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Characterizing the co-existence of metallo-β-lactamase-producing and extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates in community wastewater samples of Dhaka, Bangladesh

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ABSTRACT

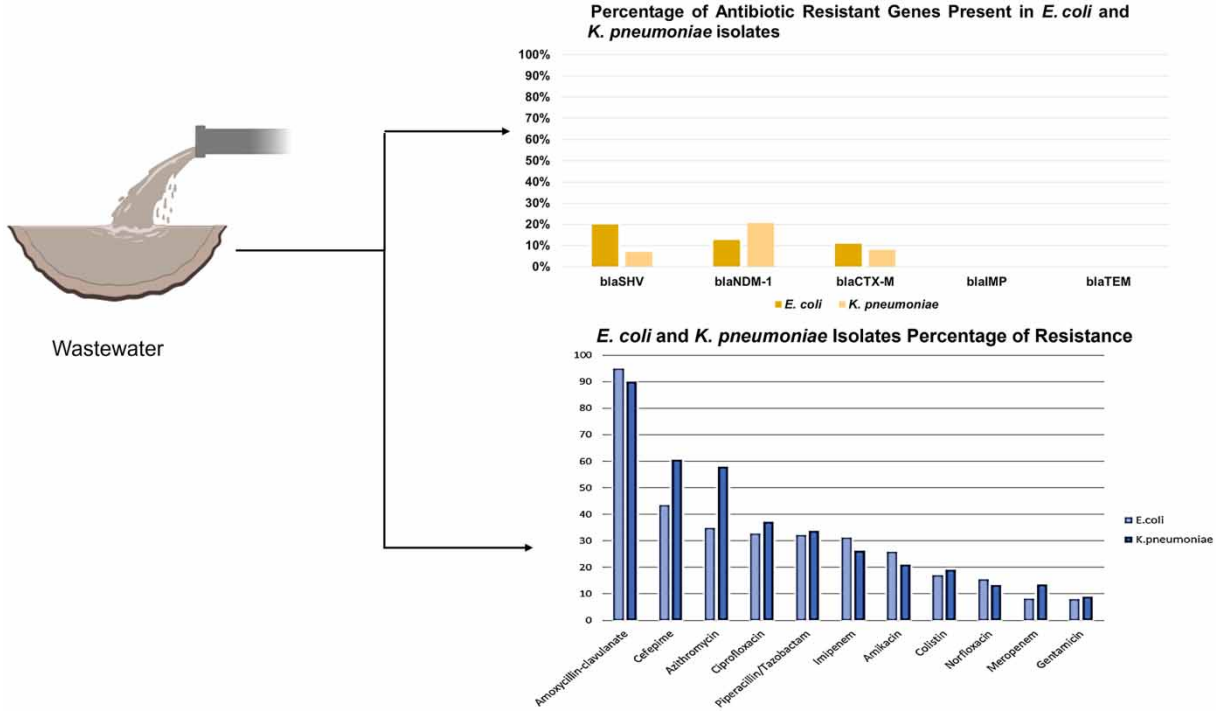
Escherichia coli and *Klebsiella pneumoniae* isolates with multiple antibiotic-resistance genes in wastewater pose serious public health risks, as they can potentially contaminate the food and water supply. The main aim of this study was to isolate and identify *E. coli* and *K. pneumoniae* from community wastewater samples, and determine their antibiotic-resistance profiles and their antibiotic-resistant genes. From the northern part of Dhaka, Bangladesh, 36 wastewater samples were collected across 11 different areas, which were then serially diluted, and cultured using selective media. Isolates were identified via polymerase chain reaction. Out of the 197 isolates identified, *E. coli* and *K. pneumoniae* accounted for 55.8% ($n = 110$) and 44.2% ($n = 87$), respectively. Antibiotic susceptibility tests revealed multidrug resistance (MDR) in 30% of *E. coli* and 35.56% of *K. pneumoniae* isolates. Among *E. coli*, the prevalence of antibiotic-resistance genes included $bla_{\text{NDM-1}}$ (8.9%), bla_{SHV} (13.9%), and $bla_{\text{CTX-M}}$ (7.6%). In *K. pneumoniae*, the percentages were $bla_{\text{NDM-1}}$ (12.8%), bla_{SHV} (4.3%), and $bla_{\text{CTX-M}}$ (5.0%). Co-existence of multiple antibiotic-resistance genes was observed in 4.54% of *E. coli* isolates ($n = 5$) and 5.74% of *K. pneumoniae* isolates ($n = 5$). This suggests the escalating issue of infectious species becoming increasingly resistant to antibiotics in wastewater systems.

Key words: antimicrobial-resistant bacteria, extended-spectrum β-lactamase (ESBL), Gram-negative bacteria, metallo-β-lactamase (MBL), multidrug resistance and resistant genes

HIGHLIGHTS

- *E. coli* and *K. pneumoniae* isolated from community wastewater samples.
- Multidrug resistance detected in 30% of *E. coli* and 35.56% of *K. pneumoniae*.
- *E. coli* antibiotic-resistance genes: $bla_{\text{NDM-1}}$ (8.9%), bla_{SHV} (13.9%), and $bla_{\text{CTX-M}}$ (7.6%).
- *K. pneumoniae* antibiotic-resistance genes: $bla_{\text{NDM-1}}$ (12.8%), bla_{SHV} (4.3%), and $bla_{\text{CTX-M}}$ (5.0%).
- Co-existence of multiple antibiotic-resistance genes: 4.54% in *E. coli* and 5.74% in *K. pneumoniae*.

