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Uncovering Carbapenem Resistance: A Molecular Look at *Klebsiella pneumoniae* in Clinical Samples

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Abstract

The rise of multidrug-resistant (MDR) *Klebsiella pneumoniae* has greatly complicated the management of infections caused by this pathogen. This study aimed to assess the antimicrobial resistance patterns and to detect the presence of β-lactamase genes in both MDR and non-MDR *K. pneumoniae* isolates obtained from a variety of clinical specimens. A total of 50 *K. pneumoniae* isolates were collected and identified using standard microbiological techniques. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Phenotypic detection of extended-spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs), and carbapenemases was carried out via the double-disk synergy test, combined disk test, and modified Hodge test, respectively. Detection of resistance and virulence genes at the molecular level was conducted using polymerase chain reaction (PCR). Multidrug resistance was observed in 50% of the isolates, with high levels of resistance to β-lactam antibiotics, carbapenems, ciprofloxacin, piperacillin, and tazobactam. Notably, 28% of the isolates also exhibited resistance to colistin. ESBL was identified phenotypically in 30% of the isolates. NDM-1 (12%), NDM-5 (2%), VIM (4%), and KPC (8%) were detected among β-lactamase genes. These findings indicate a high prevalence of MDR *K. pneumoniae* in Bangladesh, posing a serious challenge for infection control and treatment strategies in healthcare settings.

Keywords: ESBL; *Klebsiella pneumoniae*; MDR; Bangladesh; Antibiotic resistance

Introduction

Carbapenems are a group of broad-spectrum β-lactam antibiotics commonly prescribed as a last resort for treating infections caused by multidrug-resistant Gram-negative bacteria. However, the emergence of carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) has become a major concern in hospitals worldwide due to their link to severe infections and high morbidity and mortality rates (1). Clinical isolates of *K. pneumoniae* are known for producing a range of β-lactamase enzymes and are intrinsically resistant to certain antibiotics such as ampicillin and amoxicillin (2). The increasing frequency and rapid spread of resistant strains present a growing clinical challenge. Resistance to β-lactam antibiotics in this species is primarily due to the production of enzymes like extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases, and carbapenemases. CR-Kp isolates often exhibit resistance not only to carbapenems but also to multiple other antibiotic classes, including penicillins, third-generation cephalosporins,

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fluoroquinolones, and aminoglycosides. The prevalence of these resistant strains varies by region and is influenced by local infection control practices and antimicrobial stewardship efforts (3). Of particular concern are emerging pan-resistant strains of CR-Kp, which leave no effective antibiotic options and pose a serious global health threat. Understanding the molecular epidemiology and resistance mechanisms of these strains is critical for identifying potential alternative treatments and controlling their spread (4).

One of the primary mechanisms of carbapenem resistance in *K. pneumoniae* involves the production of carbapenemase enzymes (5). These enzymes are categorized into four Ambler classes—A, B, C, and D—based on their molecular structure. Among them, classes A (e.g., KPC), B (e.g., NDM, VIM, IMP), and D (e.g., OXA-48-like enzymes) are particularly relevant in clinical settings (6). It is not uncommon for a single strain to harbor multiple β-lactamase genes, which may contribute to its high adaptability and resistance profile (7). These genes are often co-located on mobile genetic elements like plasmids and transposons, facilitating their transfer and persistence across bacterial populations (5). In this study, we sought to characterize the β-lactamase genes present in carbapenem-resistant *K. pneumoniae* isolates collected from clinical samples in Bangladesh. Understanding these genetic resistance patterns is essential for informing treatment strategies and for curbing the spread of carbapenem-resistant Gram-negative bacteria.

Materials and Methods

Study Design and Setting

A cross-sectional study was carried out in the Department of Microbiology and Immunology at Dhaka Medical College Hospital (DMCH), Dhaka, Bangladesh. The research was conducted over one year, from January to December 2022.

