/46) of isolates, all of which were positive for *pmrCAB* genes. Additionally, this study identified point mutations in the *pmrC* gene, resulting in a specific amino acid alteration (Val42→Ile) in the EptA protein, located within the transmembrane region. In conclusion, this study highlights the high prevalence of MDR-XDR *A. baumannii* clinical isolates, emphasizing the urgent requirement for effective control strategies. Furthermore, *pmrC* point mutations and distinct amino acid substitutions may contribute to low-to-moderate modification of lipid A within the lipopolysaccharide, which may contribute to colistin resistance. However, direct impact on bacterial virulence requires further investigation.

Keywords: *Acinetobacter baumannii, pmrCAB* operon, mutation, MDR, XDR, colistin resistance, and EptA protein

INTRODUCTION

Antimicrobial resistance represents an enormous threat for worldwide healthcare systems by reducing the treatments that are accessible for an increasing range of bacterial infections **(Lodise *et al.*, 2025; Trebosc *et al.*, 2019)**. As a serious nosocomial infection, *Acinetobacter baumannii* (*A. baumannii*) has been associated with a number of hospital outbreaks. In patients residing in intensive care units (ICU), as well as in immunocompromised patients, it predominantly causes meningitis, pneumonia brought on by a ventilator, wounds, urinary tract and blood stream infections **(Ko, Choi and Lee, 2017; Stanley, Awanye and Ogbonnaya, 2023)**. There is growing awareness concerning the spread of extensively drug-resistant (XDR) *A. baumannii* infections **(Muhammed and Rasool, 2025; Diao *et al.*, 2024)**. Main classes of antibiotics, including carbapenems, fluoroquinolones, aminoglycosides and beta-lactams are losing their effectiveness against *A. baumannii*, leaving no viable therapeutic alternatives **(Olalekan and Olukemi, 2023; Boucher *et al.*, 2013)**. This challenge is a major obstacle for medical professionals to treat XDR *A. baumannii* infections **(Diao *et al.*, 2024; Weber *et al.*, 2019)**.

Currently, colistin (polymyxin E) remains an important treatment option for infections caused by MDR-XDR *A. baumannii*, owing to the increasing rates of resistance to most available antibiotics **(Zafer *et al.*, 2023; Bassetti *et al.*, 2019; Gales, Jones and Sader, 2011)**. Colistin resistance (CoR) reports of clinical *A. baumannii* isolates have grown infrequently **(Abbasi, Hajihashemi and Shokri, 2024; Ayoub Moubareck and Hammoudi Halat, 2020; Trebosc *et al.*, 2019)**. *A. baumannii's* resistance to colistin is thought to stem from a variety of mechanisms. The most prevalent of these is the alteration of the lipid A component within the lipopolysaccharide (LPS) layer of the outer membrane, a process facilitated by the *pmrCAB* genes **(Novović and Jovčić, 2023a)**. Colistin has an amphipathic construction with a cationic nature and mainly react with the negative charge of the lipid A component of LPS **(Moffatt, Harper and Boyce, 2019)**. Critical CoR mechanisms in *A. baumannii* are chromosomally encoded **(Kumburu *et al.*, 2019; Lima *et al.*, 2018)**. Clinical isolates of *A. baumannii* that exhibit CoR involve alteration in the *pmrCAB* operon. The addition of phosphoethanolamine transferase (EptA) to lipid A is triggered by the *pmrC* gene; *pmrC* expression is regulated by the *pmrAB* two-component system (TCS). As a result, the outer membrane's negative charge reduces, which affects colistin binding and protects the cell membrane's stability and structure intact **(Chen and Groisman, 2013; Kumburu *et al.*, 2019)**. Controlled addition of positively charged residues, such as EptA to LPS, diminishes negative charge on the bacterial surface and subsequently inhibits the adhesion of the polymyxin to the LPS **(Moffatt, Harper and Boyce, 2019)**. Some investigations indicate that *pmrAB* mutations raise *pmrC* expression, which has been related to elevated minimum inhibitory concentrations (MICs) of colistin **(Seleim *et al.*, 2022; Charretier *et al.*, 2018)**. *A. baumannii* has swiftly gained CoR through various mechanisms that are difficult to identify using conventional molecular diagnostic techniques. This poses significant challenges for the effective management of this rapidly spreading infection in the future **(Novović and Jovčić, 2023b)**.

Despite increasing global reports of CoR in *A. baumannii,* there is limited data from our region, particularly regarding mutation-specific characterization of the *pmrCAB* operon. Therefore, the present study aimed to evaluate the prevalence of MDR, XDR, and PDR strains, elucidate the mechanisms underlying CoR, and characterize mutations in the *pmrCAB* gene, including their corresponding amino acid alterations, in clinical isolates.