GRAPHICAL ABSTRACT



INTRODUCTION

Antibiotics and their metabolites are excreted into wastewater through urine and feces. In addition to the impact of antibiotics, resistant bacteria are also directly introduced into the wastewater. Sewage, particularly raw sewage and activated sludge, has a high bacterial density, providing an environment where microorganisms can access a large pool of mobile genes that transfer between bacterial cells via horizontal and vertical gene transfer. These genes spread through bacterial populations via plasmids and various mobile genetic elements, such as transposons or integrons, which carry resistance genes to other antimicrobial agents. The presence of these genes in municipal wastewater may indicate a clinical origin and is significant because wastewater treatment plants (WWTPs) serve as hotspots for antibiotic-resistant bacteria (ARB) and resistance genes. As a result, they can act as early warning indicators of the health of the human population (Korzeniewska & Harnisz 2013). Studies have shown that multidrug-resistant (MDR) bacterial strains can be found in wastewater from hospitals, urban areas, and agricultural runoff. This wastewater can contaminate rivers, lakes, and irrigation systems used for food production, facilitating the spread of resistant strains through crops or drinking water sources (Triggiano *et al.* 2020; Kusi *et al.* 2022). For instance, a review of the environmental impact of extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *K. pneumoniae* found that these pathogens can persist in agricultural environments and food supply chains, potentially affecting humans through the consumption of contaminated food or water (Husna *et al.* 2023). Infections caused by Gram-negative bacteria producing metallo-β-lactamase (MBL) and extended-spectrum *β*-lactamase (ESBL) can be particularly hazardous, given their resistance to a broad spectrum of antibiotics (Kumar *et al.* 2015). The simultaneous presence of MBL and ESBL genes in these pathogens' limits treatment options, even in case of β-lactams (Bush & Bradford 2020; Ahmed & Asghar 2021). Improperly treated wastewater, especially from hospital sources, increases the risk of exchanging antibiotic resistance genes among these microorganisms, potentially leading to environmental spread that could eventually reach communities (Korzeniewska & Harnisz 2013).

The presence of ARB and their genes within a population can be predicted by wastewater surveillance (WWS), which involves monitoring municipal sewage. This process is also cost-effective. Municipal sewage is an ideal material for monitoring ARB because it collects bacterial biomass from a variety of sources. This includes pathogens at different stages of infection, contributed through feces, urine, nasal mucus, skin, and sputum from households, hospitals, and nursing homes. Since WWS does not involve sampling individuals, it minimizes legal and ethical challenges and avoids privacy concerns.

Moreover, WWS allows for real-time monitoring and early prediction of infection outbreaks, offering insights into the prevalence of MDR pathogens at the population level. Additionally, WWS offers the potential for real-time monitoring and early prediction of infection outbreaks, providing insights into the prevalence of MDR pathogens at a population level (Tiwari *et al.* 2022).

Various studies conducted worldwide have revealed the widespread presence of *E. coli* and *K. pneumoniae* in diverse wastewater environments. In Bangladesh, these bacteria were isolated from hospital sewerage disposal points, clinical wastewater, household wastewater, and natural water bodies (Adnan *et al.* 2013; Rabbani *et al.* 2017; Rahman *et al.* 2021; Asaduzzaman *et al.* 2022). Similarly, in Poland, wastewater samples contained *E. coli* and *Klebsiella* spp. (Korzeniewska & Harnisz 2013), while in Romania, *K. pneumoniae* strains were identified in WWTPs (Surleac *et al.* 2020). Investigations in Oregon (Khorshidi-Zadeh *et al.* 2021), Texas (Mukherjee *et al.* 2021), and Ireland (Cahill *et al.* 2019) demonstrated the presence of *E. coli* in different stages of wastewater treatment, surface water samples, hospital effluent, and municipal wastewater, respectively. Moreover, South African research focused on *E. coli* isolates obtained from various points within a wastewater treatment plant and its associated waters (Mbanga *et al.* 2021).

The discovery of MBLs occurred through the detection of the bla_{IMP} gene in clinically isolated samples (Senda *et al.* 1996; Laraki *et al.* 1999). A larger threat emerged with the later discovery of the $bla_{\text{NDM-1}}$ gene that produced the New Delhi metallo β-lactamase-1 (NDM-1) enzyme, a novel β-lactamase that was usually found alongside other genes that rendered pathogens resistant to almost all antibiotics. Most commonly found in *Klebsiella pneumoniae* and *Escherichia coli,* the $bla_{\text{NDM-1}}$ gene is easily transferable between *Enterobacteriaceae* (Moellering 2010).

MBLs and ESBLs pose a threat to public health; thus, it is important to detect and prevent their environmental spread. NDM-1, originating from India, has already been disseminated globally, with a risk of high transmission levels to neighboring countries, such as Bangladesh (Kumarasamy *et al.* 2010; Islam *et al.* 2012).

Bangladesh is a densely populated country, with Dhaka being one of the most densely populated cities in the world. The water distribution system is at high risk of contamination, as it can easily be polluted by the wastewater system if the two merge. This contamination can also affect the food supply chain. Despite the significant risk of contamination to the water supply, very few studies have been conducted in Bangladesh to address this critical issue (Bashar & Fung 2020). Previous studies have been conducted in Bangladesh regarding the presence of both MBL and ESBL-producing pathogens and the presence of $bla_{\text{CTX-M-1}}$ and $bla_{\text{NDM-1}}$ genes on wastewater samples (Islam *et al.* 2012; Adnan *et al.* 2013; Rabbani *et al.* 2017; Asaduzzaman *et al.* 2022). At present there have been no studies conducted in Bangladesh to assess the presence of $bla_{\text{NDM-1}}$, bla_{IMP}, bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$ genes, as well as their combinations, in *E. coli* and *K. pneumoniae* found in community wastewater samples. This is concerning given the high prevalence of pathogenic enteric bacteria, particularly in Dhaka, where regular waste and toilet waste are often merged in the wastewater system and can affect the water supply system. The present study aimed to detect the current prevalence and characterize the antibiotic resistances of ESBL and carbapenemase-producing *K. pneumoniae* and *E. coli* in wastewater of different community areas of Dhaka, Bangladesh. It also aims to confirm the molecular presence of MBL determinants, $bla_{\text{NDM-1}}$, bla_{IMP}, as well as ESBL genes, bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$, and map out their co-existence patterns within the resistant isolates.

MATERIALS AND METHODS

Sample collection and processing

From December 2021 to April 2022, a total of 36 samples of wastewater were collected from four cluster areas, consisting of 11 sampling sites, within the Dhaka Metropolitan Area, Bangladesh. Particular attention was given to Dhaka, where wastewater and toilet waste are often combined in the drainage system, possibly contaminating the water supply. The selected areas are Kachukhet, Kafrul, Mohakhali, Mohakhali DOHS, Banani, Banani DOHS, Gulshan, Shapla Housing, Baitul Aman Housing, Korail, and Niketon. They encompass the northern part of Dhaka, and represent a diverse range of socioeconomic backgrounds. Areas with a nearby water supply system and an open drainage system were focused on to assess the potential contamination risks. All the samples have been identified depending on the closer location and divided into four different clusters (Figure 1). From each area three or four samples were taken. This has been described in detail in Supplementary material, Table S1. The samples were taken from open surface sewer drains and natural drainage canals linked to the city's rivers and lakes. A minimum of 200 ml of each sample was collected in sterile 500 ml containers by lowering them into the wastewater collection points, a process described by Cahill *et al.* (2019). These were immediately transported to

