

GENETIC CHARACTERIZATION OF PLASMID-MEDIATED COLISTIN RESISTANCE GENES IN MULTIDRUG-RESISTANT KLEBSIELLA PNEUMONIAE

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ABSTRACT

Background: Antimicrobial resistance in *Klebsiella pneumoniae* is a growing global health concern, with rising multidrug- and carbapenem-resistant strains narrowing treatment options. Colistin remains a last-resort agent, but plasmid-mediated mobile colistin resistance (*mcr*) genes threaten its efficacy through horizontal transfer.

Objective: To detect *mcr-1* and *mcr-2* genes among multidrug-resistant *K. pneumoniae* clinical isolates in Karachi, Pakistan.

Methods: In this one-year cross-sectional study (April 2025–April 2026) at Fatima Teaching Hospital and Mohammad Ali Jinnah University, 101 clinical samples (urine, blood, sputum) were processed by culture, Gram staining, and biochemical identification. Antimicrobial susceptibility was assessed by Kirby–Bauer disk diffusion (CLSI guidelines). Colistin-resistant isolates underwent plasmid DNA extraction, PCR for *mcr-1*/*mcr-2*, gel electrophoresis, and Sanger sequencing.

Results: *K. pneumoniae* was isolated from 50/101 samples (49.5%). All isolates were ampicillin-resistant (100%); resistance was also seen to ceftriaxone (62.0%), ciprofloxacin (62.0%), gentamicin (50.0%), and meropenem (44.0%). Colistin resistance occurred in 15/50 isolates (30.0%). *mcr-1* was detected in 7/15 colistin-resistant isolates (46.7%; 14.0% of all isolates); *mcr-2* was not detected. Sequencing confirmed 99.8% identity of all 7 *mcr-1* amplicons with the reference plasmid sequence (GenBank MH143576.1).

Conclusion: *mcr-1* is circulating among colistin-resistant *K. pneumoniae* in Karachi, though most colistin-resistant isolates (53.3%) were *mcr*-negative, suggesting additional chromosomal or non-*mcr* resistance mechanisms. Continued molecular surveillance, judicious colistin use, and strengthened infection control are needed to preserve its clinical effectiveness.

KEYWORDS: *Klebsiella pneumoniae*; colistin resistance; *mcr-1*; *mcr-2*; antimicrobial resistance; Karachi.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the most critical public health challenges of the 21st century. In 2021, an estimated 4.71 million deaths were associated with bacterial AMR globally, with low- and middle-income countries disproportionately affected [1]. *Klebsiella pneumoniae*, a Gram-negative opportunistic pathogen belonging to the family Enterobacteriaceae, is a leading cause of healthcare-associated and community-acquired infections, including pneumonia, urinary tract infections, and bloodstream infections, particularly among hospitalized and immunocompromised patients [2]. The organism has an exceptional ability to acquire antimicrobial resistance genes, including extended-spectrum β -lactamases (ESBLs) and carbapenemases, resulting in progressively limited therapeutic options for severe infections [3].

Colistin (polymyxin E) has emerged as a last-resort antimicrobial for the treatment of infections caused by carbapenem-resistant Gram-negative bacteria [4]. Until 2015, colistin resistance was primarily attributed to chromosomal mutations. The discovery of the plasmid-mediated mobile colistin resistance gene, *mcr-1*, demonstrated that colistin resistance could also spread horizontally between bacteria via conjugative plasmids [5]. Since then, ten mobile colistin resistance (*mcr*) gene families (*mcr-1* to *mcr-10*) have been described, with *mcr-1* remaining the most prevalent and widely distributed variant, whereas *mcr-2* has been reported less frequently, particularly among *K. pneumoniae* isolates [6]. The plasmid-mediated nature of these genes facilitates their rapid dissemination within and between bacterial species, posing a significant challenge to antimicrobial stewardship and infection prevention efforts [7].