Isolation and Identification of *Klebsiella pneumoniae*

Clinical specimens, including wound swabs, urine, wound swabs, pus, tracheal aspirates, sputum, blood, and other body fluids, submitted to the Microbiology Laboratory at DMCH were processed for bacterial isolation. A total of 50 consecutive, non-duplicate isolates of *K. pneumoniae* were obtained from hospitalized patients during the study period. Identification of *K. pneumoniae* began with observing colony morphology on MacConkey agar, followed by Gram staining and standard biochemical tests. These included catalase and oxidase activity, urease production, indole reaction, gas formation, motility testing, citrate utilization, and lactose fermentation. For quality control, the reference strain *K. pneumoniae* ATCC 700603 was used during culture, biochemical testing, and phenotypic confirmation of clinical isolates. The study received ethical approval from the institutional review board of Dhaka Medical Colleg, and written informed consent was secured from all participating patients.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility profiles of the *Klebsiella pneumoniae* isolates were determined using the standard Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Oxoid Ltd., UK) (8). A range of commercially prepared antibiotic discs was employed, including amoxicillin-clavulanic acid (20/10 µg), piperacillin-tazobactam (100/10 µg), cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), amikacin (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), tigecycline (15 µg), and aztreonam (30 µg). Interpretation of inhibition zones was carried out under guidelines established by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). To identify ESBL-producing strains, the double-disk synergy test was applied. Furthermore, isolates were categorized as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) based on criteria outlined by the Centers for Disease Control and Prevention (CDC) (9, 10).

Definition of Multidrug-Resistant (MDR) *Klebsiella pneumoniae*

In this study, *Klebsiella pneumoniae* isolates were classified as multidrug-resistant (MDR) if they demonstrated resistance to at least one antimicrobial agent in three or more distinct classes of antibiotics. These classes included, but were not limited to, antipseudomonal penicillins, aminoglycosides, carbapenems, first and second-generation cephalosporins, extended-spectrum cephalosporins, third and fourth-generation cephalosporins, fluoroquinolones, β-lactam/β-lactamase inhibitor combinations, and penicillins, following CDC-defined criteria (10).

Phenotypic Detection of Resistance Mechanisms

Detection of ESBL Production Using the Double Disk Synergy Test (DDST)

ESBL activity was evaluated phenotypically using the double disk synergy test (DDST) on Mueller-Hinton Agar (MHA). In this method, disks containing third-generation cephalosporins, specifically ceftriaxone and ceftazidime (30 µg each), were positioned 20 mm apart from a central disk of amoxicillin-clavulanic acid (20/10 µg). An enhancement of the inhibition zone around either cephalosporin disk in relation to the clavulanate-containing disk was interpreted as an ESBL production. A distinct "keyhole" or "champagne cork" shaped zone confirmed the presence of synergy between the antibiotics, supporting the ESBL positive phenotype (10) (Fig. 1).

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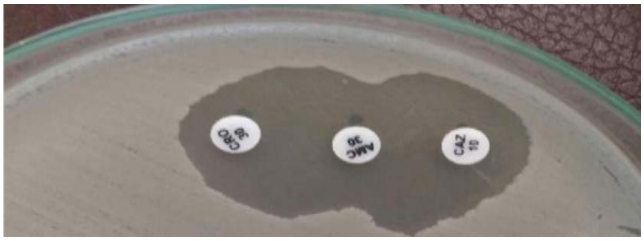

Figure 1: Detection of ESBL by double disc synergy test (DDST)

Detection of Carbapenemase Production Using the Modified Hodge Test (MHT)

The Modified Hodge Test (MHT) was employed to screen for carbapenemase activity in the *Klebsiella pneumoniae* isolates. A suspension of *Escherichia coli* ATCC 25922 was prepared and adjusted to a 0.5 McFarland turbidity standard. Using a sterile cotton swab, the suspension was uniformly spread over the surface of a Mueller-Hinton agar plate to form a lawn culture. The inoculated plate was then left at room temperature for approximately 15 minutes to allow for surface drying. An imipenem (10 µg) disk was placed at the center of the agar plate. Test isolates were streaked in a straight line from the edge of the imipenem disk outward toward the edge of the plate. After overnight incubation at 37 °C, plates were examined for enhanced growth of *E. coli* along the streaked line toward the imipenem disk. A cloverleaf-like indentation in the inhibition zone around the disk at the point of intersection was considered a positive result, indicating carbapenemase production by the test organism (11) (Fig. 2).

