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Molecular mechanisms of colistin resistance mediated by *pmrCAB* genes in *Acinetobacter baumannii* isolated from patients hospitalized in Isfahan medical centers

Original Study

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Abstract

Introduction. In recent years, colistin-resistant *Acinetobacter baumannii* (*A. baumannii*) has been found all over the world. In this current study, the main purpose was to examine the occurrence of extensively drug resistant (XDR), resistance to colistin and characterization and mutations in *pmrCAB* genes among *A. baumannii* obtained from inpatients.

Materials and Methods. A total of 108 clinical isolates of *A. baumannii* were collected from several hospitals located in Isfahan, Iran. The Kirby-Bauer assay was performed to assess the antimicrobial resistance. The Phoenix automated system was utilized to determine the minimum inhibitory concentration (MIC) of colistin for each of the bacterial isolates. Polymerase chain reaction was used to screen for *pmrCAB* genes that mediate colistin resistance, and sequencing was used to determine the amplicon's nucleotide sequence.

Results. The results revealed that all A. baumannii isolates (100%) were resistant to piperacillin tazobactam, meropenem and ciprofloxacin. All isolates were classified as XDR, with seven isolates being pan-drug resistant (PDR). Colistin resistance (CoR) was found in 6.48% (7/108) of studied isolates, all of which were positive for *pmrCAB* genes. The sequencing results showed a substitution in *pmrB* and two isolates showed a substitution in *pmrC*.

Conclusions. In conclusion, this study is the initial report of the existence and mutations of *pmrB* and C genes in the clinical isolate of *A. baumannii* in our region. This outcome highlights the necessity to explore additional mutations in the PMR operon of *A. baumannii* in forthcoming studies. Moreover, our results highlight the high occurrence of XDR-*A. baumannii* strain in Isfahan, Iran.

Keywords

MDR• XDR • colistin resistance • pmrCAB • Acinetobacter baumannii

1. Introduction

The spread of *Acinetobacter baumannii* (*A. baumannii*) infections that are resistant to multiple drugs is a growing concern [1]. Antibiotics, such as beta-lactams, aminoglycosides, fluoroquinolones and carbapenems, which are important groups of antibiotics, are becoming increasingly ineffective against *A. baumannii*, leaving no effective treatment options available [2,3]. This issue poses a serious challenge for clinicians treating MDR *A. baumannii* infections [4].

The emergence of bacterial strains exhibiting resistance to a wide range of antibiotics commonly available in the commercial market and the shortage of novel antibiotics effective MDR Gram-negative bacteria has led to the use of colistin (polymyxin E) as a valuable therapeutic option [5]. However, there have been an increasing number of reports of colistin-resistance among the *A. baumannii* stain [6].

The resistance of Gram-negative bacteria to colistin is attributed to a range of different mechanisms. Among these, the most prevalent one in *A. baumannii* is the alteration of the lipid A factor of the lipopolysaccharide layer in the outer membrane, that is primarily facilitated through the pmrCAB operon [7].

The pmrAB two-component system regulates the expression of the pmrC gene, which is accountable for the addition of phosphoethanolamine (pEtN) to lipid A. Consequently, this leads to a reduction in the negative charge on the outer membrane, thereby affecting the binding of colistin and preventing harm to the stability and structure of the cell membrane [8,9]. According to reports, mutations in *pmrAB* result in an increase in *pmrC* expression, which is associated with higher colistin minimum inhibitory concentrations (MICs) [10].

A. baumannii has the potential to rapidly acquire colistin resistance (CoR) through several mechanisms that are challenging to screen with straightforward molecular diagnostic tests. This scenario has significant implications for effectively treating this rapidly emerging pathogen in the future [7]. In addition, CoR in

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Gram-negative bacteria has been described sporadically, and new agents effective against such organisms are not available.

Due to the limited number of studies conducted in Iran, especially within the region, investigating the mechanisms of resistance to colistin, the purpose of this research was to determine the frequency of XDR, resistance to colistin, and the characterization and mutations in *pmrCAB* genes among *A. baumannii* isolated from inpatients.