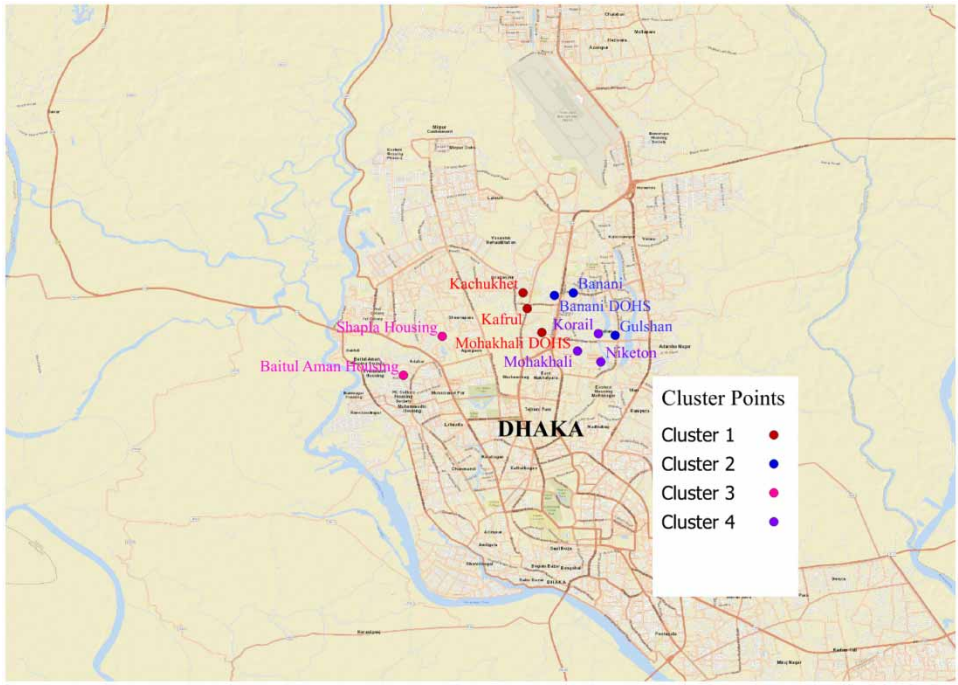


Figure 1 | Maps of the sample collection area. Samples are divided into four clusters. Red indicates Cluster 1, blue indicates cluster 2, pink indicates cluster 3, and purple indicates cluster 4.

the laboratory maintained at 4 °C to avoid any microbial growth, and kept at this temperature until processed. Within 4 h it was centrifuged to remove debris and processed for microbiological and molecular analysis.

Statistical analysis

To evaluate the significance of differences in resistance patterns across various antibiotics and regions of Dhaka, as well as between *E. coli* and *K. pneumoniae* isolates, a two-way analysis of variance (ANOVA) was conducted using SPSS 29.0 for Windows (IBM). Estimated marginal means were performed to identify statistically significant combinations. A *p*-value of less than 0.05 was considered statistically significant for all tests.

Bacterial isolation

Samples were serially diluted and plated onto HiCrome™ UTI Agar and EMB Agar for incubation at 37 °C for 24 h. Isolates were preliminarily identified based on colony morphology.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the 197 isolates was tested using the Kirby–Bauer Disk Diffusion method (Hudzicki 2012). Antibiotic discs were used for gentamicin (10 μg), amoxicillin (30 μg), piperacillin/tazobactam (100/10 μg), imipenem (10 μg), meropenem (10 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), amikacin (30 μg), cefepime (30 μg), azithromycin (15 μg), and colistin (10 μg). The tested isolates were classified as sensitive (S), intermediate (I), and resistant (R) based on inhibition zone measurements in accordance with Clinical Laboratory Standards Institute (CLSI) 2023 as shown in Supplementary material, Table S2 (Standards & Testing 2023). Colistin resistance was determined at zone diameter $\leq$11 mm and sensitive at $\geq$14 mm (a limitation is that these parameters may be subject to error) (Gales Reis & Jones 2001).

Phenotypic selection of NDM and ESBL producers

Carbapenemase production was detected by resistance to imipenem and meropenem antibiotics according to CLSI standards as shown in Supplementary material, Table S2. Isolates were cultured onto HiChrome ESBL agar, containing supplements to differentiate ESBL-producing organisms. Isolates showing growth were suspected of producing ESBL enzymes. This was phenotypically confirmed by double-disk synergy testing with cefotaxime (30 μg) and cefotaxime clauvanate (40 μg).

DNA extraction and amplification

The confirmation of both isolates *E. coli* and *K. pneumoniae* were done by polymerase chain reaction (PCR) with ECO and KP primers (Supplementary material, Table S3 and Figures S1 and S2). DNA extraction was carried out for all suspected carbapenemase and ESBL-producing isolates. These were further examined through gene amplification by conventional PCR method and identification through gel electrophoresis for MBL encoding genes, $bla_{\text{NDM-1}}$, and bla_{IMP}, as well as ESBL encoding genes, bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$ (Supplementary material, Figures S3–S5). Primers used for the study have been detailed in Supplementary material, Table S3.

RESULTS

In the study, among 36 samples from four different cluster regions, a total of 110 (55.8%) colonies were isolated from *E. coli,* and 87 (44.2%) colonies were isolated from *K. pneumoniae* (Table 1).

Both species were identified in all samples collected with differing prevalences between the regions as shown in Table 1. This table mentions the highest percentage of *E. coli* isolates were obtained from region 1, whereas the highest percentage of *K. pneumoniae* isolates were obtained from region 2. Here, *N* defines the isolate numbers of *E. coli* and *K. pneumoniae.*

Antibiotic resistance profiles

Antibiotic susceptibility tests were performed using the antibiotics previously mentioned, with the results shown in Figure 2. Table 2 presents the antibiotic resistance patterns across different areas for *E. coli*. Similarly, Table 3 shows the resistance rates across different areas for *K. pneumoniae*. The two-way ANOVA revealed a statistically significant difference, indicating that both the region and the antibiotic choice significantly affect resistance percentages for both *E. coli* and *K. pneumoniae*.