MATERIALS AND METHODS

Identification and isolation of study isolates

All 46 unique study isolates of *A. baumannii* had been identified in various clinical specimens, comprising endotracheal aspirate (ETA), burn, urine, wounds, sputum, blood, synovial fluid, and central venous line (CV-line), from March 2023 to June 2024 at several hospitals in the Sulaymaniyah governorate, Iraq. Samples were collected and identified using MacConkey agar, nutrient and blood-based agar (Himedia, India), and Acinetobacter CHROMagar (CHROMagar, Paris, France), and advanced identification was done using Phoenix (BD Diagnostic Systems, Franklin Lakes, NJ, USA). Their final confirmation was performed by PCR of the $bla_{\text{OXA-51}}$-like gene **(Charretier *et al.*, 2018)**. All isolates were molecularly confirmed by PCR targeting the $bla_{\text{OXA-51}}$-like gene. *A. baumannii* ATCC19606 was determined to serve as a positive control. The isolates were subsequently preserved at -70°C until further investigation was carried out in a Tryptone Soy Broth medium (Himedia, India) supplemented with 20% glycerol **(Sunarno *et al.*, 2021)**.

Antibiotic susceptibility test

MICs of 13 different antibiotics, including ceftazidime (CEFT), ampicillin/sulbactam (A/S), cefepime (CEF), piperacillin/tazobactam (P/T), imipenem (IPM), meropenem (MER), amikacin (AK), colistin (CL), levofloxacin (LEV), ciprofloxacin (CIP), gentamicin (GEN), tigecycline (TIG), and trimethoprim-sulfamethoxazole (SXT)—against *A. baumannii* isolates were determined using the Phoenix (BD Diagnostic Systems, Franklin Lakes, NJ, USA). In accordance with the Clinical and Laboratory Standards Institute (CLSI) 2024 recommendations, the MICs of every antibiotic were interpreted. Furthermore, the Centres for Disease Control and Prevention (CDC) and the European Centre for Disease Prevention and Control (ECDC) developed standards for antimicrobial susceptibility, which were used to classify isolates into MDR, XDR, and PDR antimicrobial resistance categories **(Falah, Shokoohizadeh and Adabi, 2019)**.

PCR screening for *pmrCAB* genes

Using the thermal shock method, the genomic DNA of bacterial isolates was extracted **(Falah, Shokoohizadeh and Adabi, 2019; Hou and Yang, 2015)**. A final volume of 25 µl was used for all PCRs. Including Taq Master Mix (addbio, Korea). Screening for the bla_{OXA-51}-like gene and CoR genes, *pmrCAB* operon (*pmrA, pmrB*, and *pmrC*), using primers and PCR conditions previously published in "Table 1." 3 µl of *A. baumannii* whole DNA extract was used as a template for the PCR, 10 µl of 2X master mix, 10 µl of PCR water, and 1 µl per primer. In a thermocycler 'Applied Biosystems, USA.' The following standard PCR amplification protocol was used: 30 cycles of amplification are performed after the initial 5-minute heating to 95°C. The three stages of each cycle were denaturation at 95°C for 30 seconds, annealing at the appropriate temperature for 30 seconds, and extension at 72°C for the appropriate duration, followed by a final elongation step of 5 minutes at 72°C. Finally, the PCR product was stored at 4°C until they were analyzed. To determine the elongation time, the length of the anticipated amplified DNA was utilized. The PCR products were electrophoresed on a 1.5% agarose gel, using ethidium bromide as a stain, and the results of the specific amplified target gene were visualized using a UV transilluminator (BIO-RAD, California, USA) **(Gerson *et al.*, 2020; Magiorakos *et al.*, 2012)**. Furthermore, capillary gel electrophoresis (CGE), as an advanced technique, was used for the separation of PCR products, and DNA was detected by fluorescent labeling **(QIAxcel Advanced, GERMANY) (Screengel, 2024)**.

Table 1 Oligonucleotide sequences and annealing temperature used in this study.

Target genes	Primer sequences (5′-3′)	Amplicon size (bp)	Annealing Temp.	Elongation time	Ref
bla_{OXA-51}	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	353bp	53°C	30 sec	**(Hou and Yang, 2015)**
pmrA	F: GATGGTTTAAATTTGGGTGCAGAT R:TTGACTCGCAAGTTGAGCTTCT	120bp	58°C	45 sec	**(Abbasi, Hajihashemi and Shokri, 2024)**
pmrB	F: GCCATTATTCGTCGTGGTTTAAA R: GCGCTCAAAAAGACGGTTCA	150bp	57°C	45 sec	**(Abbasi, Hajihashemi and Shokri, 2024)**
pmrC	F: CCATTTGGCTAGGTGCAATTT R: CCGCATAATAGGTAGCAACAAG	132bp	57°C	45 sec	**(Abbasi, Hajihashemi and Shokri, 2024)**

***pmrCAB* sequence authentication and assessment**

Sanger sequencing was used by an outside professional service provider (Macrogen, Korea) to sequence the PCR products. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI). The *pmrA, pmrB*, and *pmrC* gene sequences of the three CoR isolates were compared to the reference sequence of *A. baumannii* ATCC 19606 (GenBank: HM149345.1) attained from NCBI.

