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Emergence of Colistin Resistance in Multidrug-Resistant *Klebsiella pneumoniae* Isolates from a Tertiary Care Hospital in Dhaka, Bangladesh

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Abstract

Background: The global rise of multidrug-resistant *Klebsiella pneumoniae* poses a significant challenge to human health. Colistin, a last-resort antibiotic for multidrug-resistant infections, is losing its effectiveness due to extensive use. **Objective:** The purpose of the study was to detect colistin resistance among clinical isolates of *Klebsiella pneumoniae* and identify the colistin resistance genes by Polymerase Chain Reaction (PCR). **Methodology:** This cross-sectional study was conducted in the Department of Microbiology of Dhaka Medical College, Dhaka, Bangladesh from January 2021 to December 2021. *Klebsiella pneumoniae* was isolated and identified by standard microbiological procedures. The antibiotic susceptibility pattern of all tested antibiotics was determined by the disc-diffusion method. The Minimum Inhibitory Concentration (MIC) of colistin was determined by agar dilution method. PCR was used to identify different colistin resistance genes (*phoP, phoQ, mgrB, pmrA, pmrB, pmrC, mcr-1, mcr-2*). **Results**: Out of 55 isolated *Klebsiella pneumoniae*, 19 (34.55%) were resistant to colistin detected by agar dilution method. MIC of colistin showed a significant rise ranging from 4 to ≥256 µg/ml. A total of 36 (65.45%) *K. pneumoniae* isolates were found to be multidrug-resistant (MDR). Among 19 colistin resistant isolates, 15 (78.9%) were positive for mgrB and *pmrC*, 14(73.9%) for *pmrB*, 13(68.4%) for *pmrA*, 11(57.9%) for *phoP* and 10(52.6%) were positive for *phoQ*. **Conclusion:** This study showed a high rate of colistin resistance among multidrug-resistant *Klebsiella pneumoniae* isolates. Strict implementation of antimicrobial stewardship policies are essential to control the spread of colistin resistance.

Keywords: Colistin resistance genes; *Klebsiella pneumoniae*; Minimum inhibitory concentration; Multidrug resistance

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Introduction

Klebsiella pneumoniae is an important pathogen in nosocomial infections and is responsible for many clinical syndromes including pneumonia, bacteremia, thrombophlebitis, urinary tract infection, cholecystitis, upper respiratory tract infection, wound infection, osteomyelitis and meningitis[1].

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Currently, *Klebsiella pneumoniae* is showing a high resistance to broad spectrum of drugs including beta-lactam antibiotics, fluoroquinolones and aminoglycosides[2-3]. Carbapenems are the drug of choice for the treatment of infections caused by ESBL producing bacteria[4]. Unfortunately, bacterial resistance to carbapenems has been increased due to production of *Klebsiella pneumoniae* carbapenemase (KPC), which has forced clinicians to search for alternative agents[5-6]. Under these circumstances, colistin has re-emerged as the last-resort treatment against multidrug-resistant (MDR) *Klebsiella pneumoniae*[7]. Colistin is a cationic antimicrobial peptide that

interacts electrostatically with the anionic phosphate groups of lipopolysaccharides in the bacterial outer membrane, displacing divalent cations and disrupting membrane integrity, ultimately leading to cell lysis[8]. Nowadays, it is the most used antimicrobial against carbapenem-resistant *Klebsiella pneumoniae*. Unfortunately, colistin resistance has increasingly been reported worldwide[9]. Alteration in the genes regulating the chemical additions of L-Ara4N and pEtn to the lipid moiety are the most common and well characterized mechanisms of acquired colistin resistance in *Klebsiella pneumoniae*[10]. A key mechanism of colistin resistance involves deleterious mutations in *mgrB*, disrupting the negative feedback of the PhoP/PhoQ two-component system, which leads to overexpression of PhoP-regulated genes and excessive lipid A modifications[11]. In addition, the worldwide spread of plasmid-mediated, transferable colistin resistance (mcr) genes poses a significant threat to the clinical efficacy of colistin[12].

The high rate of colistin resistance among multidrug-resistant *Klebsiella pneumoniae* is alarming and detection method of colistin resistance is also a challenge in clinical diagnosis. Therefore, the present study aimed to determine the rate of colistin resistance among multidrug-resistant *Klebsiella pneumoniae* isolates and to detect the presence of associated colistin resistance genes.