Pakistan has reported a substantial burden of ESBL-producing and carbapenem-resistant *K. pneumoniae*, leading to increased use on colistin as salvage therapy for multidrug-resistant infections [8]. However, molecular epidemiological data on plasmid-mediated colistin resistance genes among Pakistani clinical isolates remain scarce and are limited to a few tertiary-care centers [9]. Understanding the prevalence and molecular characteristics of *mcr*-mediated resistance is essential for strengthening antimicrobial stewardship, infection prevention strategies, and national antimicrobial resistance surveillance.

Therefore, this study aimed to detect and molecularly characterize the plasmid-mediated colistin resistance genes *mcr-1* and *mcr-2* among colistin-resistant multidrug-resistant *K. pneumoniae* clinical isolates recovered from patients with pneumonia, septicemia, and urinary tract infections at a tertiary care hospital in Karachi, Pakistan. In addition, *mcr-1*-positive isolates were characterized by Sanger sequencing to evaluate sequence similarity with published reference strains.

MATERIALS AND METHODS

Study Design, Setting and Duration

This laboratory-based cross-sectional study was conducted between April 2025 and April 2026 at Fatima Teaching Hospital and Mohammad Ali Jinnah University (MAJU), Karachi, Pakistan, following approval from the Institutional Review and Ethics Board [BMU-IREB/13-2025/004] and the Board of Advanced Studies and Research, Baqai Medical University. Clinical specimens were processed at the Clinical Microbiology Laboratory, Fatima Teaching Hospital, while molecular analyses were performed at the Biotechnology Laboratory, MAJU.

Sample Collection

A consecutive sampling technique was employed, whereby all eligible clinical specimens meeting the inclusion criteria were enrolled sequentially over the study period. The sample size was calculated using the formula $n = Z^2P(1-P)/d^2$, based on an expected frequency of colistin resistance among multidrug-resistant *K. pneumoniae* isolates of 7% [10], at a 95% confidence level ($Z = 1.96$) and 5% margin of error ($d = 0.05$), yielding a minimum required sample size of 101 specimens. Specimens, including urine, blood, and sputum, were collected from adult patients (≥ 18 years) with clinically suspected urinary tract infection, septicemia, or pneumonia. Patients with prior antibiotic exposure before specimen collection were excluded.

Isolation and Identification of *Klebsiella pneumoniae*

Clinical specimens were cultured on Blood Agar and MacConkey Agar. *Klebsiella pneumoniae* isolates were identified by colony morphology, Gram staining, and standard biochemical tests, including Triple Sugar Iron (TSI), urease, indole, and citrate utilization tests.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) M100 34th Edition (2024) guidelines [11]. The antimicrobial agents tested were ampicillin (10 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), meropenem (10 µg) and colistin (10 µg). Ampicillin was included as an intrinsic resistance marker for *K. pneumoniae* rather than an indicator of acquired resistance. Multidrug resistance (MDR) was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes according to the joint ECDC/CDC consensus definition [12].

Detection of *mcr-1* and *mcr-2*

Plasmid DNA was extracted from colistin-resistant isolates by the alkaline lysis method [14] and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), with A260/A280 ratios of 1.8–2.0 accepted as indicating adequate purity. Conventional PCR was performed using previously published primers for *mcr-1* (309 bp) and *mcr-2* (1747 bp) [15]. Each 25 µL reaction contained 12.5 µL 2× PCR Master Mix, 0.5 µL each primer (10 µM), 3.0 µL template DNA, and 8.5 µL nuclease-free water. Amplification was carried out on a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA): initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 30 s, annealing at 51°C (*mcr-1*) or 53°C (*mcr-2*) for 45 s, and 72°C for 60 s (*mcr-1*) or 90 s (*mcr-2*); with a final extension at 72°C for 7 min. Each run included a verified *mcr-1*/*mcr-2* plasmid-positive control and a no-template negative control. PCR products were resolved on 1.5% agarose gel stained with GelRed, electrophoresed at 90 V for 45–60 min alongside a 100 bp DNA ladder, and visualized under UV transillumination.