Statistical analysis

Statistical analysis were conducted using SPSS version 26.0 (IBM Corp., Armonk, NY, USA) to evaluate resistance proportions, which are presented with 95% confidence intervals. The Fisher's exact test was used to compare resistance rate among the MDR, XDR, and PDR groups, with a significance threshold set at a p-value of less than 0.05. All statistical tests were performed as two-tailed.

RESULTS

The isolates (n=46) were identified as *A. baumannii* and were collected from patients in various hospital wards. The distribution of isolates included 26 (56.5%) from the Intensive Care Unit (ICU), 12 (26.1%) from the Burn and Plastic Surgery Unit, and 8 (17.4%) from the Outpatient Department (OPD). Clinical specimens included endotracheal aspirates (ETA), burn swabs, and urine samples (10 isolates each, 21.7% each), followed by wounds (7, 15.2%), sputum (6, 13.0%), and blood, synovial fluid, and central venous line (CV-line) samples (1 isolate each, 2.2%). There were 20 male patients (43.5%) and 26 female patients (56.5%), having a mean age of 46.4 years and a range of 12 to 81 years.

As shown in Fig. 1, antibiotic susceptibility testing revealed that most isolates were MDR (25, 54.3% CI: 39.8–68.1%), defined as resistance to at least one agent in three or more antibiotic classes. XDR isolates (18, 39.1% CI: 26.1–53.8%) were susceptible to only two or fewer antimicrobial categories, while PDR isolates (3, 6.5% CI: 2.2–17.5%) were resistant to all tested agents. While the difference in propositions between MDR and XDR groups was not significant (p = 0.142; OR = 1.85; 95% CI 0.81-4.22), PDR isolates were significantly less frequent than both MDR (p = 0.001; OR = 0.06; 95% CI: 0.01-0.22) and XDR groups (p = 0.003; OR = 0.11; 95% CI: 0.02-0.43) (Table 2). (CI= confidence intervals, OR= odds ratio). *A. baumannii* isolates have a high resistance rate to cefepime, ceftazidime, and piperacillin-tazobactam (97.8%); and levofloxacin (95.6%). Resistance rates to meropenem and imipenem were high, at 93.4% and 91.3%, respectively. Besides β-lactam antibiotics, the study isolates showed high resistance to the majority of antimicrobial medicines, containing trimethoprim-sulfamethoxazole (95.6%), meropenem (93.4%), and gentamicin (86.9%). Colistin showed the highest susceptibility rate (93.5%), followed by tigecycline (54.4%). The corresponding resistance rates were 6.5% for colistin and 45.6% for tigecycline. Three isolates (6.5%), which were categorized as PDR, exhibited resistance to colistin (MIC ≥ 4 mg/L).

Colistin exhibited the lowest resistance rate at 6.5% (3/46; 95% CI 2.2-1.7%), significantly lower than that of tigycycline, which was 54.4% (25/46; 95% CI 40.0-68.1%; p<1.001). Resistance to carbapenems was notably high for both meropenem at 93.4% (43/46; 95% CI: 82.1-97.8%) and imipenem at 91.3% (42/46; 95% CI 97.7-96.6%) with no statistically significant difference between the two agents (p = 0.715). Notably, all three PDR isolates (100%) exhibited resistance to coilistin whereas CoR was absent in both MDR (0/25; 0%) and XDR (0/18; 0%) categories (p<0.001 for PDR vs. MDR and PDR vs. XDR comparisons). Resistance to β-lactam antibiotics (cefepime, ceftazidime, and piperacillin-tazobactam) was uniformaly high all three categories (range: 96.0-100%), with no significant differences observed between groups (p>0.05 for all comparisons). Similarly, high resistance rate to levofloxacin (95.6% 44/46; 95% CI: 85-98.8%) and trimethoprim-sulfamethoxazole (95.6%; 44/46; 95% CI: 85.2-98.8%) were observed across all resistance categories.