Methodology

Study Settings and Population: This cross-sectional study was carried out in the Department of Microbiology of Dhaka Medical College, Bangladesh during January 2021 to December 2021. Urine, wound swab, blood and sputum samples were collected from clinically suspected infected patients of inpatient department of Dhaka Medical College Hospital (DMCH). A total of 340 samples were received in the Microbiology Department for culture and sensitivity testing. All samples were included in this study regardless of the patient's age, sex and prior antibiotic use, after obtaining written informed consent.

Identification of Klebsiella pneumoniae: *Klebsiella pneumoniae* was identified on the basis of their colony morphology, Gram staining and biochemical tests (catalase, oxidase, urease, indole test, gas production, motility, lactose fermentation and citrate utilization)[13].

Antimicrobial susceptibility tests: All bacterial isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer modified disc-diffusion technique using Mueller-Hinton agar plates following Clinical and Laboratory Standard Institute (CLSI) guideline 2021 and United States Food and Drug Administration (FDA) guideline for tigecycline[14,15]. *Escherichia coli* ATCC 25922 strain was used as control strain to assess the performance of the method. Multidrug-resistant (MDR) isolates were defined as those non-susceptible to at least one agent in three or more antimicrobial categories, according to the European Centre for Disease Control Criteria[16].

Determination of Minimum Inhibitory Concentration of Colistin: Agar dilution method was used to determine the MIC of colistin. Different concentration of colistin ranging from 2 µg/ml to 256 µg/ml were prepared and bacterial inoculum was applied on the agar surface followed by incubation at 37°c overnight.

Table 1: Primers used for detection of colistin resistance genes in this study[19]

Genes		Sequence (5'-3')	size (bp)
phoP	*F*	GAG CGT CAG ACT ACT ATC GA	942
	R	GTT TTC CCA TCT CGC CAG CA	
phoQ	*F*	CCA CAG GAC GTC ATC ACC A	1594
	R	GCA GGT GTC TGA CAG GGA TT	
pmrA	*F*	CGC AGG ATA ATC TGT TCT CCA	808
	R	GGT CCA GGT TTC AGT TGC AA	
pmrB	*F*	GCG AAA AGA TTG GCA AAT CG	659
	R	GGA AAT GCT GGT GGT CAT CTG A	
pmrC	*F*	CTC TCG CCT CGT TCT GAA	140
	R	CGG AGT GGT GTC GAG GAT A	
mgrB	*F*	ACC ACC TCA AAG AGA AGG CGT T	347
	R	GGC GTG ATT TTG ACA CGA ACA C	
mcr1	*F*	CGG TCA GTC CGT TTG TTC	309
	R	CTT GGT CGG TCT GTA GGG	
mcr2	*F*	TGTTGCTTGTGCCGATTGGA	567
	R	AGATGGTATTGTTGGTTGCTG	

Isolates having MIC of ≤2 µg/ml were considered susceptible while MIC of ≥2 µg/ml were considered resistant[17].

Detection of Colistin resistance genes: PCR was used to detect colistin resistance genes (*phoP, phoQ, mgrB, pmrA, pmrB, pmrC, mcr-1, mcr-2*). Genomic DNA extraction was done by boiling method[18].

The following cycling parameters were used: After initial denaturation at 94oC for one minute, the reaction was subjected to 32 cycles (annealing at 57oC for 45 seconds and elongation at 72oC for one minute) followed by final extension at 72oC for 10 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide and visualized under ultraviolet light (Figure 1).

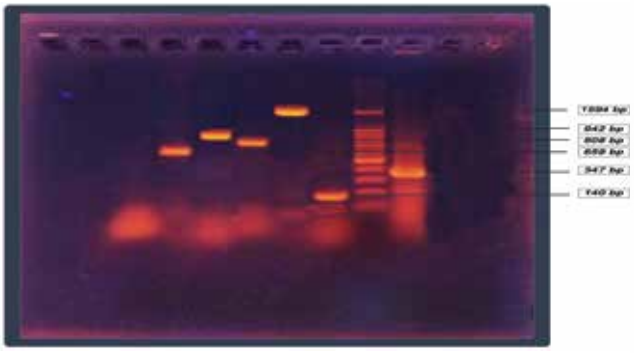

Figure I: Photograph of gel electrophoresis of colistin resistance genes showing amplified DNA of 659 bp for *pmrB* gene (Lane 4), DNA of 942 bp for phoP gene (Lane 5), DNA of 808 bp for *pmrA* gene (Lane 6), DNA of 1594 bp for *phoQ* gene (Lane 7), DNA of 140 bp for *pmrC* gene (Lane 8), 100 bp DNA ladder (Lane 9) and amplified DNA of 347 bp for *mgrB* gene (Lane 10).