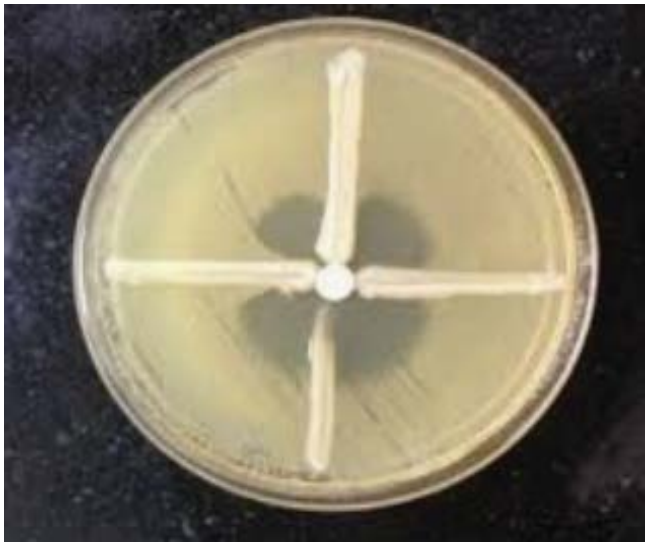

Figure 2: Detection of carbapenemase by Modified Hodge test (MHT)

Combined Disc Test with Imipenem-EDTA for Detection of Metallo-β-Lactamase (MBL)

To screen for the presence of metallo-β-lactamase (MBL) production, the combined disc test using imipenem and imipenem-EDTA was performed. The test isolate was adjusted to a 0.5 McFarland turbidity standard and uniformly inoculated onto Mueller-Hinton agar using a sterile swab. Two antibiotic discs were placed on the agar surface: one containing imipenem (10 µg) and the other containing imipenem combined with EDTA (10/750 µg). The plates were incubated at 37 °C for 18–24 hours. An increase of ≥7 mm in the diameter of the inhibition zone surrounding the imipenem-EDTA disc compared to the imipenem-only disc was interpreted as indicative of MBL production by the isolate (12, 13) (Fig. 3).

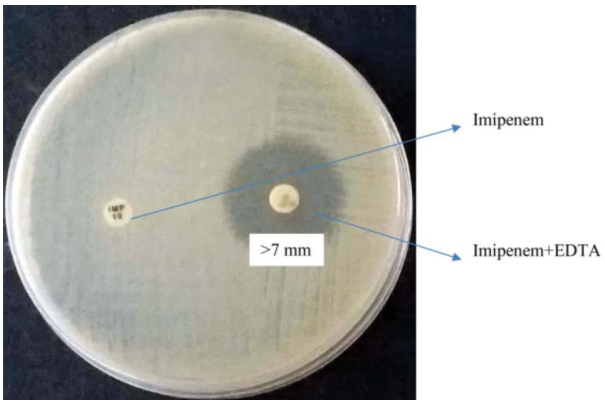

Figure 3: Detection of metallo β-lactamase by Imipenem-EDTA combined disc test

Detection of β-Lactamase Genes by Polymerase Chain Reaction (PCR)

Conventional polymerase chain reaction (PCR) was employed to detect carbapenemase (*KPC*) and metallo-β-lactamase (*NDM*, *VIM*) genes in *Klebsiella pneumoniae* isolates. Genomic DNA was extracted using the boiling method. Amplified PCR products were visualized by agarose gel electrophoresis to confirm the presence of target genes (14,15).

Data Analysis

All collected data were analyzed using IBM SPSS Statistics version 23. Categorical variables were summarized as frequencies and percentages. Graphs and visualizations were generated using the matplotlib library in Python.

Results

Out of a total of 350 clinical specimens analyzed, the majority were wound swabs and pus (n = 149), followed by urine samples (n = 91), sputum (n = 42), blood (n = 46), and endotracheal aspirates (n = 22). Of these, 226 samples (64.57%) showed positive bacterial growth upon culture, as detailed in **Table 1**. The distribution of bacterial species identified from these 226 culture-positive samples is presented in **Table 2**.