Table 2 Comparison of resistance profile across MDR, XDR, and PDR categories of *A. baumannii* isolates

Resistance Category	n (%)	95% CI	Comparison	p-value	Odds Ratio (95% CI
MDR	25 (54.3%)	39.8–68.1%	MDR vs. XDR	0.142	1.85 (0.81–4.22)
XDR	18 (39.1%)	26.1–53.8%	MDR vs. PDR	0.001	0.06 (0.01–0.22
PDR	3 (6.5%)	2.2–17.5%	XDR vs. PDR	0.003	(0.02–0.43)
Total	46 (100%	–	–	–	–

Table 2 demonstrated the distribution of MDR, XDR, and PDR *A. baumannii* clinical isolates, along with their corresponding 95% CI. MDR isolates were the most prevalent (54.3%), followed by XDR (39.1%) and PDR (6.5%). Comparative analysis indicated no statistically significant difference between MDR and XDR isolates (p = 0.142), whereas significant differences were observed between MDR and PDR (p = 0.001) and between XDR and PDR (p = 0.003), reflecting the markedly lower frequency of PDR isolates.

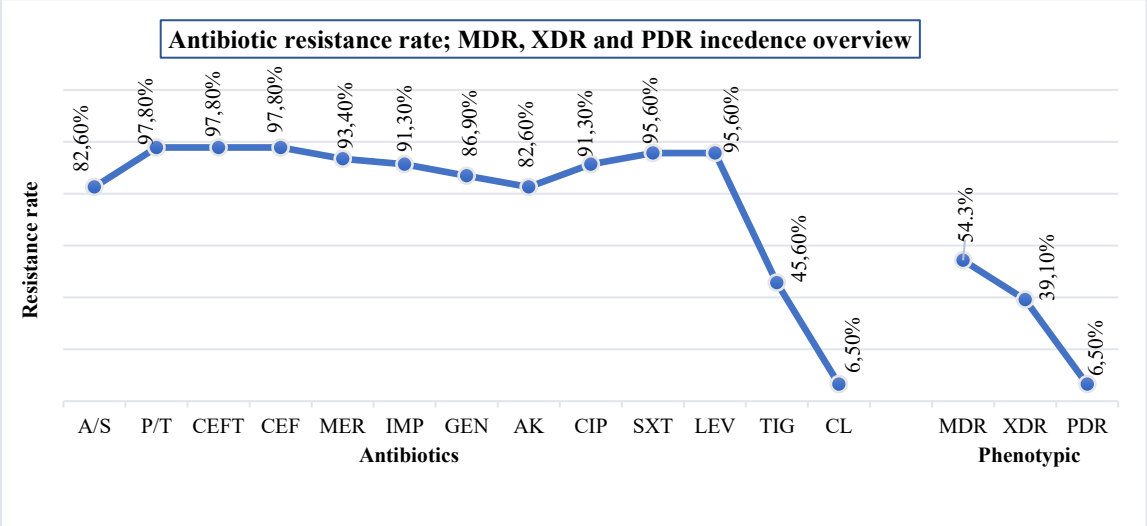

Figure 1 *A. baumannii* antibiotic resistance profile exhibited MDR (54.3%), XDR (39.1%), and PDR (6.5%). Overall, resistance was most prevalent against cefepime, ceftazidime, and piperacillin/tazobactam (97.8%). Tigecycline (45.6%) and colistin (6.5%) demonstrated the highest effectiveness. A/S: ampicillin-sulbactam; P/T: piperacillin/tazobactam; CEFT: ceftazidime; CEF: cefepime; MER: meropenem; IMP: imipenem; AK: amikacin; GEN: gentamicin; LEV: levofloxacin; CIP: ciprofloxacin; SXT: trimethoprim-sulfamethoxazole; TIG: tigecycline; and CL: colistin.

PCR screening of *pmrCAB* genes

PCR screening analysis was conducted to detect bla_{OXA-51} as a confirmatory identification of *A. baumannii* isolates and identify *pmrCAB* genes in *A. baumannii* isolates. Concerning the genes associated with colistin resistance, *pmrA, pmrB, and pmrC* were found in all three CoR *A. baumannii* isolates.

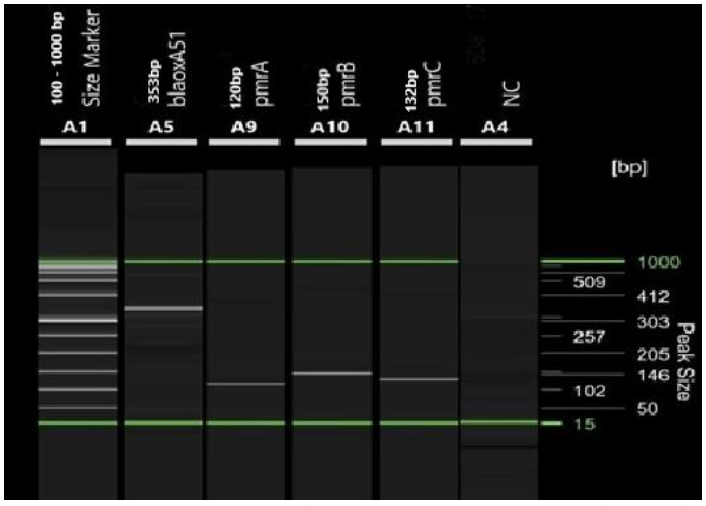

Figure 2 Capillary gel electrophoresis (QIAGEN, Germany), showing the amplified product of antibiotic resistance-related genes. A1: ladder (50 bp–1000 bp); A5: bla_{OXA-51} 353 bp; A9: *pmrA* 120 bp; A10: *pmrB* 150 bp; A11: *pmrC* 132 bp; A4: NC negative control.