The trend for resistance against the antibiotics was similar for both *E. coli* and *K. pneumoniae*, and results were recorded as shown in Figure 2. This was confirmed through statistical analysis using two-way ANOVA, which revealed no significant difference in antibiotic resistance between *E. coli* and *K. pneumoniae* for the antibiotics tested. The highest resistance observed towards amoxicillin–clavulanate (95 and 89.86%), and the least resistance for gentamicin (8.05 and 8.87%). Two carbapenems of the β-lactam class were tested where both species displayed a higher antibiotic resistance capability towards imipenem, $n = 34$ (*E. coli*) and $n = 22$ (*K. pneumoniae*), compared to meropenem, $n = 7$ and 10, which instead had the highest number of isolates that were sensitive (85.88%) (Supplementary material, Table S4).

In this study, six different classes of antibiotics were selected; β-lactams, cephalosporins, fluoroquinolones, aminoglycosides, macrolides, and polymyxins. The percentage of resistance of *E. coli* and *K. pneumoniae* isolates to different antibiotics is shown in Figure 2.

Although a full consensus has not been reached for defining MDR organisms, being resistant to three or more antimicrobial classes has been used by a majority of studies (Magiorakos *et al.* 2012; Mahato *et al.* 2019). According to this, we can conclude that 30% of *E. coli* and 35.56% of *K. pneumoniae* isolated in the study displayed MDR properties. Bacterial resistance to different classes of antibiotics and their multidrug resistance is shown in Figure 3.

Phenotypic characteristics

ESBL characteristics were observed in 20.81% of isolates tested ($n = 41$), confirmed by double-disk synergy testing. Among these isolates, *E. coli* showed a higher phenotype for ESBL production at 63.64%, while *K. pneumoniae* showed a phenotype

Table 1 | Distribution of *E. coli* and *K. pneumoniae* isolates across different sampling regions

		***E. coli* isolates**		***K. pneumoniae* isolates**	
Region	**No. of samples**	***N***	**%**	***N***	**%**
1	9	31	28.18	17	19.54
2	10	31	28.18	41	47.12
3	11	35	31.81	18	20.68
4	6	13	11.81	11	12.64
Total	36	110	55.83	87	44.16

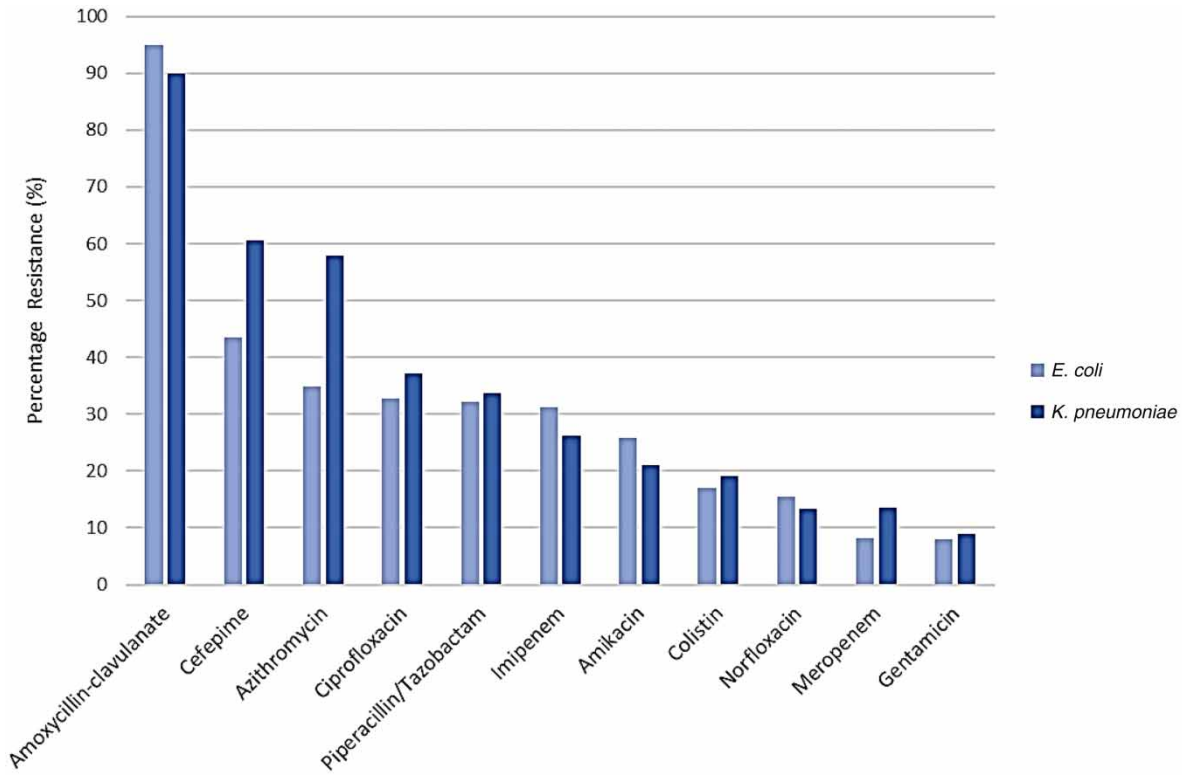


Figure 2 | Percentage of resistant *E. coli* and *K. pneumoniae* isolates for each antibiotic tested.

distribution of 50%. MBL phenotypes were determined by β-lactam resistance to either imipenem or meropenem in all the colonies showed 63.60 and 36.4% for the two species as seen in Figure 4.

Genetic profile and co-existence of genes

Among 110 isolates of *E. coli*, five showed the co-existence of genes. Five different primers were employed in the experiment, targeting ESBL and MBL-resistant genes. The investigation revealed the absence of bla_{IMP} and bla_{TEM} genes**.** Four *E. coli* isolates showed the co-existence of two genes $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M}}$ and one *E. coli* isolate showed the co-existence of three genes $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M}}$ and bla_{SHV}. Five of 87 isolates of *K. pneumoniae* showed co-existence with different types of genes. Genes $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M}}$ was found in three *K. pneumoniae* and $bla_{\text{CTX-M}}$ & bla_{SHV} was found in two of them. In total, 10 isolates co-existent with resistant genes were detected. This is described in detail in Table 4.

DISCUSSION

Raw wastewater is considered the most abundant water habitat known for antibiotic resistance genes (Vaz-Moreira Nunes & Manaia 2014).

