



Research Paper

Detection of Colistin-resistant Genes in Gram-negative
Bacilli Isolated From Patients With COVID-19Razieh Jahandar¹, Laleh Hoveida^{1*}, Soodabeh Rostami²

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ABSTRACT

Introduction: Recent reports have highlighted bacterial coinfections alongside COVID-19, increasing mortality rates. The emergence of high resistance to carbapenems and colistin mediated by mobile genetic elements poses a severe public health concern.**Materials & Methods:** In this cross-sectional study, 74 gram-negative bacterial isolates were collected from tracheal samples of COVID-19 patients admitted to Al-Zahra Hospital, Isfahan, Iran. Bacterial identification was performed using biochemical tests, and antibiotic susceptibility was determined by the Kirby-Bauer method. Colistin minimum inhibitory concentrations (MICs) were assessed by broth microdilution. The presence of *mcr-1*, *mcr-2*, *mcr-3*, and *pmrAB* genes was detected via polymerase chain reaction (PCR). Clinical isolates were obtained from COVID-19 patients admitted to the intensive care unit (ICU) (n=23), internal unit (n=23), surgical unit (n=10), and from other units (n=18).**Results:** The predominant isolates were *Acinetobacter* spp. (70%), *Klebsiella pneumoniae* (16%), *Pseudomonas aeruginosa* (7%), and *Escherichia coli* (4%). The highest resistance was observed against ampicillin (94.6%), while gentamicin and ceftazidime exhibited the lowest resistance (74.3%). Among all isolates, 31(41.9%) had MICs ≥ 4 , indicating resistance to colistin. Additionally, 20% of the isolates harbored the *pmrAB* gene, while none possessed *mcr-1*, *mcr-2*, or *mcr-3* genes.**Conclusion:** Since colistin is one of the last choices for treating severe infections, the high prevalence of colistin-resistant bacteria in this study, coupled with the detection of *pmrAB*, underscores the urgent need for continuous surveillance of colistin resistance mechanisms to inform effective clinical management and infection control strategies in COVID-19 patients. Although no horizontal transfer of resistance genes was found in this study, hospital infection control systems should routinely scan Enterobacteriaceae and non-fermentative Gram-negative bacteria, especially *Acinetobacter* spp., for colistin resistance and its mechanisms of action.

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1. Introduction

Respiratory-associated coinfections are a significant contributor to the mortality of hospitalized COVID-19 patients [1]. Factors such as prolonged intubation, extensive catheter use, and compromised immune systems in patients with respiratory complications elevate the risk of secondary bacterial and fungal infections. Consequently, antimicrobial agents were excessively utilized in critical care settings, resulting in emerging drug-resistant pathogens [2]. Infection caused by multidrug-resistant (MDR) pathogens is now a worldwide issue, considering the wide distribution of MDR isolates. SARS-CoV-2 mutations, cytokine storms following the immune response to infection, comorbidities, and the immunogenetic condition of COVID-19 patients render these patients vulnerable to secondary infections [3]. A high rate of morbidity and mortality was associated with bacterial coinfections in COVID-19 cases [4].

The surge in antimicrobial resistance among COVID-19 patients predominantly stems from the dissemination of high-risk clones, particularly gram-negative bacteria including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. [4]. Gram-negative bacteria have exhibited antibiotic resistance due to broad-spectrum beta-lactamases [5]. The widespread antibiotic resistance to first- and second-line antibiotics, such as cephalosporin resistance observed in hospital-associated infections caused by *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*, particularly carbapenemase-producing strains, presents a significant healthcare challenge [6].

Bacterial resistance leads to various complications, including urinary tract infections, septicemia, pneumonia, and intra-abdominal infections, affecting patients across different hospital departments. The emergence of high resistance to carbapenems and colistin mediated by mobile genetic elements poses a severe public health concern [7]. The *mcr* and *pmr* genes confer colistin resistance and are significantly expressed in colistin-resistant isolates [8]. Colistin is used as a last-resort antibiotic to treat infections caused by MDR gram-negative bacteria, but bacteria can develop resistance to it through various mechanisms including modification of lipopolysaccharide (LPS) structure, alteration of outer membrane proteins, activation of efflux pumps, mutation in regulatory genes, and horizontal transfer of resistance genes such as *mcr* genes. These resistance mechanisms reduce colistin's efficacy, highlighting the urgent need for prudent antibiotic use, infection control measures, and ongoing

research into alternative treatment strategies to address the public health threat posed by colistin-resistant bacteria [9]. Given the critical importance of colistin as a last-resort antibiotic and the escalating threat of resistance, this study aimed to determine the prevalence of colistin resistance, both phenotypically and genotypically, and to identify the associated *mcr-1*, *mcr-2*, *mcr-3*, and *pmrAB* genes among Gram-negative bacterial isolates from hospitalized COVID-19 patients in Isfahan, Iran.