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Table 1: Positive culture results from various clinical samples (N = 350).

Samples	Number of Samples	Culture Positive n (%)
Wound swab and pus	149	115 (77.18)
Urine	91	45 (49.45)
Sputum	42	23 (54.76)
ETA	22	19 (86.36)
Blood	46	24 (52.17)
Total	350	226 (64.57)

Table 2: The distribution of organisms isolated from various samples (*n* = 226).

Organism	*n* (%)
Escherichia coli	54 (23.89)
Pseudomonas aeruginosa	51 (22.57)
Klebsiella pneumoniae	50 (22.12)
Klebsiella oxytoca	2 (0.88)
Acinetobacter baumanii	13 (5.75)
Enterobacter cloacae	8 (3.54)
Enterobacter aerogenes	6 (2.65)
Citrobacter freundii	2 (0.88)
Citrobacter koseri	1 (0.44)
Proteus mirabilis	5 (2.22)
Proteus vulgaris	3 (1.34)
Gram-positive bacteria	31 (13.72)
Total	226 (100.00)

The antimicrobial resistance profiles of the *Klebsiella pneumoniae* isolates are summarized in **Table 3**. Of the 50 isolates tested, resistance to cefuroxime was observed in 66% (n = 33), followed closely by ceftriaxone, with 62% (n = 32) of isolates showing resistance. Fosfomycin exhibited the highest susceptibility among the tested antibiotics, with only 12% (n = 6) of isolates demonstrating resistance. Notably, resistance to colistin was identified in 28% (n = 14) of the isolates. The dissemination of resistance categories amid the isolates is presented in **Table 4**. Half of the *K. pneumoniae* isolates (n = 25, 50%) were classified as multidrug-resistant (MDR), while 18% (n = 9) met the criteria for extensively drug resistant (XDR) status. Two isolates (4%) were identified as pandrug resistant (PDR), and 15 isolates (30%) were confirmed as ESBL producers, as illustrated in **Figure 4**.

Phenotypic detection of resistance mechanisms among *Klebsiella pneumoniae* isolates is summarized in **Table 5**. Of the 50 isolates examined, extended-spectrum β-lactamase (ESBL) production was identified in 15 isolates (30.0%) using the double disc synergy test (DDST). Metallo-β-lactamase (MBL) activity was detected in 11 isolates (22.0%) through

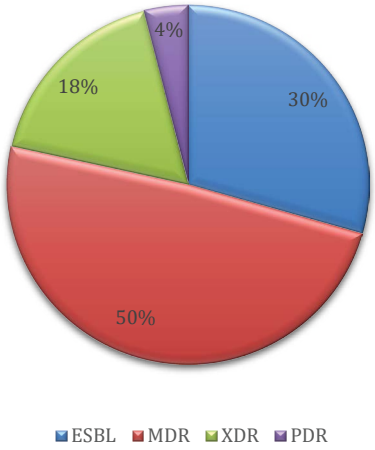

Figure 4: Antimicrobial resistance pattern

Table 3: Antimicrobial resistance pattern among isolated *Klebsiella pneumoniae* (*n*=50)

Antimicrobial drugs	Resistance, *n* (%)
Amikacin	23 (46.0)
Amoxiclav	24 (48.0)
Cefoxitin	24 (48.0)
Cefuroxime	33 (66.0)
Ceftazidime	30 (60.0)
Ceftriaxone	32 (64.0)
Cefepime	25 (50.0)
Ciprofloxacin	26 (52.0)
Piperacillin/Tazobactam	18 (36.0)
Aztreonam	31 (62.0)
Imipenem	11 (22.0)
Tigecycline	9 (18.0)
Fosfomycin	6 (12.0)
Colistin	14 (28.0)

Table 4: Classification of *Klebsiella pneumoniae* isolates by resistance profile, including MDR, XDR, PDR, and ESBL producers (n = 50)

Type of resistance	*n* (%)
ESBL	15(30.0)
MDR	25(50.0)
XDR	9(18.0)
PDR	2 (4.0)

the combined disc (CD) test with imipenem and EDTA. Additionally, carbapenemase production was confirmed in 8 isolates (16.0%) using the Modified Hodge Test (MHT).