mcr-1-positive amplicons ($n = 7$) were purified using the Wizard® PCR and Gel DNA Purification System (Promega, USA) and sequenced unidirectionally on an ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) using BigDye™ Terminator v3.1 chemistry (Macrogen Inc., Seoul, South Korea). Chromatograms were analyzed in ChromasPro (Technelysium, Australia), and consensus sequences were compared against NCBI reference sequences using BLAST.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to summarize specimen distribution, antimicrobial resistance patterns, colistin resistance, and the detection of *mcr* genes; results are presented as counts and percentages. For key proportions including colistin resistance and *mcr-1* detection among colistin-resistant isolates 95% confidence intervals were calculated using the Wilson score method to convey the precision supported by the underlying sample size, particularly for the colistin-resistant subgroup ($n = 15$).

RESULTS

A total of 101 clinical specimens, comprising 33 urine, 37 blood, and 31 sputum samples, were processed during the study period. *Klebsiella pneumoniae* was isolated from 50 of 101 specimens (49.5%; 95% CI 40.0–59.1%)

and confirmed by culture, Gram staining, and biochemical identification (Figures 1–3). The distribution of clinical specimens and confirmed *K. pneumoniae* isolates is presented in Table 1.

Table 1. Distribution of Clinical Specimens and Confirmed *K. pneumoniae* Isolates

Specimen Source	Total Specimens n (%)	Confirmed <i>K. pneumoniae</i> n (%)
Urine	33 (32.7)	9 (18.0)
Blood	37 (36.6)	23 (46.0)
Sputum	31 (30.7)	18 (36.0)
Total	101 (100.0)	50 (100.0)



Figure 1: Blood Agar showing large, grey, mucoid, non-hemolytic colonies suggestive of *Klebsiella pneumoniae*

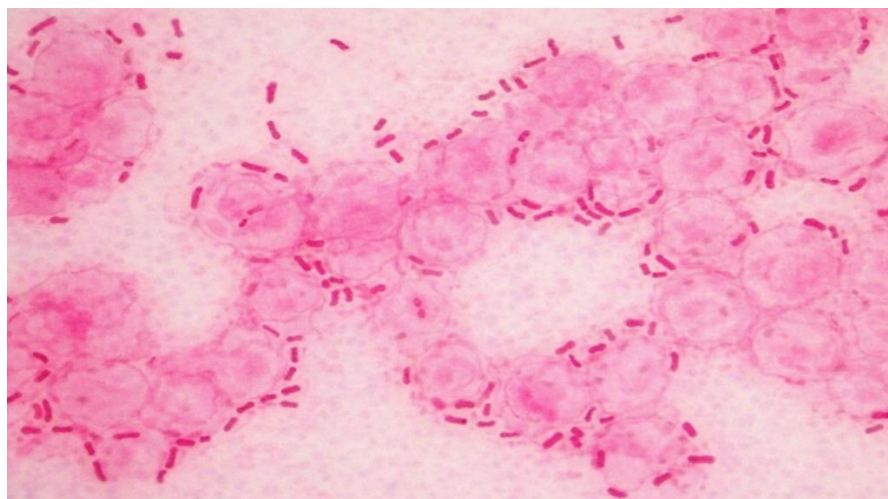


Figure 2: Gram stain of *Klebsiella pneumoniae* showing Gram-negative short rods



Figure 3: Biochemical identification of *Klebsiella pneumoniae* showing TSI (A/A, no H₂S), Citrate positive, Indole negative, and Urease positive reactions

The antimicrobial susceptibility profile of the 50 MDR *K. pneumoniae* isolates is summarized in Table 2. All isolates were resistant to ampicillin (50/50, 100.0%). Resistance to ceftriaxone and ciprofloxacin was observed in 31 of 50 isolates each (62.0%; 95% CI 48.2–74.1%), followed by gentamicin in 25 of 50 (50.0%; 95% CI 36.6–63.4%) and meropenem in 22 of 50 isolates (44.0%; 95% CI 31.2–57.7%). The colistin agar test identified colistin resistance in 15 of 50 isolates (30.0%; 95% CI 19.1–43.8%) (Figure 4 & 5).