2. Materials and Methods

2.1. Study design

This study was conducted on 74 hospitalized patients with COVID-19 who were confirmed by a positive reverse transcription polymerase chain reaction (RT-PCR) test or the presence of ground glass opacity on a computed tomography (CT) scan from different units (intensive care unit (ICU), internal, and surgical) at [Al-Zahra Hospital](#), Isfahan, Iran, in 2022. Data on the age and gender of the patients were recorded from their medical archives. All patients gave their consent to participate in the study. The [Islamic Azad University](#) Ethics Committee confirmed the study.

2.2. Bacterial isolation and identification

Seventy-four bacterial isolates from the trachea of COVID-19 patients were obtained and cultured. Identification of isolates was confirmed using biochemical tests, including TSI, Urease, Oxidase, SIM, MRP, O/F, DNase, and Simmons citrate.

2.3. Antibiotic susceptibility tests

According to the Clinical and Laboratory Standards Institute (CLSI-M100-2021) [10], the antibiotic susceptibility test followed the Kirby-Bauer protocol. The antibiotics tested (Padtan Teb Co., Iran) were cefepime (30 µg), amoxicillin-clavulanic acid (20/10 µg), ampicillin (10 µg), levofloxacin (5 µg), cotrimoxazole (1.25/23.75 µg), amikacin (30 µg), ceftazidime (30 µg), imipenem (10 µg), gentamicin (10 µg), tazobactam (10 µg), meropenem (10 µg), and ciprofloxacin (5 µg).

Colistin stock with a volume of 1 mL, a concentration of 5120 µg/mL, and a potency of 980 µg/mg was prepared to determine the minimum inhibitory concentration (MIC) of colistin through the microdilution method, according to CLSI M07-A10 [11]. Different concentrations of colistin were applied (0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 µg/mL). According to the CLSI

protocol [11], colistin resistance thresholds for Enterobacteriaceae and non-fermenting gram-negative bacilli were as follows: MIC ≤ 2 $\mu\text{g/mL}$ was considered susceptible, and MIC ≥ 4 $\mu\text{g/mL}$ was considered resistant. All tests were performed in triplicate to ensure reproducibility of results.

2.4. Detection of colistin resistance genes

Bacterial DNA was extracted using Sina Gene kit (Sina Gene Co., Iran) following the manufacturer's instructions, and the quantity of extracted DNA was measured using a NanoDrop. To evaluate the colistin resistance genes *mcr-1*, *mcr-2*, *mcr-3*, and *pmrAB*, a PCR test was performed using previously designed primers (Sina Colon Co., Iran) [12] (Table 1). Detection of *mcr-1*, *mcr-2*, *mcr-3* genes was performed using 12.5 μL of PCR master mix (Sina Gene Co., Iran) and a PCR thermocycler (Eppendorf, Germany) according to the following procedure: one cycle of initial denaturation at 95 °C for 3 min; 30 cycles including 20 sec of denaturation at 94 °C, 15 sec of annealing at 54 °C (*mcr-1*), 58 °C (*mcr-2*), and 50 °C (*mcr-3*), and 15 sec of extension at 72 °C; and one cycle for the final extension at 72 °C for 1 min. Expression of the *pmr* genes was evaluated by PCR with one cycle at 95 °C for 3 min for initial denaturation; 30 cycles for denaturation (30 sec at 94 °C), annealing (30 sec at 54 °C), and extension (45 sec at 72 °C); and one cycle for 1 min at 72 °C for the final extension. The quality of the final PCR product was confirmed by the presence of a sharp band on the gel following electrophoresis.

2.5. Statistical analysis

All data were analyzed using SPSS version 20 and reported as numbers, percentages, and Mean $\pm$ SD. An-

tibiogram results were analyzed using WHONET V.5.6 software. Sequence confirmation was performed by MacroGen (MacroGen, Inc., Korea), and the results were analyzed using the National Center for Biotechnology Information (NCBI) database, Chromas, Mega4, and GeneRunner software.

