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Molecular characterization of colistin resistance in Gram negative isolates

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ABSTRACT

Background: The increasing prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria (GNB) presents a critical global health challenge. Colistin and polymyxin B have regained prominence as last-resort antibiotics against MDR/XDR infections. However, the overuse and misuse of colistin have led to rising resistance. Colistin resistance (COR) is primarily driven by chromosomal mutations and, more concerningly, by the emergence of plasmid-mediated *mcr* genes. Here, we attempted to screen *mcr* genes (*mcr*-1 through *mcr*-9) prevalence in COR isolates. **Methods:** This study, conducted at Cairo University hospitals, aimed to investigate the prevalence of mcr genes (mcr-1 to mcr-9) in colistin-resistant isolates. Out of 380 collected clinical samples, 100 MDR/XDR and PDR isolates were selected for analysis. Phenotypic (COR) was determined using the broth microdilution (BMD) method, while genotypic detection of mcr genes were performed by multiplex PCR. **Results:** Among the 100 isolates, 68% exhibited colistin resistance, predominantly found in ICU patients (75%). Klebsiella pneumoniae was the most common resistant organism (57.4%), followed by Pseudomonas species (23.5%) and Acinetobacter species (17.6%). Notably, *mcr*-4 was detected in 5.8% (4/68) of colistin-resistant isolates, with no detection of other *mcr* genes. The *mcr*-4 positive isolates were predominantly *Acinetobacter* species (75%) and *Klebsiella pneumonia* (25%), all from ICU patients with bloodstream infections (75%) and urinary tract infections (25%). **Conclusions**: The findings highlight the alarming prevalence of colistin resistance and the emergence of mcr-4 mediated resistance. These results underscore the urgent need for enhanced antimicrobial stewardship and ongoing surveillance to monitor resistance patterns and guide treatment strategies.

Introduction

The global incidence of infections due to multi-/extensively drug-resistant Gram-negative bacteria (MDR/XDR-GNB) has substantially increased, posing a significant public health threat. The Centers for Disease Control and Prevention indicate that these pathogens demonstrate resistance to most available antibiotics, leading to treatment failures and increased mortality rates **[1].**

The increasing prevalence of antimicrobial resistance in GNB, driven by the inappropriate overuse of antibiotics, has led to an increased focus on the therapeutic use of polymyxin antibiotics. Colistin and polymyxin B have recently garnered significant interest as the final therapeutic option, coinciding with increased antimicrobial resistance in clinical infections **[2].**

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Unfortunately, the excessive use of colistin in human and veterinary medicine due to limited therapeutic options for MDR/XDR GNB infections has driven increasing resistance rates in high-risk species such as *Escherichia coli*, *Klebsiella pneumoniae, and Acinetobacter baumannii*. This trend has precipitated refractory infections with significant clinical and socioeconomic ramifications **[3].**

Chromosomal gene mutations are the predominant mechanism of colistin resistance, inducing structural modifications to lipid A within the lipopolysaccharide (LPS) of bacterial cell walls. These alterations disrupt cationic interactions between colistin and LPS, effectively diminishing drug binding **[4,5].**

Prior to 2016, polymyxins represented one of the few antibiotic classes devoid of horizontally transferable resistance mechanisms. **Liu et al. (2016)** initially characterized the mcr-1 gene, identifying that the mcr-1 gene was located on a plasmid. Research has increasingly focused on the mechanisms underlying resistance to colistin **[6].** The propagation of colistin-resistant (CoR) genes is considered the primary contributing factor to escalating colistin resistance (CoR) rates.

Several mcr genes, specifically *mcr*-2 through *mcr*-10, have been identified recently, making colistin resistance more complicated **[7,8].** These *mcr* genes confer resistance by reducing colistin binding via the inhibition of negatively charged bacterial cell membranes. Few reports were delivered from Egypt on the molecular drivers of colistin resistance and were mostly addressing the *mcr1*-gene **[9].**This study aimed to assess the prevalence of *mcr* genes (*mcr*-1 to *mcr*-9) in CoR isolates.

Materials and Methods:

The present descriptive study was conducted from April 2024 to October 2024 in the Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, Egypt. The study received approval from the Research Ethical Committee (N-361-2024).