Due to the rising resistance to cephalosporin antibiotics in *E. coli,* carbapenem usage has increased which has led to the development of carbapenem resistance with the emergence of ESBL and MBL in the community. Carbapenem resistance to *E. coli* has seriously threatened the human healthcare system. A new problem in the management of bacterial infections is the formation of NDM-1 which confers resistance to carbapenem drugs (Kumarasamy *et al.* 2010). Bangladesh is a highly populated country with limited wastewater treatment facilities allowing the spread of these Gram-negative pathogens in large numbers (Rabbani *et al.* 2017). The scientific community has made numerous mentions of how wastewater treatment facilities contribute to the spread of antimicrobial resistance and multidrug resistance (Mukherjee *et al.* 2021). The consequence of the cumulative impact of several mechanisms of resistance leads to the emergence of multidrug-resistant bacterial pathogens which can cause extremely life-threatening diseases.

Table 2 | Area-wise percentage of resistance to different antibiotics for *E. coli* isolates

	Name of antibiotics										
Name of area	**Gentamicin**	**Piperacillin/Tazobactam**	**Imipenem**	**Meropenem**	**Amoxicillin–clavulanate**	**Cefepime**	**Ciprofloxacin**	**Norfloxacin**	**Amikacin**	**Azithromycin**	**Colistin**
Banani DOHS	15.38%	0%*	15.38%*	7.69%	92.31%*	100%*	0%	0%	38.46%*	0%	0%*
Banani	16.66%	75%*	25%*	16.66%*	100%*	91.66%*	41.66%*	0%*	25%*	16.66%*	8.33%*
Gulshan	0%	16.66%*	0%*	50%	83.33%*	33.33%*	33.33%	0%	0%*	66.66%	33.33%*
Kafrul	11.11%	38.89%*	16.67%*	16.67%	100%*	38.88%*	22.22%	0%	11.11%*	33.33%	16.67%*
Kochukhet	0%	0%	0%	0%	0%*	0%	0%	16.67%	0%	0%	0%
Mohakhali DOHS	0%	57.14%*	28.57%*	14.29%	100%*	0%*	28.57%	0%	0%	14.29%	14.29%*
Shapla Housing	0%	11.11%	33.33%	0%	33.33%*	0%*	11.11%	0%	0%	0%	0%
Baitul Aman Housing	0%	8.33%*	50%	0%*	0%*	8.33%*	8.33%	0%	58.33%	8.33%	16.67%
Korail	0%	16.67%*	33.33%	0%	100%*	33.33%*	0%	0%	16.67%	0%	0%
Mohakhali	0%	100%*	0%*	0%*	100%*	100%*	0%*	0%*	0%*	0%*	100%*
Niketon	0%*	75%*	50%*	0%*	100%*	25%*	0%*	75%*	75%*	0%*	50%*

*Indicates the combinations that are statistically significant.

Table 3 | Area-wise percentage of resistance to different antibiotics for *K. pneumoniae* isolates

	Name of antibiotics										
Name of area	**Gentamicin**	**Piperacillin/Tazobactam**	**Imipenem**	**Meropenem**	**Amoxicillin–clavulanate**	**Cefepime**	**Ciprofloxacin**	**Norfloxacin**	**Amikacin**	**Azithromycin**	**Colistin**
Banani DOHS	15.38%	0%	0.154%	0.08%	0.92%*	1%	0%	0%	0.38%	0%	0%
Banani	0.17%*	75%*	25%*	16.66%*	100%*	91.66%*	41.66%*	0%*	25%*	16.66%*	8.33%*
Gulshan	0%*	16.66%*	0%*	50%*	83.33%*	33.33%*	33.33%*	0%*	0%*	66.66%	33.33%*
Kafrul	11.11%*	38.89%*	16.67%*	16.67%*	100%*	0.39%*	22.22%*	0%*	11.11%*	33.33%	0.17%*
Kochukhet	0%	0%	0%	0%	0%*	0%	0%	0.17%	0%	0%	0%
Mohakhali DOHS	0%	0.57%	0.29%	0.14%	1%*	0%	0.29%	0%	0%	0.14%	0.14%
Shapla Housing	0%	0.11%	0.33%	0%	0.33%	0%	0.11%	0%	0%	0%	0%
Baitul Aman Housing	0%	0.08%	0.50%	0%	0%*	0.08%	0.08%	0%	0.58%	0.08%	0.17%
Korail	0%	0.17%	0.33%	0%	1%*	0.33%	0%	0%	0.17%	0%	0%
Mohakhali	0%	1%	0%	0%	1%*	1%	0%	0%	0%	0%	1%
Niketon	0%	0.75%	0.50%	0%	1%*	0.25%	0%	0.75%	0.75%	0%	0.50%

*Indicates the combinations that are statistically significant.

