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RESEARCH ARTICLE

Prevalence and molecular mechanisms of colistin resistance in *Acinetobacter baumannii* clinical isolates in Tehran, Iran

AMIN KHOSHBAYAN[1], AREF SHARIATI[1], SAMANE SHAHMORADI[1], ZOHRE BASERI[2], HANIYEH MOZAFARI[1] and DAVOOD DARBAN-SAROKHALIL[1]*

[1] Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran

[2] Department of Pathology and Laboratory Medicine, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Colistin is one of the last remaining active antibiotics against multidrug resistant Gram-negative bacteria. However, several recent studies reported colistin-resistant (ColR) *Acinetobacter baumannii* from different countries. In the current study, we investigated molecular mechanisms involved in colistin resistance in *A. baumannii* isolates from different clinical samples.

A total of 110 clinical *A. baumannii* isolates were collected from two hospitals in Tehran. Minimum inhibitory concentrations (MICs) were determined by broth microdilution according to the Clinical and Laboratory Standards Institute. For the ColR isolates, mutation was detected in *pmrA*, *pmrB*, *lpxA*, *lpxC*, and *lpxD* genes using the polymerase chain reaction (PCR) and sequencing. Moreover, the relative expression of the *pmrC* gene was calculated using quantitative reverse transcription PCR. Three colistin resistant isolates were identified with MIC between 8 and 16 μg/mL and were resistant to all the tested antimicrobial agents. All the three isolates had a mutation in the *pmrB*, *pmrA*, *lpxA*, *lpxD*, and *lpxC* genes. Moreover, the overexpression of *pmrC* gene was observed in all isolates. Our results showed that the upregulation of the PmrAB two component system was the primary mechanism linked to colistin resistance among the studied colistin resistant *A. baumannii* isolates.

KEYWORDS

colistin resistance, colistin, *Acinetobacter baumannii*, PmrAB, PmrC

INTRODUCTION

Acinetobacter baumannii is an opportunistic pathogen associated with nosocomial infections such as pneumonia, bacteremia, urinary tract infections, and meningitis. *A. baumannii* outbreaks have been reported mainly in intensive care unit settings in many countries worldwide [1, 2]. This bacterium is intrinsically resistant to a different class of antibiotics. Moreover, it becomes resistant to other antibiotics, making *A. baumannii* infection treatment very challenging [3]. With the rise of multidrug resistance (MDR) and extensively drug resistant (XDR) isolates of *A. baumannii*, only few therapeutic options remain to treat infections caused by this bacterium. Colistin, a member of the polymyxin family, is one of these options that maintain its activity against MDR and XDR Gram-negative bacteria [4]. This antibiotic binds to lipid A in lipopolysaccharide (LPS) and subsequently disrupts the membrane that leads to the death of bacteria [5, 6]. However, recent studies reported colistin-resistant (ColR) *A. baumannii* [7, 8]. This phenomenon is mainly related to a mutation in *pmrA* and *pmrB* genes, which leads to the consistent activation of the PmrAB regulatory

*Corresponding author. Tel.: +98 2186703183, fax: +98 2188058649.
E-mail: darban.d@iums.ac.ir; davood_darban@yahoo.com

system. It causes *pmrCAB* overexpression and subsequently leads to the modification of LPS with phosphoethanolamine (PEtN). Moreover, this modification leads to the decreased negative charge of lipid A and eventually reduces colistin affinity to LPS [7–11].

Moreover, another ColR mechanism in *A. baumannii* is the loss of LPS core or complete loss of lipid A related to the inactivation of LPS biosynthesis genes such as *lpxC*, *lpxA*, and *lpxD* [1, 12]. Besides, in recent years the plasmid mediated colistin resistance gene, *mcr*, was identified as another mechanism of colistin resistance isolates from animals and humans [13]. In this regard, the current study was designed to investigate the possible molecular mechanism of ColR *A. baumannii* clinical isolates collected from two hospitals in Tehran.

MATERIALS AND METHODS

Bacterial isolates

A total of 110 *A. baumannii* clinical isolates were collected from two hospitals in Tehran between August 2018 and September 2019. The isolates recovered from different clinical specimens such as a wound, bronchoalveolar lavage (BAL), tracheal aspirate, ascites, abscess, sputum, and blood. The isolates were identified using conventional biochemical tests, and amplification 16S rRNA and $bla_{\text{OXA-51-like}}$ carbapenemase genes by the polymerase chain reaction (PCR) using primers, as listed in Table 1 [14].