Multi/or extensively and pan drug-resistant (MDR/XDR/PDR) Gram-negative isolates were obtained from cultured clinical samples processed in the microbiology unit for routine culture and sensitivity testing from patients suffering symptoms and signs of systemic infection admitted to Kasr-AlAiny Cairo University hospitals.

Isolates were categorized as:

Multidrug -resistance (MDR): Resistance to ≥1 agent in ≥3 antimicrobial categories.

Extensively drug-resistant (XDR): Susceptibility to ≤2 antimicrobial categories.

Pan-drug resistance (PDR): Resistance to all antimicrobial agents **[10].**

All clinical samples were cultured on conventional media, including Cysteine lactose electrolyte deficient agar (CLED) for urine, while blood, chocolate and MacConkey agar media (Oxoid Co. England) for other samples. All inoculated plates underwent 24-hour aerobic incubation at 37°C**.**

All plates underwent aerobic incubation at 37°C for 24 hours.

Bacterial identification was performed via Gram staining, followed by a series of conventional biochemical tests such as catalase, oxidase, citrate, triple sugar iron, motility-indole-ornithine, and urease tests **[11].**

The antimicrobial susceptibility was assessed using the Kirby-Bauer disc diffusion method with 0.5 McFarland turbidity suspensions for each isolate. Each suspension was streaked on Mueller-Hinton media (Oxoid Co., England), incubated aerobically at 37°C. Antibiotic sensitivity was assessed for each organisms toward the following antibiotics (potency in µg/disc) ampicillin (10 mg); amoxicillin/clavulanic acid (10 mg);cefuroxime(30mg) ceftazidime (30 mg); cefotaxime (30 mg); ceftriaxone (30 mg); cefepime (30 mg); imipenem (10 mg); meropenem (10 mg); trimethoprim/sulfamethoxazole (1.25/23.75 mg); piperacillin/tazobactam (100/10 mg); gentamicin (10 mg); amikacin (30 mg); ciprofloxacin (5 mg);Levofloxacin (5mg) ;and Ofloxacin (5mg) (Oxoid, Basingstoke, United Kingdom). The susceptibility results were released, as referred to the (**CLSI 2023**) guidelines **[12].**

Phenotypic susceptibility testing for colistin resistance:

The broth microdilution (BMD) method was performed to determine the minimum inhibitory concentration (MIC) of colistin in the tested isolates. Cation-adjusted Mueller-Hinton broth (HiMedia, India) was used according to Clinical and Laboratory Standards Institute (CLSI) guidelines (2023). Isolates were classified as intermediate (MIC ≤2 µg/mL) or resistant (MIC ≥4 µg/mL) **[12].**

Molecular detection of plasmid-encoded mcr genes:

Colistin-resistant Gram-negative isolates (per BMD results) were screened for *mcr* genes (*mcr*-1 to *mcr*-5), and (*mcr*-6 to *mcr*-9) in two separate multiplex PCR reactions. Bacterial genomic DNA was extracted and purified using the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol. Primer sequences were used as per the method of Rebelo et al. (2018) and Borowiak et al. (2020) (**Supplementary Table 1**) **[13,14].**

The two multiplex PCR reactions were conducted with 25 µL volume involving 2 µL of DNA template, 0.5 µL of individual primer solution (10 µM), 12.5 µL of PCR Master Mix (Thermo Fisher Scientific, USA). PCR conditions were optimized as follows: 25 denaturation cycles at 95°C for 30 seconds. For the annealing step, the first reaction was optimized at 58°C for 90 seconds, while the second reaction at 55◦C for 30 seconds. Afterwards, elongation for 60 s at 72◦C was done followed by final elongation step at 72°C for 10 minutes. Afterwards, gel electrophoresis was done to visualize the amplified products under UV lamp, which were then compared with a DNA ladder **[13,14].**

Statistical analysis:

Qualitative and quantitative data analysis was conducted using the SPSS software version 21. Both parametric and non-parametric significance tests were employed, specifically the Chi-square test.The level of significance was set at $p \leq 0.05$

Results

Out of 380 clinical samples collected from patients admitted to Kasr-AlAiny Cairo University hospitals, 100 samples met the criteria for MDR-, XDR-, and PDR-isolates after excluding duplicates.