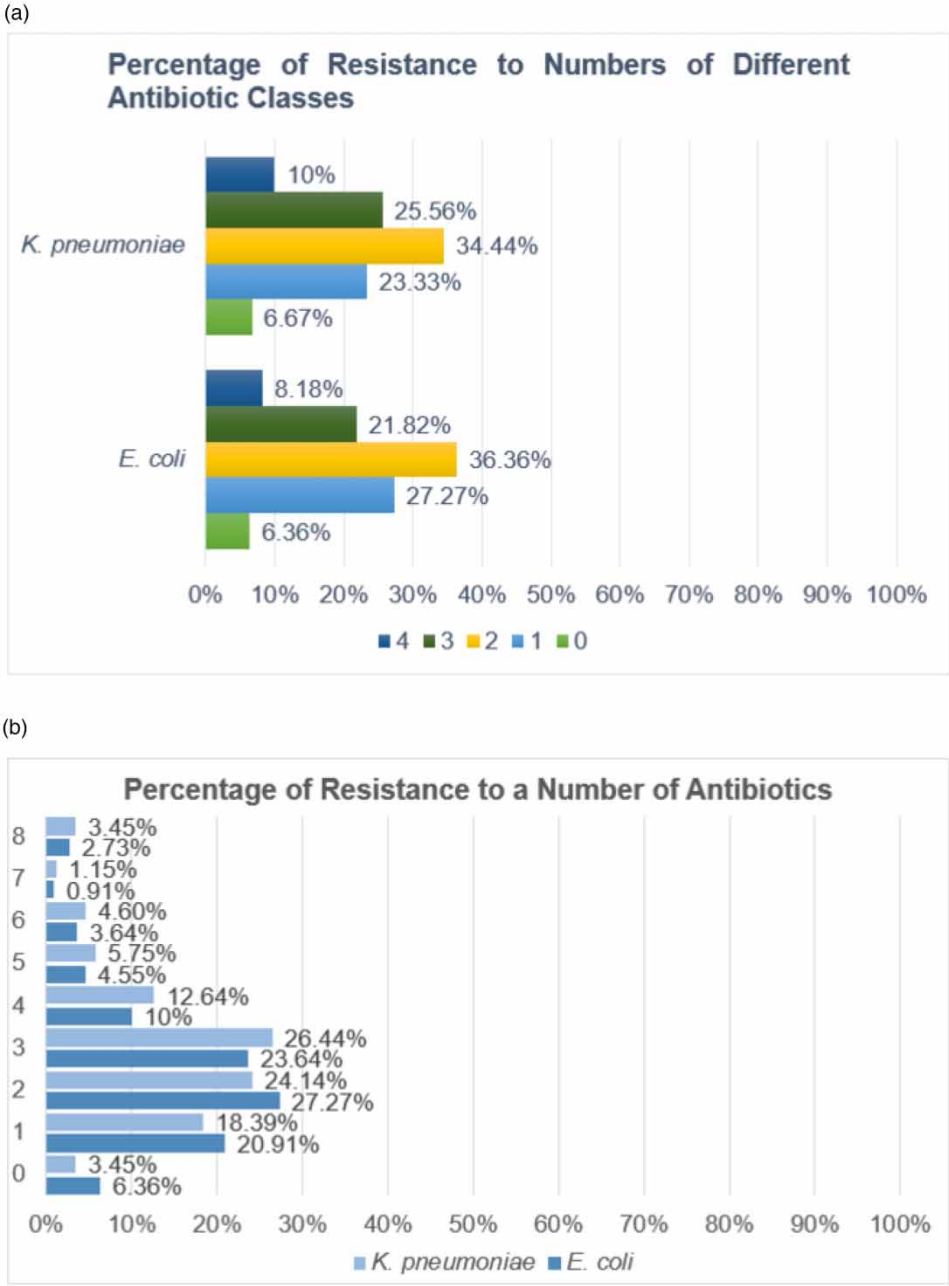


Figure 3 | The prevalence of bacterial resistance to different antibiotic classes was recorded. (a) Percentage of *E. coli* and *K. pneumoniae* resistant to different numbers of antibiotic classes that displays the relative proportion of MDR isolates. (b) Percentage of *E. coli* and *K. pneumoniae* resistant to different numbers of individual antibiotics tested.

In this study, among 36 samples from four different cluster regions, a total of 110 (55.8%) colonies were isolated from *E. coli*, and 87 (44.2%) colonies were isolated from *K. pneumoniae* (Table 1).

Antibiotic susceptibility tests in this study revealed similar resistance trends for both *E. coli* and *K. pneumoniae*, with the highest resistance observed towards amoxicillin–clavulanate (95% for *E. coli* and 89.86% for *K. pneumoniae*), and the lowest resistance towards gentamicin (8.05% for *E. coli* and 8.87% for *K. pneumoniae*) (Supplementary material, Table S4). Notably, both species exhibited higher resistance to imipenem (*E. coli*: $n = 34$; *K. pneumoniae:* $n = 22$) compared to meropenem, where the latter showed the highest sensitivity with 85.88% of isolates being sensitive. Overall, *K. pneumoniae* displayed

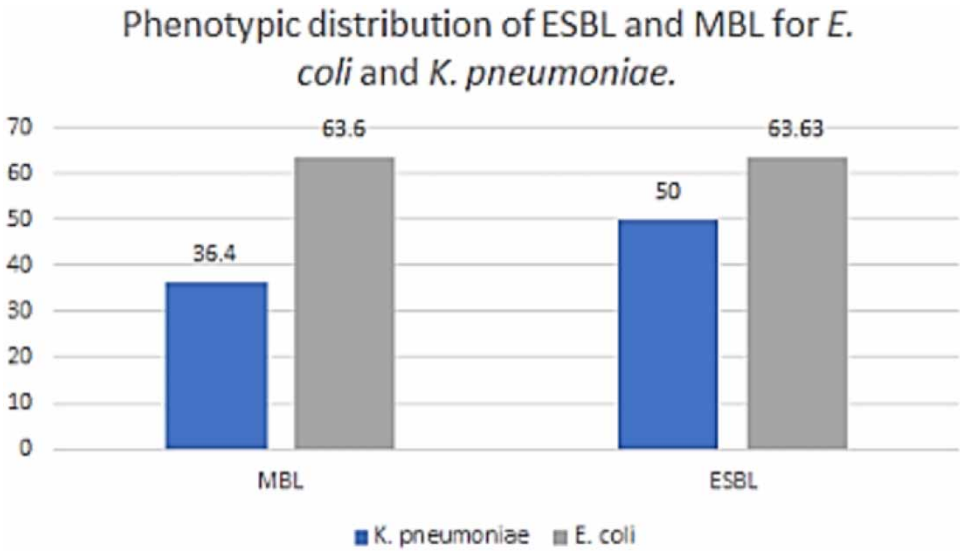


Figure 4 | Phenotypical characterization of ESBL- and MBL-producing *E. coli* and *K. pneumoniae*.

Table 4 | Co-occurrence of ESBL and MBL genes in *E. coli* and *K. pneumoniae* isolates

Isolates	bla_{NDM-1} + bla_{CTX-M}	bla_{CTX-M} + bla_{SHV}	bla_{NDM-1} + bla_{CTX-M} + bla_{SHV}
E. coli	4 (3.63%)	0 (0%)	1 (0.9%)
K. pneumoniae	3 (3.44%)	2 (2.29%)	0 (0%)
Total	7 (3.5%)	2 (1.01%)	1 (0.50%)

higher resistance percentages compared to *E. coli*, with both exhibiting elevated resistance rates to β-lactams (*E. coli*: 87.16%; *K. pneumoniae*: 89.29%) and lower resistance to polymyxin (*E. coli*: 16.98%; *K. pneumoniae*: 19.05%). Additionally, 60.49% of *K. pneumoniae* and 43.43% of *E. coli* isolates were resistant to cephalosporin (Supplementary material, Table S5).