Among the 50 *Klebsiella pneumoniae* isolates analyzed, metallo-β-lactamase (MBL) genes were detected in 11

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isolates (22.0%). Of these, six isolates (12.0%) carried the *NDM-1*gene, one isolate (2.0%) harbored *NDM-5*, and two isolates (4.0%) tested positive for the *VIM* gene. In addition, carbapenemase genes were identified in 8 isolates (16.0%), with *KPC* detected in four of them (8.0%), as detailed in **Table 6**.

Table 5: Phenotypic detection of β-Lactamase production in *Klebsiella pneumoniae* isolates (n = 50)

Detection Method	Positive Isolates, n (%)
Double Disc Synergy Test (ESBL)	15 (30.0%)
Combined Disc Assay (MBL)	11 (22.0%)
Modified Hodge Test (Carbapenemase)	8 (16.0%)

Table 6: Distribution of MBL and carbapenemase genes detected in *Klebsiella pneumoniae* isolates (n = 50)

Gene Type	Gene	Positive Isolates, n	Percentage (%)
Metallo-β-lactamase (MBL)	NDM-1	6	12
	NDM-5	1	2
	VIM	2	4
	Total (MBL)	9	18
Carbapenemase	KPC	4	8
	Total	4	8

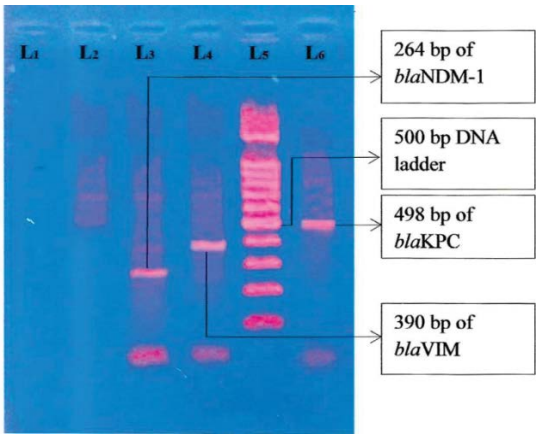
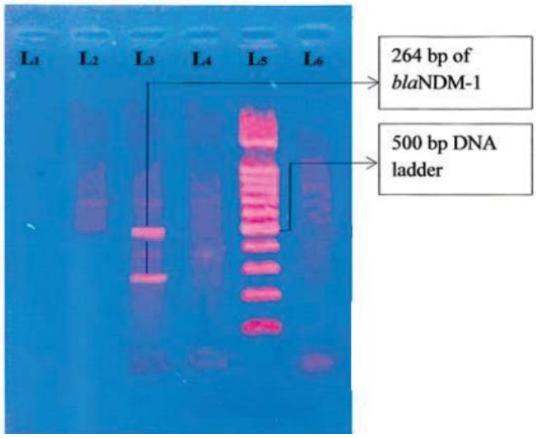


Figure 5: Photograph of gel electrophoresis; negative control without DNA (TE buffer) (lane one), negative control Escherichia coli ATCC 25922 (lane 2), amplified DNA of 264 bp for blaNDM-1 (lane-3), DNA of 390 bp of blaVIM (lane 4), DNA of 498 bp of blaKPC (lane 6) in imipenem-resistant *K. pneumoniae*, hundred bp DNA ladder (lane 5).