3. Results

3.1. Studied population and bacterial isolates

The mean age of COVID-19 patients was 53.08 $\pm$ 26.12 years (1-92 years), and 41 of the 74 isolates were collected from males (55.4%). About 31% (n=23) of patients were admitted to the ICU, 31% (n=23) to the internal unit, 13.5% (n=10) to the surgical unit, and 24% (n=18) were in other units. Among gram-negative bacilli, 80% belonged to the family of non-fermenting gram-negative bacilli, including 52(70%) isolates of *Acinetobacter* spp., 5(7%) isolates of *P. aeruginosa*, 1(1.5%) isolate of *Achromobacter denitrificans*, and 1(1.5%) isolate of *Stenotrophomonas maltophilia*. About 20% of isolates belonged to the Enterobacteriaceae, including 12(16%) isolates of *K. pneumonia* and 3(4%) isolates of *E. coli*.

3.2. Antibiotic susceptibility tests

Gram-negative bacilli showed the highest resistance to ampicillin with a frequency of 94.6% and the lowest resistance to gentamicin and ceftazidime with a frequency of 74.3%. *Acinetobacter* spp. isolates demonstrated the highest resistance to amoxicillin-clavulanic acid (98.1%), meropenem (94.5%), ampicillin, and cefepime (92.6%) and were sensitive to ceftazidime. *P. aeruginosa* isolates showed high resistance to ampicillin (100%) and were sensitive to gentamicin, amikacin, and ceftazi-

Table 1. Primer sequences used in this study to identify the colistin resistance genes *mcr-1*, *mcr-2*, *mcr-3*, and *pmrAB*

Genes	Name of Primers	Oligonucleotide Sequences	Size of the Amplified Products	Ref.
<i>mcr-1</i>	mcr-1-F	5'-CTTGGTCGGTCTGTAGGG-3'	309 bp	[12]
	mcr-1-R	5'-CGGTACAGTCCGTTTGTTTC-3'		
<i>mcr-2</i>	mcr-2-F	5'-AGATGGTATTGTTGGTTGCTG-3'	215 bp	[12]
	mcr-2-R	5'-TGTTGCTTGTCGCCGATTGGA-3'		
<i>mcr-3</i>	mcr-3-F	5'-TTAACGAAATTGGCTGGAACA-3'	732 bp	[12]
	mcr-3-R	5'-TTGGCACTGTATTTGCATTT-3'		
<i>pmrA&B</i>	pmrAB-F	5'-CATTTCCGCGCA CTG TCT GC-3'	808 bp	[12]
	pmrAB-R	5'-CAG CTT TCA GTT GCA AAC AG-3'		

dime. Among *E. coli* isolates, the highest resistance was observed to ampicillin, trimethoprim-sulfamethoxazole, and amoxicillin-clavulanic acid (100%), and the lowest resistance to levofloxacin, piperacillin-tazobactam, amikacin, and gentamicin was observed. *K. pneumoniae* isolates were resistant to ampicillin (100%) and sensitive to amikacin, gentamicin, and meropenem (Table 2).

The mean MIC for colistin was 13.7 µg/mL. According to our findings, 20(64.5%) isolates of *Acinetobacter* spp., 6(19.3%) isolates of *K. pneumoniae*, 4(12.9%) isolates of *P. aeruginosa*, and 1(2.3%) isolate of *A. denitrificans* were resistant to colistin (MIC ≥4 µg/mL), while no isolates of *E. coli* or *S. maltophilia* showed resistance to colistin (Table 3).

3.3. Molecular detection

Genotyping results showed that 20% (n=6) of the isolates had the *pmrAB* gene, and none of the gram-negative bacillus isolates had *mcr-1*, *mcr-2*, or *mcr-3* genes. Among the isolates resistant to colistin, 8(26.6%) isolates expressed *pmrA* (five isolates were *Acinetobacter* spp., and three isolates were *P. aeruginosa*), and *pmrB* was detected in six (20%) isolates (four isolates were

Acinetobacter spp., and two isolates were *P. aeruginosa*). None of the *K. pneumoniae* and *E. coli* isolates demonstrated any of the colistin resistance genes. A total of 10 isolates with high resistance to colistin and a sharp band on gel electrophoresis of PCR products of the *pmrAB* gene were sequenced and confirmed by Gene Fanavaran company (FNM Co., Iran).

4. Discussion

The emergence of the COVID-19 pandemic has brought unprecedented challenges to global healthcare systems, with a profound impact on patient management and treatment strategies. Among the numerous complications associated with COVID-19, secondary antibiotic-resistant infections have emerged as significant clinical concerns, particularly among hospitalized patients [13, 14]. Colistin, a last-resort antibiotic, has been increasingly relied upon for managing MDR bacterial infections. However, reports of colistin-resistant bacteria isolated from the trachea of COVID-19 patients underscore the urgency of addressing antimicrobial resistance in this global health crisis [14, 15].