The age of the patients varied from 0.03 to 78 years, with a mean of 36.18 years. There were 70 (70%) males and 30(30%) females.

Specimen types included respiratory tract sputum/tracheal aspirates (13%), blood (22%), body fluids (4%), urine (22%), and wound swabs (39%). Most isolates originated from intensive care units (ICUs) (73%), with 27% from other wards. The predominant isolates were *Klebsiella pneumoniae* (55%), followed by *Pseudomonas species* (27%), *Acinetobacter species* (16%), and *E. coli* (2%).

The majority of isolates exhibited extensive drug resistance (67%), while 3% demonstrated a multidrug-resistant (MDR) pattern, and 30% were classified as pan-drug-resistant (PDR).

The antibiotic susceptibility pattern of the tested isolates demonstrated high sensitivity to amikacin and gentamicin (8%), followed by imipenem (4%), as illustrated in Table (2).

Among the 100 isolates tested, 68% exhibited resistance to colistin, whereas 32% showed intermediate susceptibility. As shown in Table 3, the majority of CoR isolates were obtained from ICU patients, accounting for 75%, with *Klebsiella species* identified as the most prevalent pathogen at 57.4%.

Multiplex PCR detected the mcr-4 gene in 5.8% (4/68) of CoR isolates. No other *mcr* genes were identified. Among the four *mcr*-4-positive isolates, Acinetobacter spp. constituted the majority (75%), while *Klebsiella spp.* accounted for 25%. All positive isolates originated from ICU patients, with 75% isolated from bloodstream infections and 25% from urine specimens.

Table 1. The sequences of used primer **[13,14]**.

Target gene	Primer sequences (5'-3')	Size (bp)
mcr-1 (40-359)	mcr1_fw AGTCCGTTTGTTCTTGTGGC	320
	mcr1_rev AGATCCTTGGTCTCGGCTTG	
mcr-2 (401-1115)	mcr2_fw CAAGTGTGTTGGTCGCAGTT	715
	mcr2_rev TCTAGCCCGACAAGCATACC	
mcr-3 (17-945)	mcr3_fw AAATAAAAATTGTTCCGCTTATG	929
	mcr3_rev AATGGAGATCCCCGTTTTT	
mcr-4 (38-1153)	mcr4_fw TCACTTTCATCACTGCGTTG	1116
	mcr4_rev TTGGTCCATGACTACCAATG	
mcr-5 (1-1644)	mcr5_fw ATGCGGTTGTCTGCATTTAT	1644
	mcr5_rev TCATTGTGGTTGTCCTTTTCT	
Mcr-6	mcr-6_fw AGCTATGTCAATCCCGTGAT	252
	mcr-6_rev ATTGGCTAGGTTGTCAATC	
Mcr -7	mcr-7_fw GCCCTTCTTTTCGTTGTT	551
	mcr-7_rev GGTTGGTCTCTTTCTCGT	
Mcr-8	mcr-8_fw TCAACAATTCTACAAAGCGTG	865
	mcr-8_rev AATGCTGCGCGAATGAAG	
Mcr-9	mcr-9_fw TTCCCTTTGTTCTGGTTG	1011
	mcr-9_rev GCAGGTAATAAGTCGGTC	

Table 2. Antimicrobial susceptibility results of isolated pathogens,

	AP	CXM	CTX	CRO	CAZ	FEP	TZP	AMC	CIP	LEV	OFX	GM	AK	MEM	IPM	SXT
S	0	0	0	0	0	0	2	2	0	1	2	8	8	1	4	1
I	0	0	0	0	0	0	1	0	0	1	4	0	1	0	1	0
R	100	100	100	100	100	100	97	98	100	98	94	92	91	99	95	99
TOTAL	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