Statistical analysis: Statistical analysis was performed with SPSS software, versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY:IBM Corp.). Continuous data that were normally distributed were summarized in terms of the mean, standard deviation, median, minimum, maximum and number of observations, Categorical or discrete data were summarized in terms of frequency counts and percentages.

Ethical Clearance: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration) and with the ethical guidelines of the Institutional research ethics. This research protocol was approved by the Research Review Committee and Ethical Review Committee of Dhaka Medical College (Reference number: MEU-DMC/ERC/2021/144). All participants were informed about the procedure and purpose of the study and assured of the confidentiality of the information provided.

Results

A total of 340 samples were studied, of which 226(66.5%) samples yielded growth in culture. Fifty-five (24.3%) Klebsiella pneumoniae were identified out of 226 culture positive samples. Among 55 isolated Klebsiella pneumoniae, 19(34.6%) were resistant to colistin, of which 7(36.8%) were from wound swab followed by 6(31.6%) from urine, 4(21.1%) from sputum and 2(10.5%) from endotracheal aspirates (Figure II).

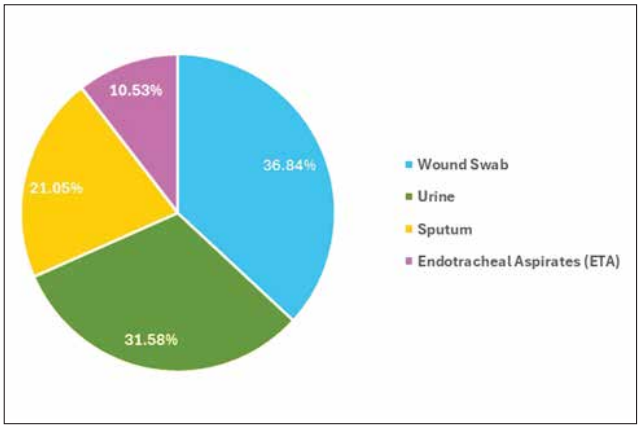

Figure II: Distribution of Colistin resistant *K. pneumoniae* among different samples

Out of 19 colistin-resistant Klebsiella pneumoniae, MIC of colistin ranged from 4 to ≥ 256 µg/ml, of which 4 had MIC ≥ 256 µg/ml, 2 had 128 µg/ml, 4 had 64 µg/ml, 3 had 32 µg/ml and 4 had 16 µg/ml (Table 2).

Table 2: MIC of Colistin among Colistin-Resistant *Klebsiella pneumoniae* (N=19)

MIC of colistin (µg/ml)	Frequency	Percent
≥ 256	4	21.1
128	2	10.5
64	4	21.1
32	3	15.8
16	4	21.1
8	1	5.3
4	1	5.3
≤ 2	0	0.0
Total	**19**	**100.0**

Among 55 isolated *Klebsiella pneumoniae*, 36(65.5%) were detected as MDR, of them all were resistant to cephalosporins, amoxiclav, aztreonam and ciprofloxacin followed by 91.7% to amikacin, 58.3%

to imipenem, 52.8% to colistin and 33.3% to tigecycline (Table 3).

Table 3: Antimicrobial resistance pattern of the isolated MDR *Klebsiella pneumoniae* (N= 36)

Antimicrobial drugs	Frequency	Percent
Amikacin	33	91.7
Amoxiclav	36	100.0
Cefepime	36	100.0
Ceftriaxone	36	100.0
Ceftazidime	36	100.0
Cefuroxime	36	100.0
Ciprofloxacin	36	100.0
Piperacillin-Tazobactam	30	83.3
Aztreonam	36	100.0
Meropenem	23	63.9
Colistin	19	52.8
Tigecycline	12	33.3

Out of 19 colistin-resistant isolates, 15 (78.9%) were positive for mgrB and pmrC, 14 (73.7%) were for pmrB, 13(68.4%) for pmrA, 11 (57.9%) for phoP and 10 (52.6%) were positive for phoQ. No plasmid mediated mcr genes were found in any isolates (Table 4).