Nucleotide sequence analysis of *pmrCAB*

Mutations were identified through pairwise alignment with the corresponding *A. baumannii* ATCC 19606 (HM149345.1). Gene sequences can be accessed via the NCBI, BLAST tool at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequence analysis conducted in this study demonstrated an identity rate of 98-100% when compared to the reference strain. Furthermore, BLAST analysis revealed all three CoR *A. baumannii* isolates shared a *pmrC* substitution (124 G>A) and a *pmrB* substitution (2877 C>A) compared with the reference strain, as shown in figure 3. While mutations in *pmrA* were not found in any of the three isolates. The accession numbers for the *pmrCAB* genes we submitted to GenBank, pertaining to the nucleotide sequences of the CoR *A. baumannii* isolate, are as follows: PQ780766, PQ780773, PQ788525, PQ780767, PQ780768, PQ780769, PQ780770, PQ780771, and PQ780772.

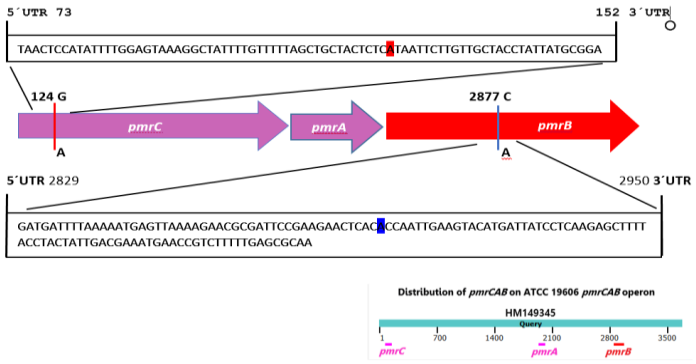

Figure 3 schematic representation of the *pmrCAB* operon. The overall gene map is adapted from previously published studies (Gerson *et al.*, 2020)**.** point mutations identified in the present study are overlaid on this map. Mutations highlighted in red represent unique transition mutations, while transversion mutations, indicated in blue, are detected in all three CoR isolates. The highlighted mutations represent the original finding of this study, whereas the operon structure is derived from the literature.

Amino acid sequencing and substitutions in *pmrCAB*

Regarding the amino acid sequence analysis of *pmrCAB*, present results indicate high amino acid sequence similarity with *pmrCAB* originating from ATCC 19606. Sequence analysis of *pmrC* showed one amino acid substitution (42 valine to isoleucine) was detected in all three CoR isolates as a result of point mutation in *pmrC*. While the *pmrA* and *pmrB* displayed exact sequence identity regardless of amino acid substitutions, as depicted in table 3.

3D structure modeling of EptA in *A. baumannii* Cor isolates

The amino acid sequence of the EptA from *A. baumannii* was submitted to Swiss-Model for homology-based 3D structure prediction (https://swissmodel.expasy.org/) **(Waterhouse *et al.*, 2018)**. The selected template showed a 99.25% sequence identity, demonstrating a strong evolutionary and functional similarity. The predicted structural model of EptA was evaluated using various validation tools in the Swiss-Model workspace, yielding quality parameters indicative of a good model. The QMEAN Z-score was measured at -1.35, indicating a global model quality within the expected range for similar structures. The QMEAN global score was recorded at 0.78 ± 0.05, with a Ramachandran plot showing 94.2% of residues in favored regions, 4.6% in allowed regions, and 1.2% as outliers. Additional metrics included 2.8% rotamer outliers, a Cβ deviation of 0.6%, and a 99.25% sequence identity with the template. With a GMQE value of 0.92 and template coverage of 98.5%, these results collectively support that the EptA model is of high quality and suitable for analyzing the V42I mutation.

Table 3 Alignment of the *pmrCAB* amino acid sequences among isolates, using ATCC 19606 as a reference. Amino acid substitutions are indicated in red (Val to Ile) at the position of 42.