Antimicrobial susceptibility testing

The BD (New Jersey, USA) and Mast (Liverpool, UK) antibiotic disks were used to determine susceptibility to colistin and other antimicrobials agents using the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. First, screening was performed to detect MDR isolates using the colistin (10 µg), imipenem (10 µg), cefepime (30 µg), and piperacillin/tazobactam (100/10 µg). The MDR phenotype was defined based on the International Expert proposal for the Interim Standards Guidelines [16]. Furthermore, the susceptibility of colistin-resistance (ColR) isolates to ciprofloxacin (5 µg), cefotaxime (30 µg), gentamicin (10 µg), trimethoprim/sulfamethoxazole (25 µg), cefixime (5 µg), and tetracycline (30 µg) was evaluated.

The minimum inhibitory concentration (MIC) of colistin

The MIC of colistin was determined by colistin sulfate (Sigma-Aldrich) using the microdilution method according to Wiegand et al. and the CLSI guidelines. Isolates with MIC >4 µg/mL were categorized as resistance following the CLSI guidelines [15, 17, 18]. Furthermore, *A. baumannii* ATCC 19606 was used as a standard control for antimicrobial susceptibility testing.

The molecular characterization of ColR isolates by PCR

DNA was extracted by the boiling method using Tris-EDTA buffer as previously described to investigate the leading cause of ColR [19]. To detect the possible role of chromosomal mutations in ColR isolates, the *pmrA*, *pmrB*, *lpxA*, *lpxC*, and *lpxD* genes were amplified by PCR and the amplicons were analyzed by electrophoresis on 1% gel agarose containing safe stain (Yekta Tajhiz Azma, Iran). Furthermore, the amplicons were sent to the Bioneer Company (Seoul, South Korea) for DNA sequencing. The used primers are listed in Table 1.

The *pmrC* gene transcriptional analysis by reverse transcription PCR (RT-PCR)

To investigate the possible association of *pmrCAB* operon upregulation with colistin resistance, the expression level of the *pmrC* gene was evaluated [14]. All ColR isolates and *A.*

Table 1. Nucleotide sequences of primers used in this study

Primer name	Sequence (5′ to 3′)	Size of product (bp)	Reference
$bla_{\text{OXA-51-like}}$-F	TAATGCTTTGATCGGCCTTG	353	[21]
$bla_{\text{OXA-51-like}}$-R	TGGATTGCACTTCATCTTGG		
pmrA-F	ACTGGACATGTTGCACTCTTGT	757	[14]
pmrA-R	ATGCACTTTTATGAAGTCCCGA		
pmrB-F	TCGGGACTTCATAAAAGTGCAT	1,336	[14]
pmrB-R	GCGATTTGTATTCATCGTTTTG		
lpxA-F	GCTAGCGTTGACGGCATTGT	857	[14]
lpxA-R	TTAGCGCACAATTCCACGCT		
lpxC-F	GCGTTCTTCGCCAATGAGTA	1,217	[14]
lpxC-R	CGACCCTAAGCTTAGCCAA		
lpxD-F	GACTGTTGCCTATGACGCTA	1,171	[14]
lpxD-R	GGTGTAGTAGACTCGGTCAT		
pmrC-F	CCATTTGGCTAGGTGCAATTT	132	[14]
pmrC-R	CCGCATAATAGGTAGCAACAAG		
16S_rRNA-F	CAGCTCGTGTCGTGAGATGT	150	[22]
16S_rRNA-R	CGTAAGGGCCATGATGACTT		

baumannii ATCC 19606 as colistin-susceptible strain were cultured in tryptic soy broth medium (Conda lab, Spain) until the middle of the logarithmic phase. Then, total RNA was extracted by the SinaPure TM RNA extraction kit (Sinaclone, Iran). Genomic DNA was removed using DNaseI, RNase-free enzyme (Sinaclone, Iran), and the product purification was performed based on the manufacturer's instructions. The first-strand cDNA synthesis was performed using the PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, China). Real-time PCR amplification was performed by SYBR Green Master Mix (Ampliqon, Denmark) with Rotor-Gene Q (QIAGEN, Germany) in the following thermo cycling program: 95 °C for 12 min, 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s. Notably, the melting curve was analyzed to assure single amplicon produce at the end of each run. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ formula with the 16s rRNA gene as an internal control [20].

GENBANK ACCESSION NUMBER

The nucleotide sequences of the altered *pmrB*, *pmrA*, *lpxC*, *lpxD*, and *lpxA* genes were deposited in the GenBank database under the accession numbers of MW036524 and MW557307 to MW557310.

