Detection of Colistin resistant genes in Gram-negative bacilli isolated from patients with COVID-19

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Abstract

Recent reports have highlighted bacterial coinfections alongside COVID-19, increasing mortality rates. The emergence of high resistance to carbapenems and colistin within mobile genetic elements poses a severe public health concern. 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 *pmr*AB genes was detected via polymerase chain reaction (PCR). Clinical isolates were obtained from COVID-19 patients admitted to intensive care unit (ICU) (n=23), internal unit (n=23), surgical unit (n=10), and from other units (n=18). The predominant isolates were *Acinetobacter* spp (70%), *Klebsiella pneumoniae* (*K. pneumoniae*) (16%), *Pseudomonas aeruginosa* (*P. aeruginosa*) (7%), and *Escherichia coli* (*E. 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 MIC ≥4, indicating resistance to colistin. Additionally, 20% of the isolates harbored the *pmr*AB gene, while none possessed *mcr*-1, *mcr*-2,

or *mcr*-3 genes. Since colistin is one of the last choices for treating sever infections, the high prevalence of colistin-resistant bacteria in this study, coupled with the detection of *pmr*AB, underscores the urgent need for continuous surveillance of colistin resistance mechanisms to inform effective clinical management and infection control strategies in COVID-19 patient. Although no horizontal transfer of resistance genes was found in this study, hospital infection control system should routinely scan Enterobacteriaceae and non-fermentative Gram-negative bacteria, especially *Acinetobacter* spp, for colistin resistance and its mechanisms of action.

Keywords: Gram-negative bacteria, Colistin resistance, COVID-19, Enterobacteriaceae

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) is now a worldwide issue, considering the wide distribution of MDR isolates. SARS-CoV-2 mutations, cytokine storm following immune response to infection, comorbidities, and immunogenetic condition of COVID-19 patients vulnerable these patients 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* (*A. baumannii*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Pseudomonas aeruginosa* (*P. 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 within 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 multidrug-resistant 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 *pmr*AB genes among Gram-negative bacterial isolates from hospitalized COVID-19 patients in Isfahan, Iran.

2. Methods and materials

2.1.Study Design

This study was conducted on 74 hospitalized patients with COVID-19 that were confirmed by a positive RT-PCR test or presence of ground glass opacity in the CT-scan from different units (intensive care unit (ICU), internal, and surgical) in 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 [IR.IAU.FALA.REC.1401.006].

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 Simon citrate.

2.3.Antibiotic Susceptibility Tests

According to the Clinical and Laboratory Standards Institute (CLSI-M100-2021), the antibiotic susceptibility test followed the Kirby-Bauer protocol. Administrated antibiotics (Padtan Teb Co., Iran) were

cefepime (30µg), amoxicillin-clavulanic acid (20/10µg), ampicillin (10µg), levofloxacin (5µg), cotrimoxazole (1.25/23.75µg), amikacin (30µg), ceftazidime (30µg), imipenem (10µg), gentamicin (10µg), tazobactam (10µg), meropenem (10µg), and ciprofloxacin (5µg).

Colistin stock with a volume of 1 mL, a concentration of 5120 µg/mL, and a 980 µg/mg potency was prepared to find the desirable minimum inhibitory concentration (MIC) for colistin through the microdilution method, according to the CLSI M07-A10. Different concentrations of colistin were applied (0.5, 1, 2, 4, 8, 16, 32, 64, 18, and 256 µg/mL). According to the CLSI protocol, colistin resistance thresholds for Enterobacteriaceae and non-fermenting Gram-negative bacilli were as follows: MIC ≤2 µg/mL were considered intermediate resistance, and MIC ≥4 µg/mL were 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 NanoDrop (22,23). To evaluate colistin resistance genes *mcr-1, mcr-2, mcr-3,* and *pmrAB*, PCR test was performed using previously designed primers (Sina Colon Co., Iran) (10) (Table 1). *mcr-1, mcr-2, mcr-3* genes detection using 12.5 µl master mix (Sina Gene Co., Iran) PCR (Eppenorf, Australia) was performed following procedure: one cycle for initial denaturation at 95°C for 3 min; 30 cycles included 20 sec for denaturation at 94°C, 15 sec annealing at 54°C (*mcr-1*), 58°C (*mcr-2*), and 50°C (*mcr-3*), and extension for 15 sec at 72°C; and one cycle for the final extension at 72°C for one min. *pmr* genes expression was evaluated by PCR for 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 7°C); and one cycle for one min at 72°C for the final extension. The quality of the final PCR product was confirmed due to the formation of a sharp band produced by the gel through gel electrophoresis.