Discussion

Infections caused by multidrug-resistant Klebsiella pneumoniae is a major public health concern. The increasing rate of resistance to colistin deteriorates the condition more. In this study, 19(34.5%) colistin-resistant *Klebsiella pneumoniae* were identified. Previous studies in Dhaka Medical College Hospital (DMCH) reported 10.67% colistin resistance in 2017 and 31.8% resistance in 2019 among *Klebsiella pneumoniae*[20-21]. These findings indicate a significant rise of colistin resistance which may be due to increased use of these drugs in clinical settings and the horizontal transfer of resistance genes[21]. This ongoing increase reinforces the need for strict monitoring of colistin resistance. However, most laboratories in Bangladesh still use the disc diffusion method which is not the recommended method whereas agar dilution method is accurate and validated by CLSI guideline[22].

In this study, most of the colistin-resistant organisms were found from wound swab (36.8%) followed by urine (31.6%), sputum (21.05%) and endotracheal aspirates (10.5%) which was in accordance with the study by Pawar et al[23] who reported colistin resistance mostly from pus (42.3%) followed by urine (16.7%) and endotracheal aspirate (9.1%).

All urine samples yielding colistin-resistant *Klebsiella pneumoniae* were collected from catheterized patients in this study. Notably, a previous study reported a higher prevalence of colistin resistance (65.2%) among catheterized patients[24]. According to Richter et al[25] indwelling catheter is one of the risk factors for colistin resistance.

Among the 19 colistin-resistant *Klebsiella pneumoniae* isolates, all exhibited MICs of 4 to ≥256 µg/ml, indicating an increase compared to a previous study at DMCH, where MICs ranged from 4 to 64 µg/ml[20]. In this study, a total of 36(65.5%) multidrug-resistant *Klebsiella pneumoniae* isolates were recovered from various clinical samples, including wound swabs, urine, sputum, ETA and blood. Previous studies have reported high rates of multidrug-resistant *Klebsiella pneumoniae*, including 63.0% in Pakistan[26] and 70.5% in Nepal[27], which are consistent with the findings of the present study. The high level of multidrug resistance may be due to co-existence of extended spectrum beta-lactamases, carbapenemase and colistin resistance genes. Furthermore, prior antibiotic use in hospitals or through self-medication, overuse of antibiotics in livestock and fish farming and poor

Table 4: Distribution of Colistin Resistance Genes Among Colistin-Resistant Klebsiella pneumoniae Isolates Detected by PCR in Different Samples (N= 19)

Genes	W/S* N=7	Urine N=6	Sputum N=4	ETA N=2	Total N=19
mgrB	6(85.7%)	5(83.3%)	2(50.0%)	2(100.0%)	15(78.9%)
PmrA	5(71.4%)	5(83.3%)	2(50.0%)	1(50.0%)	13(68.4%)
pmrB	6(85.7%)	4(66.7%)	2(50.0%)	2(100.0%)	14(73.7%)
pmrC	6(85.7%)	5(83.3%)	2(50.0%)	2(100.0%)	15(78.9%)
phoP	5(71.4%)	4(66.7%)	1(25.0%)	1(50.0%)	11(57.9%)
PhoQ	5(71.4%)	3(50.0%)	1(25.0%)	1(50.0%)	10(52.6%)
mcr1	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
mcr2	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)

N= Total number of colistin-resistant K. pneumoniae in different samples; n= Number of different samples positive for different colistin resistance genes; *W/S= Wound swab; *ETA = Endotracheal aspirates; Here, multiple genes were present in one sample

infection control in health care facilities contribute to the exacerbation of multidrug resistance[28].

In the present study, there is high prevalence of *phoP, phoQ, pmrA, pmrB, pmrC and mgrB among* colistin-resistant *K. pneumoniae* whereas only *mgrB* and *phoQ* were detected in a previous study of DMCH[20]. Plasmid mediated mcr genes were not found in any isolates in this study. In spite of their absence, the isolates were resistant to colistin indicating the clinical importance of chromosomal mutations in the lipid A modifications pathway[29].

Conclusion

The emergence of colistin resistance in multidrug-resistant Klebsiella pneumoniae is alarming, as colistin remains one of the few treatment options. Focus should be placed on infection control, surveillance programs and strong antimicrobial stewardship to prevent the spread of multidrug resistance.

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Conflict of Interest
The authors have no conflicts of interest to disclose.

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Authors' contributions
Farhana Akter conceived and designed the study, analyzed the data, interpreted the results and wrote up the draft manuscript. SM Shamsuzzaman contributed to the analysis of data. Md. Shafayat Hossain Mazumdar and Fouzia Akter were involved in critical review of the manuscript. All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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