Discussion

In this study, 226 out of 350 non-duplicate clinical specimens (64.57%) showed positive bacterial growth. Among the culture-positive cases, *Escherichia coli* was the most frequently isolated organism, accounting for 23.89%,



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followed by *Pseudomonas aeruginosa* (22.57%) and *Klebsiella pneumoniae* (22.12%). This distribution aligns with similar findings from a study in India, where 56.9% of clinical samples yielded bacterial growth (16). The prevalence of *K. pneumoniae* observed in this study is consistent with earlier reports from Bangladesh, which documented rates of 24% in the northeastern region and 19.72% in the southeast (17,18). Among the 50 *K. pneumoniae* isolates analyzed, half (n = 25; 50%) were identified as multidrug-resistant (MDR). This proportion is comparable to findings from a study in Indonesia by Nirwati *et al*., where 54.49% of isolates were MDR (19). However, a considerably higher MDR prevalence (84.37%) was reported in Iraq by Aljanaby *et al.* (20), indicating geographical variability in resistance patterns. The resistance profile of the isolates showed elevated resistance to second through fourth-generation cephalosporins, aztreonam, and ciprofloxacin. Similar resistance trends were previously reported in Bangladesh, where *K. pneumoniae* isolates displayed high resistance to cefuroxime, ceftriaxone, ceftazidime, cefepime, and ciprofloxacin (21,22).

In our study, the rates of resistance were notable: cefuroxime (66%), ceftriaxone (64%), aztreonam (62%), ceftazidime (60%), ciprofloxacin (52%), and cefepime (50%). These findings are in line with results from Kawser et al., who reported comparable resistance rates (23). Resistance to last-resort antibiotics, including colistin (28%), imipenem (22%), tigecycline (18%), and fosfomycin (12%), was also observed. Colistin resistance, in particular, has shown an upward trend in Bangladesh, with a previous systematic review reporting median resistance rates of 18.8%, ranging from 0% to 21.4% (22,24). This increase is likely influenced by the clinical overuse of colistin and horizontal transfer of resistance genes via mobile genetic elements. Phenotypic screening

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for extended-spectrum β-lactamase (ESBL) production using the double-disc synergy test (DDST) identified 15 isolates (30%) as ESBL producers. This result aligns with a study by Chakraborty *et al.* in Bangladesh, which reported a 45% ESBL rate in *K. pneumoniae* isolates(25), while a lower rate of 17% was observed in a study from Pakistan by Riaz *et al* (26). Metallo-β-lactamase (MBL) production was detected in 11 isolates (22%) through the combined disc assay. This is notably lower than the 85.7% MBL detection rate among imipenem-resistant isolates reported by Farzana *et al* (27). The discrepancy may be due to the inclusion of all *K. pneumoniae* isolates in the current study, rather than only imipenem non-susceptible ones, which may have lowered the overall detection rate.

Carbapenemase activity was identified in 8 isolates (16%) using the Modified Hodge Test (MHT). This detection rate is lower than those reported in studies by Metwally and Eftekhar, which found carbapenemase production in 70% and 90% of isolates, respectively (28,29). The sensitivity and specificity of the MHT can vary by region and testing conditions, and its limitations have been noted in previous literature, including by Nordmann *et al* (30). Molecular analysis revealed that 22% of the isolates carried MBL genes. Specifically, *NDM-1* was detected in 12% of isolates, and *NDM-5* in 2%. These findings are consistent with previous studies conducted in Bangladesh, where Farzana *et al*. and Rahman *et al.* reported *NDM* gene presence in 22.86% and 20.51% of isolates, respectively (31,32). The *VIM* gene was found in 4% of isolates. As previously described by Queenan *et al., VIM* and *IMP* genes are more frequently associated with *P. aeruginosa* and are less commonly detected in Enterobacteriaceae (33). Carbapenemase gene detection revealed that 8 isolates (16%) harbored such genes, with *KPC* identified in 4 isolates (8%). This is somewhat lower than the 25% prevalence reported by Sattar et al. in a study of *K. pneumoniae* isolates (34).

Conclusion

Most of the *Klebsiella pneumoniae* isolates were multidrug-resistant in this study. There are limited therapeutic options to treat MDR *Klebsiella pneumoniae,* and this manifests a gloomy scenario for the management of hospital infections in Bangladesh; it further bears the necessity of necessary surveillance system to deal with the inevitable healthcare disaster in the offing.

Ethical Clearance

Ethical approval for this study was granted by the Institutional Review Board.

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Conflicts of Interest

The authors declare no conflicts of interest.

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