***pmrA* amino acid sequences**	
ATCC 19606 ADK46864.1	100 IKPYEFDELLARIHALLRRSGVEAQLASQ 128
Suli-1 XOL43308.1	 29
Suli-10 XOL43309.1	 27
Suli-17 XOL43307.1	 26
***pmrB* amino acid sequences**	
ATCC 19606 ADK46865.1	172 DDFKNELKERDSEELTPIEVHDYPQELLPTIDEMNRLFER 211
Suli-1 XOL43310.1	 40
Suli-10 XOL43311.1	 39
Suli-17 XOL43312.1	 33
***pmrC* amino acid sequences**	
ATCC 19606 ADK46863.1	19 YHQVHTLTPYFGVKAILFLAATLVILVATYYA 50
Suli-1 XOL43313.1	.....**I**..... 28
Suli-10 XOL43315.1	.....**I**..... 25
Suli-17 XOL43314.1	.....**I**..... 25

This model provides a solid foundation for analyzing the Valine (Val) 42 to Isoleucine (Ile) mutation, enabling further investigation into its effects on enzymatic activity and colistin resistance. According to our predicted structure, Val to Ile is a conservative substitution. Assuming that Val42 is not located directly in the active site, the likelihood of modification is considered low to moderate. Moreover, the mutation occurs within the transmembrane region, which may affect membrane anchoring or orientation.

The structural model of EptA from *A. baumannii* is annotated to illustrate its functional and topological features. The catalytic domain, which is crucial for EptA transfer activity, is highlighted in brown and oriented toward the periplasm. The transmembrane helices are depicted in blue, which anchor the protein within the inner membrane. Additionally, conserved motifs and anticipated substrate entry pathways are annotated to enhance understanding of the enzyme's role in colistin resistance (Figure 4). Based on the analysis of the image and the nature of the substitution, the mutation is likely categorized in a low- to moderate-probability tier for affecting colistin resistance—unless modeling indicates secondary effects such as changes in orientation or membrane packing tension.

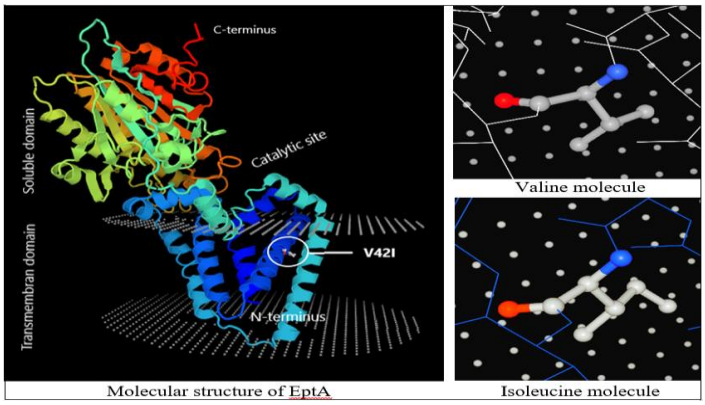

Figure 4 EptA's molecular structure is shown with the amino terminus in blue and the carboxyl (soluble) domain in red. The white circle is the V42I position (A): Rainbow representation of mutant EptA; (B): valine molecule; and (C): isoleucine molecule in CoR *A. bauamnnii* isolate created by Swiss-model platform.

DISCUSSION

Currently, colistin represents the last available treatment for various infections due to the appearance of XDR bacterial isolates **(Mousavi *et al.*, 2025; Ezadi *et al.*, 2020)**. Overuse and abuse of antibiotics are among the many factors contributing to the worldwide development of *A. baumannii* drug resistance **(Ezadi *et al.*, 2020; Kyriakidis *et al.*, 2021; Shi *et al.*, 2020)**. The findings of present study show that MDR and XRD *A. baumannii* clinical isolates are highly prevalent (54.3% and 39.1%, respectively), which is consistent with findings from other earlier investigations **(Abbasi, Hajihashemi and Shokri, 2024; Shi *et al.*, 2020)**. Current study emphasizes the importance and crucial nature of XDR in *A. baumannii,* especially in ICU patients. The predominance of isolates obtained from ICU patients (56.5%) and those from endotracheal aspirates (21.7%) emphasizes the significance of ventilator-associated pneumonia in the transmission of *A. baumannii*. Notably, two isolates that were resistant to colistin came from the ICU, and another came from the emergency burn unit, indicating that there may be different selection pressures for resistance in the ICU and burn wounds, possibly due to variations in antibiotic usage and infection control practices in these settings. This investigation showed that carbapenem resistance was prevalent, which is consistent with those documented globally. They illustrate the higher probability of carbapenem failing to treat infections caused by *A. baumannii* **(Singh *et al.*, 2024; Rezaei *et al.*, 2018)**. The rising CoR is concerning since this antibiotic is commonly used as a final-option treatment for XDR *A. baumannii* infection **(Gerson *et al.*, 2019)**. Nowadays, one of the biggest challenges in the clinical management of *A. baumannii* infections is the emergence of the CoR strain. Therefore, determining the processes underlying CoR will be critical for both research objectives and the identification of prospective novel targets for the next generation of antimicrobial drugs **(Islam *et al.*, 2024; Seethalakshmi *et al.*, 2023)**. Colistin showed the lowest resistance (6.50%) in present investigation, and all three colistin-resistant *A. baumannii* isolates (MIC ≥ 4 µg/ml) were susceptible to it. All other antibiotics, such as ciprofloxacin, tigecycline, meropenem, and piperacillin/tazobactam, were ineffective against the clinical isolates. Present study showed no significant difference between the proportions of MDR isolates ($p = 0.142$). However, PDR isolates were significantly less frequent than both MDR ($p = 0.001$) and XDR ($p = 0.003$) isolates. This suggests that while *A. baumannii* exhibits multi-drug resistance in our regions, pandrug resistance, including CoR, remains relatively uncommon (6.5%; 95% CI: 2.2-17.5%). The wide confidence intervals for PDR proportions, due to the small number of PDR isolates, warrant cautious interpretation of comparisons involving PDR. CoR was exclusively found in PDR isolates (100%) and absent in MDR and XDR isolates ($p < 0.001$), suggesting that, in our setting, it arises from the accumulation of multiple resistance determinants rather than as an isolated event. This aligns with the previous finding that colistin resistance typically develops late in the acquisition of multidrug resistance, often after prolonged antibiotic exposure (Gerson *et al.*, 2019; Trebosc *et al.*, 2019). Also, this result indicates that colistin persists as the most effective treatment for *A. baumannii* infections when compared to other antibiotics. Studies reveal that approximately 6% of isolates of *A. baumannii* exhibit CoR **(Abbasi, Hajihashemi and Shokri, 2024; Bostanghadiri *et al.*, 2024; Behera, Swain and Chandra, 2017)**.