2. Materials and methods

2.1. Patients, sample collection and identification

A total of 108 nonduplicated clinical isolates of *A. baumannii* have been obtained from different teaching hospitals in Isfahan, Iran, during 2021 to 2022. Different clinical samples, such as urine, wound, blood and respiratory secretions, were collected to obtain the clinical isolates. Furthermore, the specimens were promptly transferred in a refrigerated environment to the laboratory of Nobel Laboratory Research Center Isfahan, Iran, for further analysis. The samples were inoculated on Eosin Methylene Blue, Müller Hinton Agar, and Blood agar media (Himedia, India) and were incubated for 24 hours at 37 °C. Based on morphological and standard biochemical tests, all isolates were identified as *A. baumannii*. The isolates were then preserved in a Brain Heart Infusion (BHI) Broth medium from Oxoid, UK, supplemented with 20% glycerol, and stored at -70 °C until additional examination.

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility of all isolates was evaluated using the Kirby-Bauer disc diffusion technique on Muller Hinton agar (MHA) plates, as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [11]. Different antibiotics including gentamicin and amikacin, ciprofloxacin, cefepime and ceftazidime, meropenem, piperacillin/tozabactam, ampicillin-sulbactam, and trimethoprim-sulfamethoxazole were applied to characterize the antibiotic susceptibility profile of *A. baumannii* isolates. The MIC of colistin was detected by the automated system (Phoenix; BD Diagnostic Systems, Franklin Lakes, NJ, USA). *Escherichia coli* ATCC 25922 was utilized as the control. Antimicrobial susceptibility of strains was evaluated according to the CDC and ECDC definition, and categorized as MDR or XDR.

2.3. DNA extraction and visualization

All isolates that were confirmed to be resistant to colistin based on their phenotype were selected to the standard Genomic DNA extraction for subsequent molecular analysis. [12]. The quality of the DNA extraction was assessed on a 1.5% agarose gel.

2.4. PCR analysis of *pmrCAB* genes

According to Table 1, the *pmrCAB* genes were amplified via conventional PCR with gene-specific primers The PCR process involved the following cycling settings: 1 cycle at 94 ºC for 10 minutes, 25 cycles at 94 ºC for 45 seconds, 58 ºC (for *pmrA*) and 57 ºC (for *pmrBC*) for 45 seconds, 72 ºC for 45 seconds, and a finally at 72 ºC for 10 minutes.

On a 1.5% agarose gel stained with Safe Stain and viewed under an ultraviolet transilluminator, the results of the amplified target of the specific gene were examined.

2.5. *pmrCAB* sequence confirmation and analysis

An external expert service provider (Macrogen, Korea) utilized Sanger sequencing to perform the sequencing of the PCR products. The NCBI's Basic Local Alignment Search Tool was used to compare the sequences, and Oligoanalyzer software was used to analyze their properties and the possibility of forming secondary and dimer structures. The eight isolates' *pmrA*, *pmrB*, and *pmrC* gene sequences were compared to the reference sequence of *A. baumannii* ATCC 19,606 (GenBank: CP045110.1) available at the National Center for Biotechnology Information website.

3. Results

A total of 108 Acinetobacter isolates were obtained from teaching hospitals, with 70% of the strains being from hospitalized patients in ICUs and comprising 58 females and 50 males with an average age of 53 ± 21 years. PCR amplification of the $bla_{\text{OXA-51}}$-like gene confirmed that all of the strain were *A. baumannii*.

The *A. baumannii* isolates were derived from various sources, including blood (41.9%), urine (29.03%), respiratory secretions (19.35%), and ulcer swabs (9.6%). The antibiotic susceptibilities of *A. baumannii* isolates are presented in Figure 1. Overall, all isolates showed resistance to at least one antibiotic, with piperacillin/tazobactam, meropenem, and ciprofloxacin being the most commonly resistant antibiotics (100%). The two most effective antibiotics were colistin (84.5%) and amikacin (9.25%). In addition to β-lactam drugs, the isolates exhibited high resistance to most antimicrobial agents, including meropenem (100%), ceftazidime (98.1%), and gentamicin (94.4%). All isolates were classified as XDR, with seven isolates being pan-drug resistant (PDR). Out of 108 isolates, 7 (6.5%) were resistant to colistin, with a MIC breakpoint of ≥ 4 mg/L (CLSI). Despite the use of this antibiotic as the last line of treatment, all patients died during hospitalization.

PCR screening of the *pmrCAB* genes was performed on seven *A. baumannii* isolates that were resistant to colistin (Figure 2). The results showed that all seven colistin resistant isolates were positive for *pmrCAB* genes, which Nucleotide BLAST results showed the 97–100% sequence similarity to the sequences of the *pmrCAB* genes present in GenBank. In addition, four isolates showed a substitution (3276848 T>G) in *pmrB* and two isolates showed a substitution (3279660 G>A) in *pmrC*.