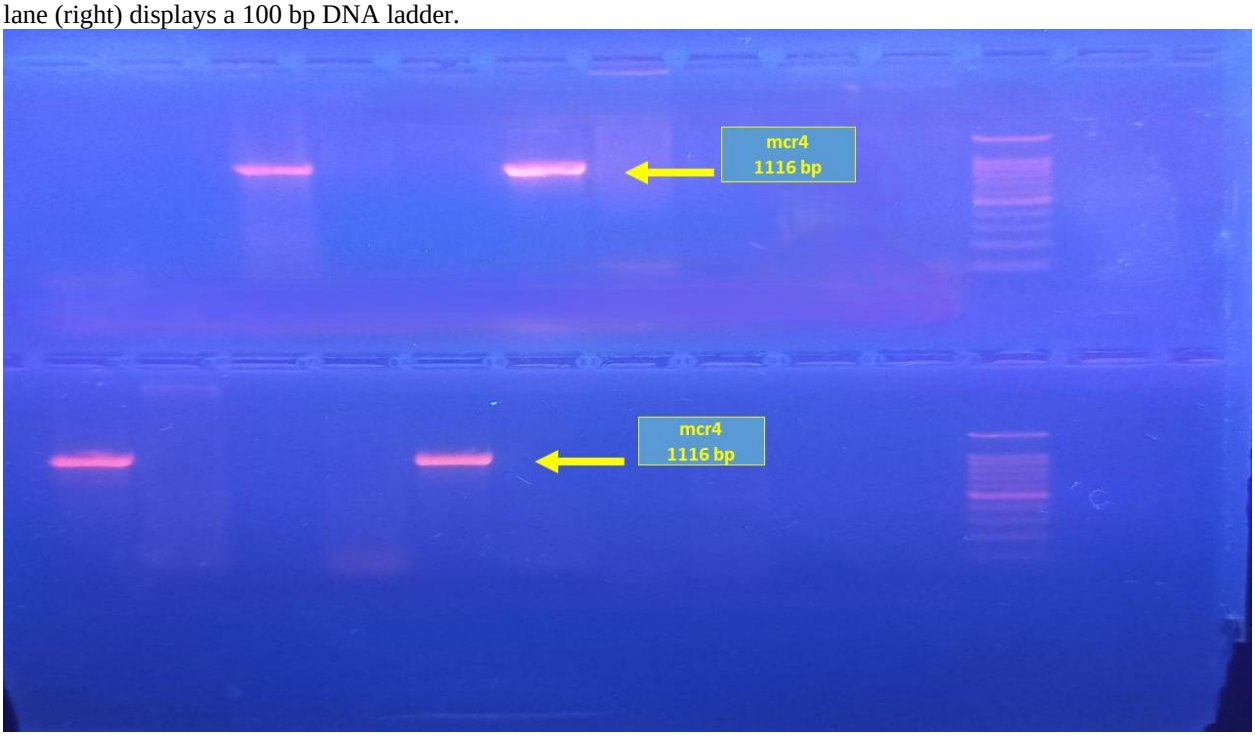
AP (Ampicillin), CXM (cefuroxime), CTX (ceftaxime), CRO (ceftriaxone), CAZ (ceftazidime), FEP (cefipime), TZP (piperacillin-tazobactam), AMC (amoxicillin/clavulanic acid), CIP (ciprofloxacin), LEV (levofloxacin), OFX (ofloxacin), GM (gentamycin), AK (amikacin), MEM (meropenem), IPM (imipenem), SXT (trimethoprim-sulfamethoxazole). S: sensitive, I: intermediate, R: resistance

Table 3. Characterization of CoR isolates.

		BMD result				
		R		**I**		**P value***
		Count	**%**	**Count**	**%**	
gender of patient	**Male**	50	73.5%	20	62.5%	0.086
	Female	18	26.4%	12	37.5%	
site of isolated samples	**ICU**	51	75.0%	22	68.8%	0.511
	Department	17	25.0%	10	31.3%	
rganism resistance	***Acinetobacter***	12	17.6%	4	12.5%	0.443
	E. coli	2	2.9%	0	0.0%	
	Klebsiella	39	57.4%	16	50.0%	
	Pseudomonas	16	23.5%	11	34.4%	

BMD: broth microdilution, ICU: intensive-care unit, R: resistance, I: intermediate, (*): chi-square test

Figure 1. Visualization of amplified products for the mcr-4 gene (1116 bp) via gel electrophoresis. The first lane (right) displays a 100 bp DNA ladder.