Researches has shown that the mechanisms of resistance to colistin in *A. baumannii* are often associated with mutations in the *pmrCAB* operon **(Trebosc *et al.*, 2019; Olaitan, Morand and Rolain, 2014)**. *pmrCAB* genes have an essential impact on the emergence of CoR in *A. baumannii* **(Gerson *et al.*, 2019; Olaitan, Morand and Rolain, 2014)**. Understanding the role of *pmrCAB* genes in *A. baumannii* colistin resistance is essential for developing novel strategies to fight antibiotic resistance **(Poirel, Jayol and Nordmann, 2017; Yang *et al.*, 2021)**. However, investigators found that CoR mechanisms in *A. baumannii* are more complicated than explained by one pathway **(Gerson *et al.*, 2020)**. Researchers are investigating various techniques to target these genes, including phage therapy and small biological inhibitors, in an effort to renovate the effectiveness of colistin to treat infections caused by *A. baumannii* **(Altamirano *et al.*, 2022)**.

Therefore, it is necessary to evaluate strain-specific polymorphisms in colistin resistance-related genes, such as mutations in the *pmrCAB* operon, besides the contribution of extra undetermined reasons linked with CoR in *A. baumannii* **(Gerson *et al.*, 2019)**. This study suggests that mutations in the *pmrCAB* gene within CoR isolates may contribute to the development of CoR strains. In present research, we identified that all three *A. baumannii* clinical isolates possessed the *pmrCAB* gene. In addition, we performed genome sequencing to search for mutations related to CoR and antibiotic resistance genes. Through applying this method, this study succeeded in identifying a point mutation in *pmrC,* which caused an alteration in the EptA amino acid. This alteration is predicted to affect protein function and colistin resistance **(Misic *et al.*, 2018; Casino, Rubio and Marina, 2010)**. Our prediction indicates that the Val→Ile substitution is conservative. Both residues are hydrophobic; however, isoleucine is somewhat bulkier. The potential impact of this change is assessed to be low to moderate, as such substitutions typically do not significantly alter hydrophobic interactions. Furthermore, we identify one silent mutation in *pmrB* due to the redundancy of the

genetic code across all three CoR isolates. It is in line with prior studies that have shown a connection between mutations in the *pmrCAB* genes and increased expression of *pmrC* **(Seleim *et al.*, 2022; Gerson *et al.*, 2019)**. Moreover, researchers indicated that colistin resistance may be exacerbated by a combination of *pmrB* and *pmrC* mutations **(Gerson *et al.*, 2019)**.