Table 2. Antimicrobial Susceptibility Profile of *K. pneumoniae* Isolates (N=50)

Antibiotic	Resistant n (%)	95% CI	Susceptible n (%)
Ampicillin	50 (100.0)	92.9–100.0	0 (0.0)
Ceftriaxone	31 (62.0)	48.2–74.1	19 (38.0)
Ciprofloxacin	31 (62.0)	48.2–74.1	19 (38.0)
Gentamicin	25 (50.0)	36.6–63.4	25 (50.0)
Meropenem	22 (44.0)	31.2–57.7	28 (56.0)
Colistin	15 (30.0)	19.1–43.8	35 (70.0)



Figure 4: Antimicrobial profile among *Klebsiella pneumoniae* isolates

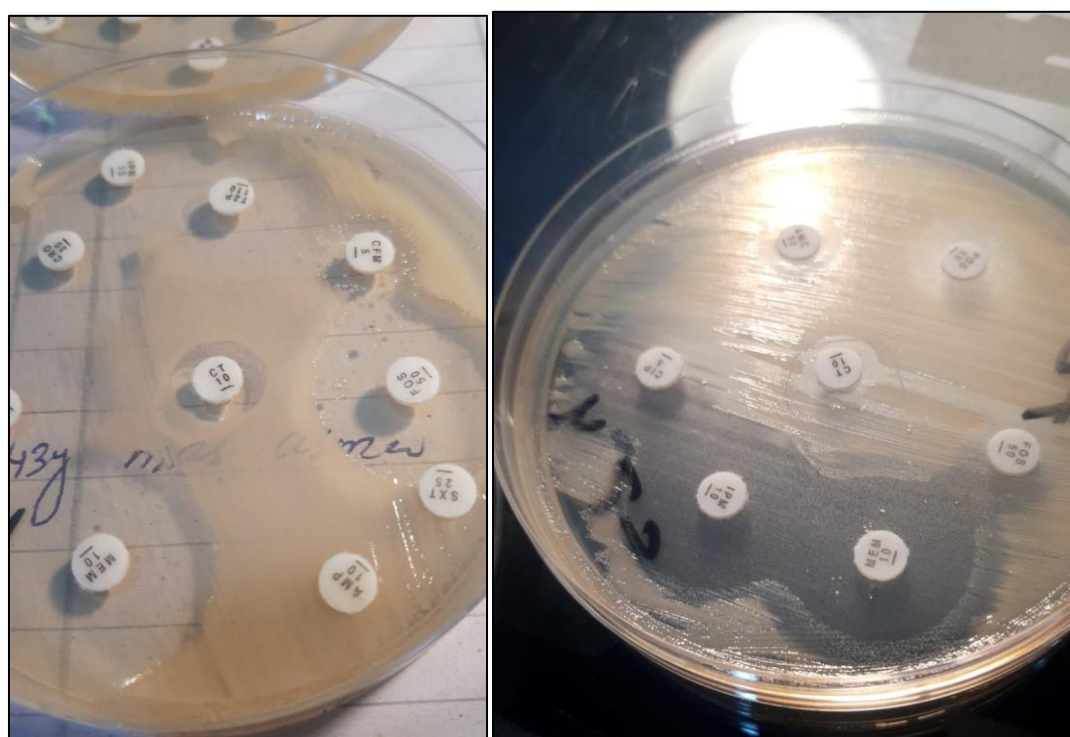


Figure 5: Colistin Resistance among *Klebsiella pneumoniae* isolates

PCR analysis was performed on the 15 phenotypically colistin-resistant *K. pneumoniae* isolates. The *mcr-1* gene (309 bp) was detected in 7 of 15 isolates (46.7%; 95% CI 24.8–69.9%), whereas 8 of 15 isolates (53.3%; 95% CI 30.1–75.2%) were negative (Table 3). No isolate yielded the expected 1,747 bp *mcr-2* amplicon (0/15, 0.0%; 95% CI 0.0–20.4%). No amplification was observed in the no-template control, confirming assay specificity (Figure 6). Given the small subgroup size ($n = 15$), these confidence intervals are wide and the point estimates should be interpreted as indicative rather than precise.