Table 1. Primer sequences that are used in this study to identify colistin resistance genes *mcr1, mcr2, mcr3,* and *pmrAB*

Genes	Name of Primers	Oligonucleotide sequences	Size of the amplified products	References
***mcr*-1**	mcr-1-F	5'-CTTGGTCGGTCTGTAGGG-3'	309 bp	10
	mcr-1-R	5'-CGGTCAGTCCGTTTGTTC-3'		
***mcr*-2**	mcr-2-F	5'-AGATGGTATTGTTGGTTGCTG-3'	215 bp	10
	mcr-2-R	5'-TGTTGCTTGTGCCGATTGGA-3'		
***mcr*-3**	mcr-3-F	5'-TTAACGAAATTGGCTGGAACA-3'	732 bp	10
	mcr-3-R	5'-TTGGCACTGTATTTTGCATTT-3'		
***pmr*A&B**	pmrAB-F	5'-CATTTCCGCGCA CTG TCT GC-3'	808 bp	10

	pmrAB-R	5'-CAG CTT TCA GTT GCA AAC AG-3'

2.5. Statistical analysis

All data was analyzed using SPSS version 20 and reported by number, percentages, and mean ± standard division (SD). Antibiogram results were analyzed using WHONET V.5.6 software. The sequence confirmation was performed by Macrogen (Macrogen, Inc. Korea), and the results were analyzed using the 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±26.12 years (1-92 years), and 41 of 74 isolates were collected from males (55.4%). About 31% (n=23) of patients were admitted to the ICU, 31.0% (n=23) in the internal unit, 13.5% (n=10) in the surgical unit, and 24.0% (n=18) were in other units. Among Gram-negative bacilli, 80% belonged to the family of non-fermenting Gram-negative bacilli, including 52 (70.0%) isolates of *Acinetobacter* spp., 5 (7.0%) isolates of *P. aeruginosa*, 1 (1.5%) isolate of *Achromobacter denitrificans*, and 1 (1.5%) were *Stenotrophomonas maltophilia*. About 20% of isolates belonged to the Enterobacteriaceae, including 12 (16.0%) isolates of *K. pneumonia* and 3 (4.0%) 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 represented a high resistance to ampicillin (100%) and were sensitive to gentamicin, amikacin, and ceftazidime. Among *E. coli* isolates, the highest resistance to ampicillin, trimethoprim-sulfamethoxazole, and amoxicillin-clavulanic acid (100%), and the lowest resistance to levofloxacin, piperacillin-tazobactam, amikacin, and gentamicin were reported. *K. pneumonia* isolates were resistant to ampicillin (100%) and sensitive to amikacin, gentamicin, and meropenem (Table 2).

153 Table 2. Frequency of antibiotic sensitivity and resistance pattern according to Gram-negative bacillus
154 isolates in patients with COVID-19.

155 R: resistance; I: Intermediate; S: Sensitive

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Class of Antibiotics	**Antibiotics**	***Acinetobacter* spp. %**			***K. pneumoniae* %**			***E. coli* %**			***P. aeruginosa* %**		
		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
Aminoglycosides	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
	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	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
Agents	Piperacillin-Tazo Bactam	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

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

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165 Table 3. The MIC pattern of sensitivity to the antibiotic colistin in Gram-negative bacteria of the
166 Enterobacteriaceae family and non-fermenting isolated from patients with COVID-19.