3.1. Nucleotide accession numbers

The accession numbers for the deposition of nucleotide sequences of pmrCAB genes in *A. baumannii* to GenBank are as follows: ON777415, ON820656, ON820662, ON820657,

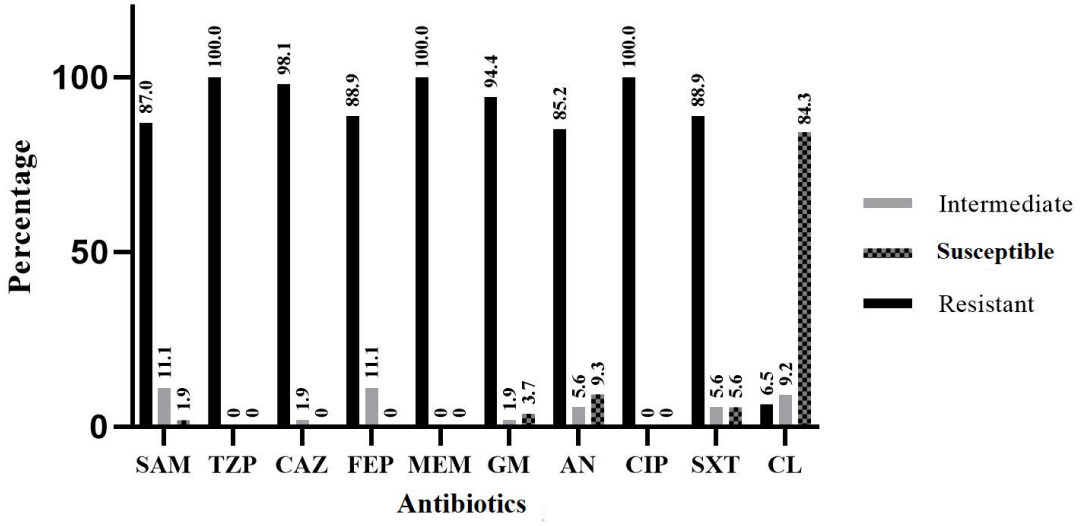

Figure 1. The results of antimicrobial susceptibility of A. baumannii to the antibiotics investigated in this study. Overall, piperacillin/tazobactam, meropenem, and ciprofloxacin were the most commonly resistant antibiotics (100%). The two most effective antibiotics were colistin (84.5%) and amikacin (9.25%). SAM: Ampicillin-sulbactam; TZP: piperacillin/tozabactam; CAZ: Ceftazidime; FEP: cefepime; MEM: Meropenem; GM: Gentamicin; AN: amikacin; CIP: ciprofloxacin; SXT: Trimethoprim-sulfamethoxazole; and CL: Colistin

Table 1. Oligonucleotide sequences used in this study

Primer	Primer sequence (5' to 3')	Amplicon size	Annealing temperature
PmrA(F)	GATGGTTTAAATTTGGGTGCAGAT	120	58
PmrA(R)	TTGACTCGCAAGTTGAGCTTCT		
PmrB(F)	GCCATTATTCGTCGTGGTTTAAA	150	57
PmrB(R)	GCGCTCAAAAAGACGGTTCA		
PmrC(F)	CCATTTGGCTAGGTGCAATTT	132	57
PmrC(R)	CCGCATAATAGGTAGCAACAAG		

ON820663, ON820659, ON820660, ON820658 ON820661, ON820664, ON820665, ON820666, grp 8647008, ON921023, ON959503, ON959504, ON959505, ON959506, ON959507, ON959508, ON959509, ON971458, and ON971457.

4. Discussion

The resistance of *A. baumannii* to drugs is spreading rapidly worldwide, and there are multiple factors that contribute to this phenomenon, including the misuse and overuse of antibiotics [13]. Colistin is one of the few available treatments for a variety of infections due to the emergence of carbapenem-resistant isolates [14]. Although, colistin is a last choice agent used to treat infections caused by MDR strains of *A. baumannii*, the emergence and rise of CoR has become a major challenge in the clinical management of *A. baumannii* infections [15]. In Iran, the increasing prevalence of *A. baumannii* poses a critical threat to healthcare, and colistin has become a last-line antibiotic [16-18].