RESULTS

The molecular profiles of the colistin resistance isolates

Out of the 110 isolates, three (2.72%) ColR *A. baumannii* were detected, and based on $bla_{\text{OXA-51-like}}$ and 16S rRNA, all the ColR isolates were confirmed as *A. baumannii*. The MIC range was between 8 and 16 µg/mL, and all the ColR isolates were resistant to imipenem, cefepime, piperacillin/tazobactam, ciprofloxacin, cefotaxime, gentamicin, trimethoprim/sulfamethoxazole, cefixime, and tetracycline. Moreover, all the ColR isolates had GGT > GAT (G260D) mutation in the *pmrB* gene. One mutation, D10E (GAT > GAG), detected in *pmrA* gene in X9 isolate. Furthermore, C120R (TGT > CGT) mutation in *lpxC*, E117K (GAA > AAA) mutation in *lpxD*, and Y131H (TAT > CAT) mutation in *lpxA* were detected by Sanger sequencing in all the ColR isolates (Table 2).

The overexpression of *pmrCAB* operon

There was an association between resistance to colistin and the overexpression of the *pmrCAB* operon. The transcription level of *pmrC* raised 5.24 to 31.55 fold in all the ColR *A. baumannii* isolates compared to the susceptible isolate.

DISCUSSION

Since the first report of ColR *Acinetobacter* strains back in 1999 in the Czech Republic, the number of reports about this issue have increased year by year [23, 24]. However, colistin and tigecycline are usually considered as few remaining antimicrobial agents with activity against XDR *A. baumannii* [14, 25]. Here, we identified three ColR *A. baumannii* isolates (2.72% resistance rate), which is similar to the result of a recent meta-analysis study that reported a 4.8% resistance rate from Iran [26]. Moreover, all the ColR isolates showed total resistance against all the tested antibiotics and one type of mutation in each of *pmrB*, *pmrA*, *lpxC*, *lpxD* and *lpxA* genes.

Similarly, a recent study reported three ColR *A. baumannii* isolates collected from the Imam Khomeini Hospital in Tehran with the same mutation profile in the *lpxC* (C120R), *lpxA* (Y131H), and *lpxD* (E117K) genes [14]. Another study from Iran reported nine colistin-heteroresistant among 44 carbapenem-resistant *A. baumannii* isolates with the MIC of 6–8 µg/mL [27]. Furthermore, in another study, the authors demonstrated that mutation in genes involved in encoding the two-component signaling proteins PmrA and PmrB were associated with resistance to colistin in *A. baumannii* [7].

We found a mutation in the *pmrB* and *pmrA* genes in the ColR isolates. Moreover, *pmrC* overexpression was observed in all the resistant isolates. These results are in agreement with Beceiro et al. study, in which ColR in *A. baumannii* occurred following the three genetic events, I) one point mutation in pmrB at least, II) the upregulation of the *pmrAB* system, and III) the addition of PEtN to lipid A, following the expression of *pmrC* [28]. It had been implicated that the upregulation of the PmrAB two-component system is observed in ColR isolates, however, amino acid substitution doesn't seem essential for resistance to colistin [29]. Furthermore, it has been demonstrated that mutation in LPS biosynthesis genes such as *lpxC*, *lpxA*, and *lpxD* leads to susceptibility to other antimicrobial agents. The reason is that the outer membrane usually is the main obstacle in the

Table 2. Characterization of the ColR *Acinetobacter baumannii* collected in present study

Isolate	Hospital and year	Ward, and source of isolation	Colistin MIC (µg/mL)	Relative expression level of *pmrC*
X3	Hospital A/2018	Abscess/General surgery	8	31.55 ± 2.1
X9	Hospital B/2018	Tracheal aspirate/ICU	16	5.24 ± 3.04
X10	Hospital A/2018	Tracheal aspirate/ICU	8	8.31 ± 0.1

ICU: Intensive care unit.

successful penetration of many antibiotics, like a *lpxA* mutant strain *A. baumannii*, showing increased sensitivity to other antimicrobial agents [1]. By the way, because of absolute resistance to the investigated antibiotics in ColR isolates, it is unlikely that the loss of LPS is the main reason for resistance to colistin.

In summary, it appears that the upregulation of the PmrAB two-component system had an essential role in developing ColR in *A. baumannii* isolates in the current study, and the alternation in the LPS biosynthesis genes was in a subsidiary position. Moreover, the overexpression of the *pmrCAB* operon led to the addition of positively charged PEtN to LPS and eventually, it reduced colistin affinity and the creation of colistin resistance phenotype in the studied *A. baumannii* isolates.

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