In this study, *E. coli* and *K. pneumoniae* isolates detected from wastewater showed gentamicin resistance levels of 8.05 and 8.97%, respectively. Previous research in Dhaka found resistance rates of 28% for *E. coli* and 10% for *K. pneumoniae* in wastewater (Rabbani *et al*. 2017). However, in study carried out in Noakhali, it was observed that none of the *E. coli* isolates from wastewater showed resistance to gentamicin but among the *Klebsiella* spp. isolates, 60% were found to be resistant to gentamicin (Rahman *et al.* 2021). A Texas study found 3% gentamicin resistance in *E. coli* from wastewater (Mukherjee *et al.* 2021). In Ireland, carbapenemase-producing *K. pneumoniae* from wastewater showed 75% resistance to gentamicin, while carbapenemase-producing *E. coli* was fully susceptible (Cahill *et al.* 2019). Finally, in Burkina Faso, gentamicin resistance rates were 8.1% for *E. coli* and 7.4% for *K. pneumoniae* from wastewater which corresponds with the findings of our study (Kagambèga *et al.* 2024).

In this study, the prevalence of resistance to piperacillin/tazobactam among *E. coli* and *K. pneumoniae* isolates from wastewater was notable, with 32.11 and 33.72% resistance rates, respectively. Comparatively, a study conducted in Romania reported an alarming 100% resistance rate among *K. pneumoniae* strains isolated from wastewater (Surleac *et al.* 2020). On the other hand, a study from Burkina Faso revealed much lower resistance rates, with only 1.4% of *E. coli* and 1.5% of *K. pneumoniae* isolated from wastewater showing resistance to piperacillin/tazobactam (Kagambèga *et al.* 2024).

In the current study, it was found that 31.19% of *E. coli* and 26.19% of *K. pneumoniae* isolates from wastewater exhibited resistance to imipenem. In a study conducted in Dhaka, lower resistance rates were observed, with 12% of *E. coli* and 7% of *K. pneumoniae* showing resistance to imipenem (Rabbani *et al.* 2017). In another study conducted in Bangladesh it was observed that all *Escherichia* spp. isolates were sensitive to imipenem (Adnan *et al.* 2013). Conversely, a study from Romania reported substantially higher resistance rates, with 82.35% *K. pneumoniae* isolates from wastewater being resistant to

imipenem, (Surleac *et al.* 2020). In contrast, studies from Oregon (Khorshidi-Zadeh *et al.* 2021) and Texas (Mukherjee *et al.* 2021) revealed much lower resistance rates among *E. coli* isolates, with only 0.27 and 0% resistance to imipenem, respectively. Similarly, studies from Burkina Faso reported no resistance to imipenem among *E. coli* and *K. pneumoniae* isolated from wastewater (Kagambèga *et al.* 2024).

In the current study, resistance rates to meropenem were recorded at 8.24% for *E. coli* and 13.51% for *K. pneumoniae*, indicating a noteworthy but not overwhelming level of resistance within these bacterial populations. Comparatively, a study from Dhaka reported a slightly higher resistance rate among *E. coli* isolates, with 13% showing resistance to meropenem (Rabbani *et al.* 2017). In Romania, significantly elevated resistance rates were observed, with 88.23% of *K. pneumoniae* isolates from wastewater, being resistant to meropenem (Surleac *et al.* 2020). Additionally, in Ireland, all *E. coli* and *K. pneumoniae* isolates showed resistance to meropenem (Cahill *et al.* 2019).

In this study, 95% of *E. coli* and 89.86% of *K. pneumoniae* isolates showed resistance to amoxicillin–clavulanate. These results are consistent with findings from Bangladesh, where all *Escherichia* spp. isolates detected from wastewater displayed resistance to amoxicillin, highlighting a widespread resistance issue (Adnan *et al.* 2013). Similarly, in Romania, high resistance rates were observed among *K. pneumoniae* isolates (88.23%) from wastewater, (Surleac *et al.* 2020). However, studies from South Africa and Burkina Faso reported lower but still significant resistance rates among *E. coli* and *K. pneumoniae* isolates. In South Africa, 33.33% of *E. coli* isolates were resistant (Mbanga *et al.* 2021), while in Burkina Faso, 8.1% of *E. coli* and 17.9% of *K. pneumoniae* isolates showed resistance (Kagambèga *et al.* 2024). Additionally, in Nigeria, 58.3% of *E. coli* isolated from wastewater were resistant to amoxicillin–clavulanate (Adekanmbi *et al.* 2020).

In this study, concerning levels of resistance were reported, to cefepime with 43.43% of *E. coli* and 60.49% of *K. pneumoniae* isolates demonstrating resistance. These results coincide with observations from Bangladesh, where high resistance rates were noted among *E. coli* isolates (74%) and *K. pneumoniae* isolates (38–45%) to cephalosporins (Rabbani *et al.* 2017). In another study conducted in Bangladesh, it was noted that all *E. coli* isolates from wastewater were resistant to cefepime, while 20% of *Klebsiella* spp. were resistant to the same antibiotic (Rahman *et al.* 2021). Furthermore, a separate study from Bangladesh reported a 50% resistance rate among *Escherichia* spp. isolates to cephalosporins (Adnan *et al.* 2013). Similarly, previous research from Romania documented alarming rates of resistance among *K. pneumoniae* isolates to cefepime, reaching 100% from wastewater (Surleac *et al.* 2020). In contrast, studies from Texas (Mukherjee *et al.* 2021) and Ireland (Cahill *et al.* 2019) revealed significant levels of resistance among *E. coli* (95–100%) and *K. pneumoniae* (75%) isolates to cephalosporins. Notably, in Burkina Faso, substantial resistance rates were observed, with 51.4% of *E. coli* isolates and 67.4% of *K. pneumoniae* isolates being resistant to cefepime (Kagambèga *et al.* 2024). Moreover, in Nigeria, high resistance rates ranging from 75 to 100% were reported among *E. coli* isolates to cephalosporins (Adekanmbi *et al.* 2020).

In this study *E. coli* and *K. pneumoniae* isolates detected from wastewater was 32.76 and 37.25% resistant to ciprofloxacin, respectively. In Bangladesh, one study noted that 71% of *E. coli* and 48% of *K. pneumoniae* isolates were resistant to ciprofloxacin (Rabbani *et al.* 2017). In another study conducted in Bangladesh, it was found that *E. coli* isolated from wastewater showed an 83.33% resistance rate to ciprofloxacin, while *Klebsiella* spp. exhibited 100% resistance to the same antibiotic (Rahman *et al.* 2021). Another study in Bangladesh found that 50% of *Escherichia* spp. isolated from wastewater exhibited resistance to ciprofloxacin (Adnan *et al.* 2013). Similarly, in Romania, research indicated high levels of resistance, with 82.35% of *K. pneumoniae* isolates from wastewater being resistant to ciprofloxacin (Surleac *et al.* 2020). Contrastingly, studies in Oregon and South Africa reported lower resistance rates among *E. coli* isolates, with 20.5 and 16.6% resistance, respectively (Khorshidi-Zadeh *et al.* 2021; Mbanga *et al.* 2021). Further variations were observed in Texas, where only 4% of *E. coli* isolates from wastewater were resistant to ciprofloxacin (Mukherjee *et al.* 2021), and in Burkina Faso, with 32.4% of *E. coli* and 34.7% of *K. pneumoniae* showing resistance to the antibiotic (Kagambèga *et al.* 2024). Additionally, a study in Nigeria found that 20.8% of *E. coli* isolated from wastewater exhibited resistance to ciprofloxacin (Adekanmbi *et al.* 2020).