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Bacterial isolates	**Numbers of isolates**	**Colistin resistance n (%)**		**MIC (µg/mL)**									
		Yes	**No**	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**
Acinetobacter spp.	52	20 (64.5)	32 (74.5)	2	1	0	0	5	7	5	7	15	10
P. aeruginosa	5	4 (12.9)	1 (2.3)	0	0	0	1	2	0	1	0	1	0
K. pneumonia	12	6 (19.3)	6 (13.9)	0	0	1	0	0	2	3	5	1	0
Achromobacter denitrificans	1	1 (3.2)	0 (0.0)	0	0	0	0	0	1	0	0	0	0

E. coli	3	0 (0.0)	3 (7.0)	0	0	0	0	0	0	0	2	1	0
Stenotrophomonas maltophilia	1	0 (0.0)	1 (2.3)	0	0	0	0	0	0	0	1	0	0

Number: n, Minimum Inhibitory Concentration: MIC

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,* and *mcr-3* genes. Among the isolates resistant to colistin, in 8 (26.6%) isolates expressed *pmrA* (5 isolates were *Acinetobacter spp.*and three isolates were *P. aeruginosa*), and in six isolates (20.0%) was detected *pmrB* (4 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 number of 10 isolates with high resistance to colistin and sharp band in the 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 (11, 12). 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 (12,13).

The most detected isolates from the trachea were from ICU, of which *Acinetobacter spp.*and *P. aeruginosa* from non-fermenting Gram-negative bacilli and *K. pneumonia* from Enterobacteriaceae had the higher 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 (14). Costa et al. reported a high prevalence of bacterial infection from 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 (15). Shah et al.

demonstrated a high prevalence of bacterial infection in the respiratory system, bloodstream, and other sterile body parts. 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 (16).

We observed that most *Acinetobacter spp.*, *P. aeruginosa*, *K. pneumoniae,* and *E. coli* isolates were resistant to ampicillin. Musaza 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 (17). Studies reported a high prevalence of individuals with COVID-19 receiving antibiotic treatment, including broad-spectrum regimens, without conclusive evidence of secondary bacterial infection (18), 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 (19). 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* isolated showed a high resistance to ampicillin (20).

The emergence of highly resistant strains significantly challenges the management of Gram-negative bacterial infections, and colistin has become one of the considerable treatments, particularly for nosocomial infections (21). 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% (22). 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 μg/mL. Among these *E. coli* and *K. pneumoniae* isolates, about 1.7% of them expressed *mcr-1* gene (23). Among *mcr-1*, *mcr-2*, *mcr-3*, and *pmrAB* resistance genes, we observed that 20% of the isolates carried the *pmrAB* gene, with the majority of *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, in which they expressed *pmrA* and *pmrB* genes (24). Osama et al. demonstrated that among 30 carbapenems-resistant isolates, five isolates were resistant to colistin. The results of genotyping for *mcr-1*, *pmrB* and *pmrA* genes showed that one isolate carried *pmrA gene*, one isolate had *mcr-1*, *pmrA*, and *pmrB genes*, while three isolates carried *pmrA* and *pmrB* genes (25).

Since we did not find any *mcr* genes among isolates, the resistance to colistin in these isolates may be caused by other mutations, other bacterial resistance mechanisms, and resistance mechanisms associated with *pmrAB* efflux pomp. The lower resistance to aminoglycoside antibiotics in these samples suggested 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.

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.*, exhibit resistance to colistin. Continuous research is essential to address these challenges and develop effective treatment strategies.

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Author Contributions

Study concept and design: L.H

Acquisition of data: RJ, LH, SR,

Analysis and interpretation of data: LH, SR

Drafting of the manuscript: L.H

Critical revision of the manuscript for important intellectual content: SR

Statistical analysis: LH, SR

Administrative, technical, and material support: LH, SR

Study supervision: LH, SR

Ethics

The Islamic Azad University ethics committee confirmed the study [Code: IR.IAU.FALA.REC.1401.006].

Conflict of Interest

The authors declare that there is no conflict of interest.

Data Availability

Data is available by request to the author.

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