Discussion

The emergence of resistance to colistin, mediated by *mcr* genes, poses a critical challenge in managing multidrug-resistant MDR-GNB infections.

In this study, 68% of tested isolates exhibited colistin resistance, which is significantly higher than that reported by **Dawoud et al.,** which found that 46.8% of isolated *Acintobacter baumannii* from hospitalized patients in Egypt were colistin-resistant **[15].**

A further investigation by **Seleim et al.** indicated that 49% of isolated *Acinetobacter baumannii* exhibited resistance to colistin **[16].**

A meta-analysis by **Pormohammad et al.** found that the increasing rates of colistin resistance in countries of the Middle East and South Asia exceeded other geographical areas in the world. Therefore, it is essential to regulate antibiotic usage in these regions by following appropriate therapeutic guidelines **[17].**

The increased rate of resistance observed may indicate the excessive use of colistin as an antimicrobial or last resort in treating critically ill patients, particularly in ICUs, where 51% of the colistin-resistant isolates were collected.

Consistent with our findings, other studies have highlighted the ICU as a high-risk environment for drug-resistant infections due to prolonged hospitalization, invasive procedures, and intense antibiotic pressure. This highlights the urgent need for optimized antibiotic stewardship protocols specifically tailored to ICU environments. A prior systematic review conducted by **Uzairue et al.** indicated that bloodstream *Klebsiella pneumoniae* from ICU patients exhibited a higher rate of colistin resistance (11.5%) compared to non-ICU patients (3.03%) **[18].**

In this study, *Klebsiella pneumoniae* was the predominant pathogen, accounting for 55% of all isolates, with 57.4% exhibiting colistin resistance. This aligns with global research identifying *K. pneumoniae* as a leading cause of colistin resistance in healthcare settings. A study conducted in Iran by **Narimisa et al.** identified an overall colistin resistance rate of 6.9% in clinical *K. pneumoniae* **[19].**

Our genetic analysis revealed the detection of the *mcr-4* gene in 5.8% of CoR isolates; however, other tested mcr genes (*mcr-1* to *mcr-3*, *mcr-5* to *mcr-9*) were absent.

This finding is notable, as *mcr-4* is less frequently reported compared to *mcr-1*, the most globally prevalent mcr variant. For instance, Dawoud et al. detected *mcr-1* in only 2/44 (4.5%) CoR *Acinetobacter baumannii* isolates, with *mcr-2, -3, -4, and -5* entirely absent **[15].** Similarly, **Ajlan et al.** in Egypt identified *mcr-1* in 3/43 (6.98%)

colistin-resistant GNB, with no other *mcr* genes observed **[20].**

A comprehensive analysis done by **Jiping et al**. on global *E. coli* genomes identified *mcr* genes across 25 countries. Among 778 *mcr*-positive isolates, *mcr-1* predominated (86.1%), followed by *mcr-9* (5.7%), *mcr-5* (4.4%), and *mcr-3* (3.0%) **[21].** These findings highlight geographical disparities in mcr gene distribution, underscoring the the need for ongoing surveillance to track their spread.

Conclusion:

The observed high colistin resistance rate and *mcr-4* detection emphasize the urgent need for robust antimicrobial stewardship programs and stringent infection control policies. Regional variations in mcr gene prevalence reinforce the importance of continuous global surveillance to elucidate resistance mechanisms and guide targeted interventions. Future studies should explore alternative therapeutic options, such as combination therapies, and investigate the environmental and agricultural role in the propagation of antimicrobial resistance.

Limitation

Our study lacks detailed patient clinical data in terms of date of admission, and antibiotic uptake leading to inability to identify hospital or community acquired infections or to relate colistin resistance rates with colistin therapeutic intake. In addition, further advanced technologies such as molecular sequencing may be required to investigate other mechanisms of colistin resistance in *mcr* genes-negative isolates, and to characterize potentially existing circulating resistant strains

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Competing interests

No conflict of interest

Financial disclosure

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Data availability

All data are avaliable on request

Authors' contribution

NSS: development of the study design and conducting the practical part. **MMB:** conduct all the statistical analysis. **YAE:** writing the article and submitting for publication All authors reviewed the article.

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