There were no mutations in *pmrA* in current results in CoR isolates, which is consistent with earlier research suggesting that there is no relationship between CoR and the occurrence of the *pmrA* gene mutation **(Abbasi, Hajihashemi and Shokri, 2024; Farajnia *et al.*, 2022; Haeili, Kafshdouz and Feizabadi, 2018)**. In comparison to *pmrA*, the *pmrB* gene was more variable, and several point mutations in this particular gene have been detected in our investigation. It is in line with earlier studies **(Olaitan, Morand and Rolain, 2014)**. Indicating that *pmrB* mutations may have a greater effect on *pmrCAB* expression and, finally, resistance to colistin **(Gerson *et al.*, 2020)**. Previous research found the greatest number of mutations in the *pmrC* region **(Zafer *et al.*, 2023; Haeili, Kafshdouz and Feizabadi, 2018)**. The present study indicates a unique substitution was detected in the *pmrC*. Additionally, combined *pmrB* and *pmrC* mutations correlated with increased colistin MIC, indicating that mutation accumulation is the mechanism underlying high-level resistance **(Gerson *et al.*, 2019)**. The correlation between *pmrCAB* mutation and elevated MIC values in the studied isolates suggests a potential relationship between these genetic changes and phenotypic resistance. These mutations may affect the structure and function of the *pmrCAB* regulatory system, ultimately leading to lipid A modification and decreased susceptibility to colistin. However, it is crucial to note that not all alterations in amino acids result in colistin resistance **(Zafer *et al.*, 2023; Gerson *et al.*, 2019)**. This study suggests that further investigation is essential to achieve a comprehensive understanding of how *pmrCAB* mutations affect *pmrCAB* overexpression, lipid A modification, and the resulting colistin susceptibility phenotype. Additionally, due to the limited number of CoR isolates, our finding regarding mutation prevalence and evolutionary should be considered preliminary. Larger multicenter studies are necessary to validate these observations.

Depending on the findings (Table 3), our prediction (Figure 4) suggests that a point mutation in the EptA protein, specifically the substitution of Val to Ile at position 42 within the transmembrane domain, represents a conservative change between two hydrophobic amino acids. Both Val and Ile are nonpolar amino acids that preferentially associate with membrane regions. This suggests that the mutation is unlikely to disrupt the overall folding or integration of the protein within the membrane **(Sun and Palzkill, 2021)**. Conversely, Isoleucine has a bulkier side chain because of an additional methyl group, which could subtly affect local packing interactions within the lipid bilayer or between neighboring transmembrane helices **(Chen *et al.*, 2023)**. While such alterations are typically accepted in membrane-embedded regions, they may impact the dynamic behavior of the protein, especially if position 42 is near areas involved in conformational transitions or substrate translocation **(Gao *et al.*, 2016)**. Notably, because this residue is not located within the catalytic domain or known substrate-binding sites, the mutation is not anticipated to significantly impair enzymatic activity of EptA **(Sun and Palzkill, 2021)**. However, slight effects on protein stability, membrane localization, or interactions with lipid A components cannot be completely ruled out **(Chen *et al.*, 2023; Gao *et al.*, 2016)**. Specifically, recent structural studies of membrane proteins indicate that isoleucine interacts more extensively with lipid environments compared to valine, which may affect local membrane packing and protein dynamics **(AL Mughram *et al.*, 2023)**. They necessitate additional analysis through structural modeling and functional assays.

Nevertheless, it is likely that other mechanisms of CoR besides *pmrC* overexpression exist and should not be ignored **(Yang *et al.*, 2021; Ezadi *et al.*, 2020)**. Previous research has indicated that outer membrane alterations that occur as a result of mutations in the plasmid-mediated colistin resistance *mcr* gene are the key to CoR in *A. baumannii* or other mechanisms unrelated to modifications in the outer membrane, including the function of efflux pumps and increased sensitivity to osmotic pressure (**Ilsan *et al.*, 2021)**. These arguments suggest that resistance of *A. baumannii* is continuously evolving. Therefore, to control hospital-acquired bacterial infections, it is essential to establish a detailed strategy and an adequate treatment plan.

CONCLUSION

In conclusion, a high prevalence of MDR, XDR *A. bauamnnii* was observed, emphasizing the need for effective control strategies. Colistin remained active against most isolates (93.5%). To the best knowledge, this study provides the first regional characterization of *pmrCAB* mutations and identifies a novel *pmrC* substitution (Val42→Ile) that suggests a low- to moderate-likelihood modification of the lipid A component within the lipopolysaccharide in colistin-resistance isolates. These findings highlight the necessity for further investigation into the molecular basis of colistin resistance.

Ethical approvals and statements: The study was approved by the Scientific and Research Ethics Committee, College of Science, University of Sulaimani (2024-04-29). The research has concluded in conformity with the applicable criteria. The study's proposal was handed over to them, and formal authorization was acquired to collect samples from different Sulaymaniyah City hospitals. After receiving assurances that their identities would remain anonymous, all patients agreed to participate in the trial.

Conflicts of Interest: The authors declare no conflicts of interest.

Author Contributions: Sirwan M. Muhammed Ameen conceived and designed the research. Aram H. Rasool conducted all laboratory experiments and performed data analysis. All authors contributed to drafting and revising the manuscript.

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