Table 3. PCR Detection Results for Colistin-Resistant *Klebsiella pneumoniae* Isolates ($n = 15$)

Gene Target	Expected Amplicon	Positive n (%)	95% CI	Negative n (%)	95% CI
<i>mcr-1</i>	309 bp	7 (46.7)	24.8–69.9	8 (53.3)	30.1–75.2
<i>mcr-2</i>	1747 bp	0 (0.0)	0.0–20.4	15 (100.0)	79.6–100.0

All seven *mcr-1*-positive PCR products were confirmed by Sanger sequencing. BLAST analysis showed 100% query coverage and 99.8% nucleotide identity with the reference *mcr-1*-carrying plasmid (GenBank accession MH143576.1), with no nucleotide substitutions, insertions, or deletions detected.

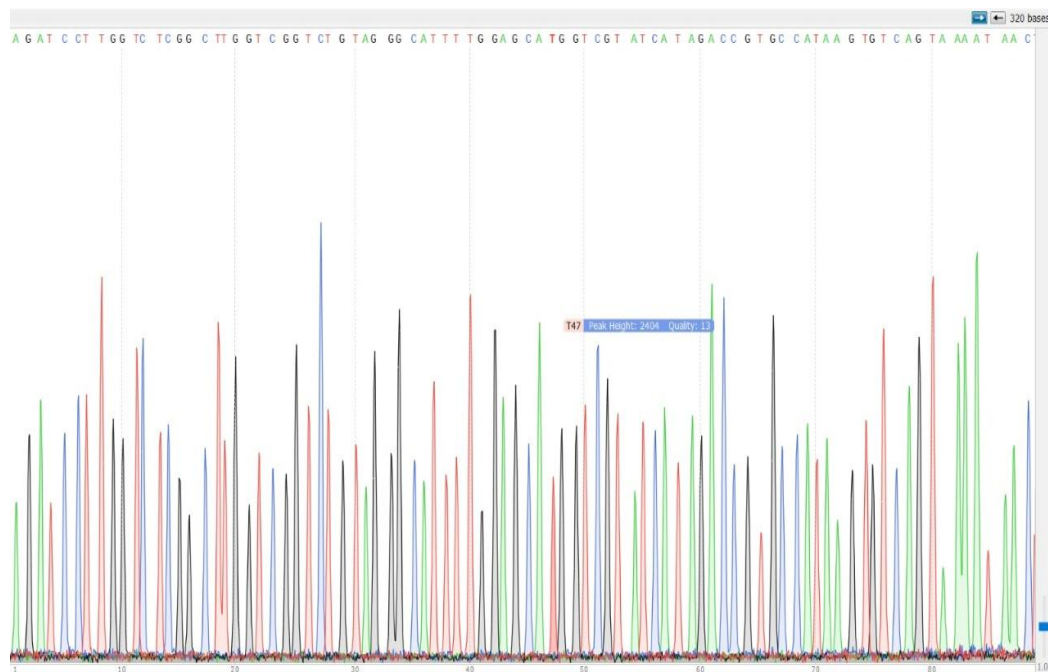


Figure 6: Agarose gel electrophoresis showing PCR amplification of the *mcr-1* gene (309 bp). No amplification was detected for *mcr-2* (1,747 bp)