The results of our research indicate that there is a high prevalence of XRD-*A. baumannii* isolates in Iran, which is in line with other studies conducted previously [19,20]. Additionally, our study emphasizes the severity and importance of XDR in *A. baumannii*, particularly in ICU patients, as most of the isolates tested showed antimicrobial resistance.

The study found that carbapenem resistance was common, with all isolates showing resistance to the tested carbapenems. These findings are consistent with those reported in different parts of the world, and they demonstrate the increased risk of carbapenem treatment failure in *A. baumannii* infections [21-23].

The lowest frequency of resistance was assigned to colistin (6.5%), which belonged to seven PDR *A. baumannii* with MIC values ≥4 µg/ml. None of piperacillin/tazobactam, meropenem, ciprofloxacin were susceptible in treatment of the isolates. In comparison to other antibiotics tested, colistin is still the most effective treatment for *A. baumannii* infections, according to research [24]. Our findings are in line with studies that show about 7% of *A. baumannii* isolates are resistant to colistin [25,26]. Nonetheless, a different study reported that CoR was observed in 57% (12/21) of the *A. baumannii* isolates, with MIC levels ranging from 4 µg/ml to over 128 µg/ml (9).

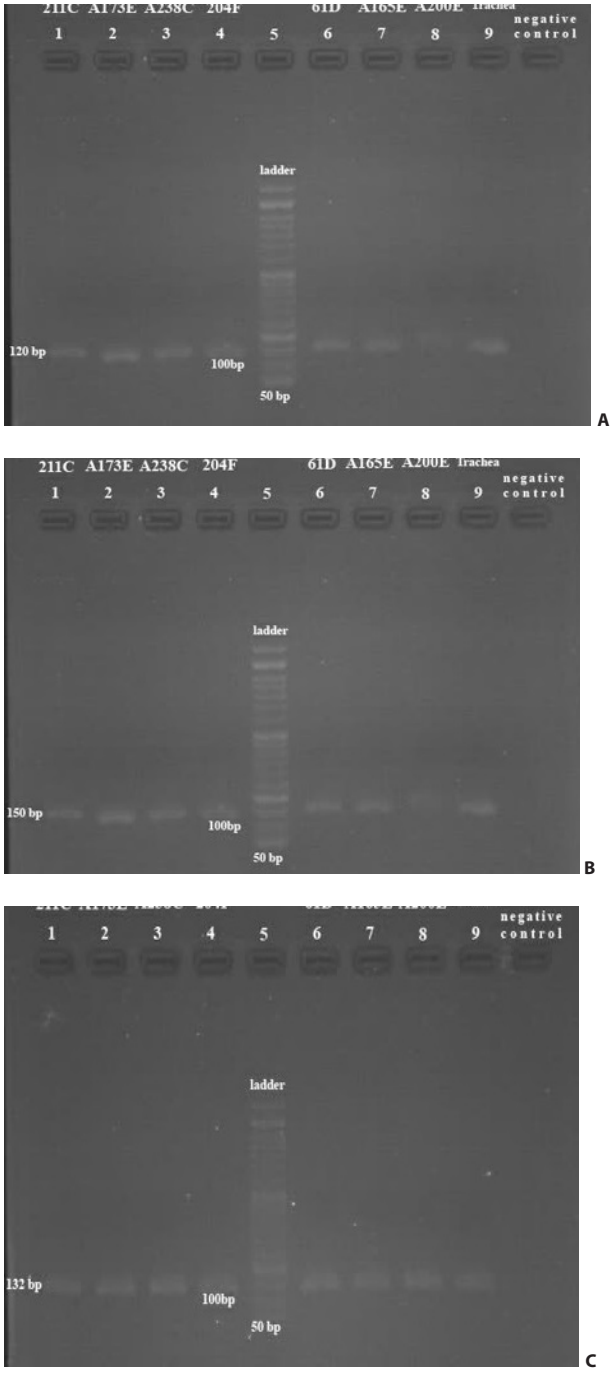

Figure 2 - PCR amplification of the PmrA, B and C genes. Ladder: 100 bp-2kb ladder, lane 1: positive control for PmrA (120bp), B (150bp) and C (132bp) genes; lane 2-9: positive results and negative control

Studies conducted earlier have suggested that the primary reason for CoR in *A. baumannii* is due to changes in the outer membrane caused by mutations in genes such as *pmr*, *lpx*, *lpsB*, *lptD*, and vacJ, the presence of *mcr* genes on plasmids, or other mechanisms not related to outer membrane changes such as enhanced sensitivity to osmotic pressure or the action of efflux pumps [27,28].