Table 2. Frequency of antibiotic sensitivity and resistance patterns according to gram-negative bacillus isolates in patients with COVID-19

Class of Antibiotics	Antibiotics	%											
		<i>Acinetobacter</i> spp.			<i>K. pneumoniae</i>			<i>E. coli</i>			<i>P. aeruginosa</i>		
		R	I	S	R	I	S	R	I	S	R	I	S
Penicillin	Ampicillin	92.63	0.00	7.40	100	0.00	0.00	100	0.00	0.00	100	0.00	0.00
	Amikacin	84.93	0.00	15.11	50.00	0.00	50.00	33.40	0.00	66.60	60.00	0.00	40.00
Aminoglycosides	Gentamicin	83.43	0.00	16.60	50.00	0.00	50.00	33.34	0.00	66.60	60.00	0.00	40.00
Carbapenems	Imipenem	94.53	0.00	5.50	50.00	0.00	50.00	66.60	0.00	33.40	80.00	0.00	20.00
	Meropenem	83.35	1.85	14.80	50.10	8.30	41.60	66.60	0.00	66.40	80.00	0.00	20.00
Cephems	Cefepime	92.65	1.85	5.50	66.70	8.30	25.00	66.60	0.00	33.40	40.00	20.00	40.00
	Ceftazidime	81.53	0.00	18.50	50.10	16.60	33.30	33.40	33.30	33.30	80.00	0.00	20.00
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	85.23	0.00	14.80	75.00	0.00	25.00	100	0.00	0.00	80.00	0.00	20.00
B-Lactam combination agents	Amoxicillin-clavulanic acid	98.15	0.00	1.85	75.00	0.00	25.00	100	0.00	0.00	80.00	0.00	20.00
	Piperacillin-tazobactam	87.04	0.00	1.96	75.00	0.00	25.00	33.40	0.00	66.60	80.00	0.00	20.00
Quinolones	Ciprofloxacin	83.40	0.00	16.60	66.70	0.00	33.30	66.60	0.00	33.40	80.00	0.00	20.00
	Levofloxacin	83.40	0.00	16.60	58.40	0.00	41.60	33.40	0.00	66.60	80.00	0.00	20.00

Abbreviations: R: Resistant; I: Intermediate; S: Sensitive.

Table 3. The MIC pattern of susceptibility to colistin among Enterobacteriaceae and non-fermenting gram-negative bacteria isolated from patients with COVID-19

Bacterial Isolates	Numbers of Isolates	No. (%)		MIC (µg/mL)									
		Colistin Resistance											
		Yes	No	256	128	64	32	16	8	4	2	1	0.5
<i>Acinetobacter</i> spp.	52	20(64.5)	32(74.5)	2	1	0	0	5	7	5	7	15	10
<i>P. aeruginosa</i>	5	4(12.9)	1(2.3)	0	0	0	1	2	0	1	0	1	0
<i>K. pneumonia</i>	12	6(19.3)	6(13.9)	0	0	1	0	0	2	3	5	1	0
<i>Achromobacter denitrificans</i>	1	1(3.2)	0(0)	0	0	0	0	0	1	0	0	0	0
<i>E. coli</i>	3	0(0)	3(7)	0	0	0	0	0	0	0	2	1	0
<i>S. maltophilia</i>	1	0(0)	1(2.3)	0	0	0	0	0	0	0	1	0	0

Abbreviations: N: Number; MIC: Minimum inhibitory concentration.

The most detected isolates from the trachea were from ICU patients, of which *Acinetobacter* spp. and *P. aeruginosa* from non-fermenting gram-negative bacilli and *K. pneumoniae* from Enterobacteriaceae had the highest prevalence among detected isolates. Viral respiratory infections, including COVID-19 and influenza, disrupt the host's innate and adaptive immune defenses, leading to secondary infections. These secondary infections are often linked to more severe outcomes, particularly in debilitated patients with underlying comorbidities [16]. Costa et al. reported a high prevalence of bacterial infection associated with ventilator and tracheitis among hospitalized COVID-19 patients in the ICU. Among those with secondary infections after hospitalization (29.8%), *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* were more prevalent than others. Over half of the *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae* isolates were MDR [17]. Shah et al. demonstrated a high prevalence of bacterial infection in the respiratory system, bloodstream, and other sterile body sites. The most isolated bacteria from the respiratory system were *P. aeruginosa*, *K. pneumoniae*, and *E. coli*. They reported an increased mortality rate in COVID-19 patients with bacterial infections [18].