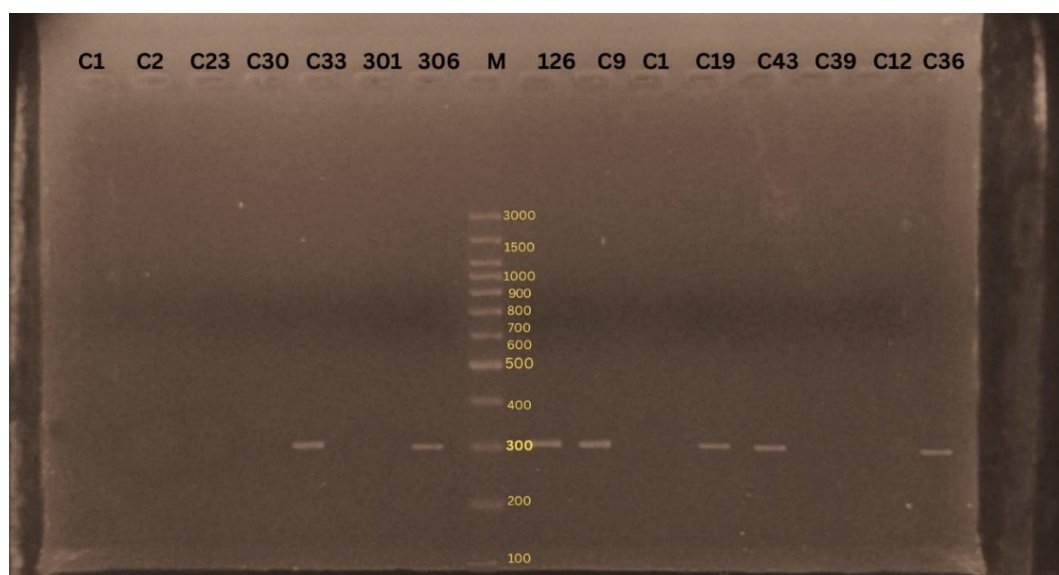


Figure 7: Sanger sequencing chromatogram of an *mcr-1*-positive *Klebsiella pneumoniae* isolate showing well-resolved nucleotide peaks

DISCUSSION

Half of the confirmed *K. pneumoniae* isolates in this study originated from blood, consistent with the organism's established role as a major cause of healthcare-associated bloodstream infection, particularly among hospitalized

and immunocompromised patients [2]. Universal ampicillin resistance reflected intrinsic chromosomal β -lactamase production, while high resistance to ciprofloxacin and ceftriaxone (31/50, 62.0% each) reflects sustained selective pressure from empirical fluoroquinolone and cephalosporin use in this setting [16].

Resistance to gentamicin and meropenem was moderate, with more than half of isolates remaining susceptible to meropenem; nonetheless, emerging carbapenem resistance in *K. pneumoniae* is well documented to be associated with treatment failure and increased mortality [17]. Colistin resistance was detected in 15 of 50 isolates (30.0%; 95% CI 19.1–43.8%), a notable finding given colistin's role as a last-resort agent for carbapenem-resistant Gram-negative infections, and broadly consistent with, though higher than, colistin resistance rates reported in other Pakistani tertiary-care settings [18, 19]. This variation may reflect differences in patient populations, prior antibiotic exposure, and local colistin usage patterns.

Molecular analysis identified the plasmid-mediated *mcr-1* gene in 7 of 15 colistin-resistant isolates (46.7%; 95% CI 24.8–69.9%), corresponding to 7 of 50 (14.0%; 95% CI 7.0–26.2%) of all confirmed isolates, while *mcr-2* was not detected in any of the 15 isolates tested, consistent with global surveillance data showing *mcr-2* to be rarer than *mcr-1* in *K. pneumoniae* [6]. As both subgroup estimates are drawn from a small sample ($n = 15$), they should be read as indicative rather than precise population estimates. Sequencing confirmed 99.8% homology to a previously characterized *mcr-1*-carrying plasmid, with no nucleotide substitutions, insertions, or deletions detected. Consequently, no nonsynonymous mutations were identified, indicating complete conservation of the *mcr-1* determinant relative to the reference sequence and suggesting a horizontally acquired rather than locally evolved variant.