The development of CoR in *A. baumannii* is significantly influenced by the *pmrCAB* genes. Understanding the role of *pmrCAB* genes in colistin-resistant *A. baumannii* is critical for developing new strategies to combat antibiotic resistance [29,30]. Researchers are exploring different approaches to target these genes, such as the use of small molecule inhibitors or phage therapy, in order to restore the efficacy of colistin and other antibiotics against *A. baumannii* infections [31].

In our study, we detected all seven *A. baumannii* isolates carrying the *pmrCAB* gene. Furthermore, we conducted genome sequencing to investigate the existence of antibiotic resistance genes and mutations that are associated with colistin resistance. Using this approach, we found some mutations in *pmrB* and *pmrC* among seven isolates.

On the other hand, earlier research has documented that mutations in *pmrCAB* genes are linked to heightened expression of *pmrC*, and some of these mutations emerged while undergoing colistin therapy [32,33]. The *pmrA/pmrB* system, which consists of two components, controls the expression of *pmrC* and mutations in *pmrAB* which may cause an increase in *pmrC* expression. However, it is possible that other mechanisms of CoR are present and should not be disregarded [34,35].

Additionally, the findings of the sequence similarity search in this study revealed a similarity rate of 97–100% when compared to the reference strains, specifically ATCC 19606. Overall, in this research, it was determined that the presence of *pmrCAB* gene mutations in Iranian patient-derived CoR isolates may be responsible for the emergence of colistin-resistant strain. In our study, there were no mutations in *pmrA* consistent with the previous studies indicating a lack of correlation between CoR and incidence of mutation in the *pmrA* gene [18]. Additionally, Babaei et al. demonstrated that there were no mutations detected in the *pmrCAB* genes when the PCR amplicon was analyzed [17].

Similar to our results, Seleim et al. also documented the occurrence of the same substitution in a colistin-resistant isolate of *A. baumannii* [32]. Gerson et al. observed that only the colistin-resistant bacteria had five amino acid substitutions that are unique and specific to the pmrB gene, namely S128R, T42P, L153F, A28V, T42P, and P170L. These substitutions were found to be linked to colistin MIC levels ranging from 8 mg/L to ≥256 mg/L[36]. Also, previously in Iran, Farajnia et al. had earlier reported the presence of the V162A substitution within the pmrB gene in two bacterial isolates from Iran [18].

Based on these findings, it appears that the resistance of *A. baumannii* is constantly evolving. Therefore, it is crucial to implement an appropriate treatment plan and a precise strategy in order to prevent infections that originate from this bacterium in hospital settings. The PDR susceptibility profile data of *A. baumannii* emphasizes the critical importance of a comprehensive and widespread national program for the study

of antimicrobial drug resistance in Iran, which is responsible for monitoring *A. baumannii* isolates from diverse regions of the country for a serious nationwide effort to control and manage the outbreak of PDR *A. baumannii* [37].

4.1. Limitations of the study

This study is subject to certain limitations: the primary limitation pertains to the incomplete availability of comprehensive patient history background information. Furthermore, it is imperative to acknowledge that the isolation of *A. baumannii* strains was confined to different hospitals, necessitating a cautious approach to the interpretation of the results. Second, the impossibility of using typing methods such as pulsedfield gel electrophoresis (PFGE), therefore, to identify the source for pathogen transmission and take preventive measures in a hospital setting, molecular analysis of clinical specimens was required.

5. Conclusions

In conclusion, this study is the initial report of the existence and mutations of *pmrB* and C genes in a clinical isolate of *A. baumannii* in our region. This outcome highlights the necessity to explore additional mutations in the PMR operon of *A. baumannii* in forthcoming studies. Moreover, our results highlight the high occurrence of XDR-*A. baumannii* isolates in Isfahan, Iran, and the urgent need for actual strategies to prevent and control the spread of these infections.

Authors' contributions

Conceived and designed the experiments: DSh and AA; Performed the experiments: AA and BH; Performed statistical and spatial analyses and interpreted all the results: DSh and AA; Contributed to the writing of the manuscript and revised the final version manuscript: AA, BH, and DSh; All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Ethics approval

Ethics Committee approval was not required. Investigated clinical samples were leftover materials from other studies.

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