We observed that most *Acinetobacter* spp., *P. aeruginosa*, *K. pneumoniae*, and *E. coli* isolates were resistant to ampicillin. Musuza et al. found that 24% of COVID-19 patients had coinfections with gram-negative bacteria, a factor significantly associated with adverse outcomes such as prolonged hospitalization or increased mortality [19]. Studies reported a high prevalence of individuals with COVID-19 receiving antibiotic treatment, including broad-spectrum regimens, without conclusive evidence of secondary bacterial infection [20], which resulted in

emerging MDR isolates. In the study by Ahmed et al., *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, and gram-positive bacteria were more prevalent among isolates from COVID-19 patients, respectively. They reported that most *E. coli* and *K. pneumoniae* isolates were resistant to ampicillin, while *P. aeruginosa* isolates were resistant to ciprofloxacin, and *A. baumannii* isolates represented a wide spectrum of resistance to amikacin, ciprofloxacin, ceftazidime, levofloxacin, cotrimoxazole, piperacillin-tazobactam, and tetracycline [21]. Another study by Pourajam et al. reported that about 10% of respiratory samples from COVID-19 patients demonstrated bacterial infections. All *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* isolates were resistant to ciprofloxacin, and *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates showed a high resistance to ampicillin [22].

The emergence of highly resistant strains significantly challenges the management of gram-negative bacterial infections, and colistin has become one of the main treatment options, particularly for nosocomial infections [23]. In the current study, *Acinetobacter* spp. illustrated a high resistance (64.5%) to colistin, followed by *K. pneumoniae* (19.3%), *P. aeruginosa* (12.9%), and *Achromobacter denitrificans*, respectively, while *E. coli* and *Stenotrophomonas maltophilia* were sensitive to colistin. Studies reported various rates of gram-negative bacteria resistance to colistin. Colistin-resistant isolates were highly prevalent in Asia and Europe, from 0.2% to 17.5% [24]. The variation in reported findings could stem from variances in geographic regions, methodologies for studying resistance, diversity in sample types, sample size, patient health statuses, antibiotic prescription practices, and adherence to infection control protocols.

Moosavian et al. reported that 13.6% of Enterobacteriaceae isolates were resistant to colistin with MIC values $>2 \mu\text{g/mL}$. Among these *E. coli* and *K. pneumoniae* isolates, about 1.7% of them expressed the *mcr-1* gene [25]. Among the *mcr-1*, *mcr-2*, *mcr-3*, and *pmrAB* resistance genes, we observed that 20% of the isolates carried the *pmrAB* gene, with the majority being *A. baumannii* isolates, followed by *P. aeruginosa*. Rout et al. reported that among *A. baumannii* strains isolated from hospital infections, 5.9% of isolates were resistant to colistin, and these isolates expressed *pmrA* and *pmrB* genes [26]. Osama et al. demonstrated that among 30 carbapenem-resistant isolates, five isolates were resistant to colistin. The results of genotyping for *mcr-1*, *pmrB*, and *pmrA* genes showed that one isolate carried the *pmrA* gene, one isolate had *mcr-1*, *pmrA*, and *pmrB* genes, while three isolates carried *pmrA* and *pmrB* genes [27].

Since we did not find any *mcr* genes among the isolates, the resistance to colistin in these isolates may be caused by other mutations, other bacterial resistance mechanisms, and resistance mechanisms associated with the *pmrAB* efflux pump. The lower resistance to aminoglycoside antibiotics in these samples suggested that they could serve as viable alternatives to beta-lactam antibiotics. The study's strengths lie in providing a comprehensive perspective on colistin-resistant gram-negative bacterial isolates, antibiotic resistance patterns, and associated genes. However, limitations include the potential impact of the study's sample size on generalizability, its single-center design limiting broader applicability, and possible biases in sample collection and patient selection. While genotyping provides molecular insights, its coverage may not encompass all resistance mechanisms, and the absence of comparison groups hinders contextualization within broader epidemiological trends.

5. Conclusion

This study highlighted the prevalence of antibiotic resistance among gram-negative bacilli, emphasizing the need for careful antibiotic prescription. While certain antibiotics showed lower resistance rates, significant proportions of isolates, notably *Acinetobacter* spp., exhibited resistance to colistin. Continuous research is essential to address these challenges and develop effective treatment strategies.

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Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of Falavarjan Branch, Islamic Azad University, Isfahan, Iran (Code: IR.IAU.FALA.REC.1401.006).

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Authors' contributions

Conceptualization, study design, and writing the original draft: Laleh Hoveida; Review and editing: Soodabeh Rostami; Statistical analysis, data acquisition, experiments, data interpretation, supervision, administrative, technical, and material support: All authors.

Conflict of interest

The authors declared no conflict of interest.

Data availability

Data are available upon request from the author.

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