Notably, phenotypic colistin resistance (15/50, 30.0%) exceeded *mcr-1* prevalence (7/50, 14.0%), indicating that mechanisms beyond plasmid-mediated *mcr* genes contribute to the resistant phenotype in this cohort. Chromosomal mutations affecting the *pmrAB*, *phoPQ*, and *mgrB* regulatory systems are well-documented independent contributors to colistin resistance in *K. pneumoniae* [20], and additional plasmid-mediated variants (*mcr-3* to *mcr-10*) have been implicated in global transmissible resistance [21]. As chromosomal loci were not sequenced in this study, this remains an important direction for follow-up work rather than a confirmed explanation.

These findings carry direct clinical relevance; because *mcr* genes disseminate via horizontal gene transfer rather than clonal spread alone, their presence in this cohort raises the possibility of inter-patient or environmental transmission within the hospital setting [22]. As colistin and carbapenems both represent last-resort therapeutic options, the confirmed presence of transferable colistin resistance alongside high carbapenem and cephalosporin resistance underscores an urgent need for combined phenotypic-molecular surveillance, judicious colistin use, and reinforced infection-control practices.

Limitations

1. This study was conducted at a single tertiary-care facility, which may limit generalizability.
2. Molecular analysis was restricted to *mcr-1* and *mcr-2*; other *mcr* variants (*mcr-3* to *mcr-10*) and chromosomal resistance loci (*pmrAB*, *phoPQ*, *mgrB*) were not investigated.
3. Sequencing was unidirectional rather than bidirectional or whole-genome, limiting variant-detection sensitivity.
4. Plasmid incompatibility typing and conjugation/transferability experiments were beyond the scope of this study.
5. The colistin-resistant subgroup was small ($n = 15$), and the *mcr-1* detection subgroup smaller still (7 positive, 8 negative); the resulting percentages (e.g., 46.7%, 53.3%) carry wide 95% confidence intervals (roughly 25–70% and 30–75%, respectively) and should be interpreted as indicative rather than precise estimates of true prevalence.

Recommendations

1. Future studies should be conducted across multiple tertiary-care centers to improve generalizability of findings.
2. Molecular screening should be expanded to include additional *mcr* variants (*mcr-3* to *mcr-10*) and chromosomal resistance loci (*pmrAB*, *phoPQ*, *mgrB*) to provide a more comprehensive resistance profile.
3. Bidirectional sequencing or whole-genome sequencing should be employed in future work to improve variant-detection sensitivity.
4. Plasmid incompatibility typing and conjugation/transferability experiments should be undertaken to better characterize the mobility and transmission potential of resistance determinants.
5. Continued surveillance of colistin resistance is recommended to guide antimicrobial stewardship and inform treatment protocols for multidrug-resistant infections, ideally with larger colistin-resistant cohorts to yield more precise *mcr* prevalence estimates.

CONCLUSION

Plasmid-mediated *mcr-1* is circulating among colistin-resistant *K. pneumoniae* clinical isolates in Karachi, Pakistan. Among 50 confirmed isolates, 15 (30.0%; 95% CI 19.1–43.8%) demonstrated phenotypic colistin resistance by the colistin agar test, and the plasmid-mediated *mcr-1* gene was detected in 7 of these 15 (46.7%; 95% CI 24.8–69.9%); *mcr-2* was not detected in any isolate. However, the majority 8 of 15 (53.3%; 95% CI 30.1–75.2%) of colistin-resistant isolates were *mcr*-negative, indicating that non-plasmid, chromosomal, mechanisms remain an important and under-characterized contributor to colistin resistance locally; given the small subgroup size, this estimate warrants confirmation in larger cohorts.

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Conflict of Interest

The authors declare no conflict of interest.

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Ethical Approval and Consent to Participate

This study was approved by the Institutional Review and Ethics Board, Baqai Medical University, Karachi, Pakistan [BMU-IREB/13-2025/004], and by the Board of Advanced Studies and Research, Baqai Medical University. Written informed consent was obtained from all patients prior to specimen collection. The study was conducted in accordance with the Declaration of Helsinki.

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