In this study *E. coli* and *K. pneumoniae* isolates detected from wastewater was 15.38 and 13.33% resistant to norfloxacin, respectively. Comparatively, a study in South Africa reported lower resistance rates, with *E. coli* exhibiting a resistance rate of 4.9% to norfloxacin (Adefisoye & Okoh 2016), while *K. pneumoniae* showed a slightly higher resistance rate of 13.8% (Okafor & Nwodo 2023). In Iran, a higher resistance rate of 30% was noted among *E. coli* isolates from wastewater (Ranjbar & Farahani 2017). However, a study in Brazil presented a contrasting finding, reporting no resistance among *K. pneumoniae* isolates detected from wastewater (Nakamura-Silva *et al.* 2023).

The current study revealed resistance rates of 25.74% for *E. coli* and 20.99% for *K. pneumoniae* to amikacin. A previous study conducted in Bangladesh showed that *E. coli* isolated from wastewater exhibited a resistance rate of 33.33% to

amikacin, while *Klebsiella* spp. showed a resistance rate of 40% to the same antibiotic (Rahman *et al.* 2021). Notably, a study in Bangladesh, along with studies conducted in Burkina Faso and Brazil, reported no resistance among *Escherichia* spp. isolates to amikacin (Adnan *et al.* 2013; Nakamura-Silva *et al.* 2023; Kagambèga *et al.* 2024). Similarly, studies in South Africa indicated low resistance rates among *E. coli* (0.5%) (Adefisoye & Okoh 2016) but higher rates among *K. pneumoniae* (39.3%) (Okafor & Nwodo 2023). However, findings from Romania painted a different picture, showing high levels of resistance, with 76.47% of *K. pneumoniae* isolates from wastewater being resistant (Surleac *et al.* 2020).

In this study, resistance to azithromycin was observed in both *E. coli* and *K. pneumoniae* isolates detected from wastewater, with rates of 34.78 and 57.89%, respectively. Similarly, in a study conducted in Bangladesh, high levels of resistance were found, with *E. coli* and *Klebsiella* spp. isolated from wastewater demonstrating resistance rates of 66.67 and 80%, respectively (Rahman *et al.* 2021). Another study in Bangladesh reported a resistance rate of 25% among *Escherichia* spp. isolated from wastewater (Adnan *et al.* 2013). Contrastingly, a previous study conducted in South Africa found a lower resistance rate, with only 8.33% of *E. coli* isolates from wastewater being resistant to azithromycin (Mbanga *et al.* 2021). Additionally, in a study conducted in Nepal, *E. coli* isolated from wastewater showed no resistance to azithromycin while *Klebsiella* spp. showed a resistance rate of 16.7% (Mahato *et al.* 2019). Finally, in Burkina Faso, *E. coli* and *K. pneumoniae* isolated from wastewater exhibited resistance rates of 68.69 and 35.59%, respectively, to azithromycin (Garba 2023).

In this study, *E. coli* and *K. pneumoniae* isolates detected from wastewater was 16.98 and 19.0% resistant to colistin, respectively. This finding is notable given previous research conducted in South Africa. One study observed a lower resistance rate of 6.3% among *E. coli* isolated from wastewater (Adefisoye & Okoh 2016), while another reported a significantly higher resistance rate of 58.51% (Igwaran *et al.* 2018). Additionally, a separate study found an even higher resistance rate of 76.5% among *E. coli* isolates from wastewater (Adegoke *et al.* 2020). The situation appears somewhat different in Pakistan, where a study observed lower resistance rates among *E. coli* (7.69%) and *K. pneumoniae* (7.14%) isolated from community wastewater (Rizwan *et al.* 2022).

Although a full consensus has not been reached for defining MDR organisms, being resistant to 3 or more antimicrobial classes has been used by a majority of studies (Magiorakos *et al.* 2012; Mahato *et al.* 2019). According to this, our findings revealed that 35.63% ($n = 31$) of the *K. pneumoniae* and 30% ($n = 33$) of the *E. coli* isolates showed MDR characteristics. WHO classifies MDR *K. pneumoniae* as a priority pathogen because it spreads widely in the environment and causes nosocomial and opportunistic infections in hospitals. In Bangladesh, a previous study reported alarming rates of MDR, with 61% of *E. coli* and 52% of *K. pneumoniae* isolates from wastewater exhibiting multidrug resistance (Rabbani *et al.* 2017). Similarly, another study in Bangladesh found notable MDR rates of *E. coli* (20%) and *Klebsiella* spp. (16.67%) in wastewater samples (Rahman *et al.* 2021). Moving to other regions, a study in Oregon observed a concerning 27.2% of *E. coli* isolates with MDR properties (Khorshidi-Zadeh *et al.* 2021). In Texas, a study reported a lower MDR rate of 3% among *E. coli* isolated from wastewater (Mukherjee *et al.* 2021). A study in Burkina Faso documented high MDR rates of 83.33% for E. *coli* and 24.4% for *K. pneumoniae* in untreated wastewater (Kagambèga *et al.* 2024). Another study in Burkina Faso further emphasized the universal multidrug resistance among both *E. coli* and *K. pneumoniae* isolates from wastewater (Garba 2023). In Nigeria, a study reported a substantial 63.15% of *E. coli* isolated from wastewater exhibiting multidrug resistance (Adekanmbi *et al.* 2020). Meanwhile, a study in South Africa revealed that a staggering 82.7% of *K. pneumoniae* isolates from wastewater were resistant to three or more classes of antibiotics (Okafor & Nwodo 2023). A study in Romania also highlighted the severity of the issue, reporting about 55.4% of *K. pneumoniae* isolates exhibiting MDR characteristics in wastewater samples (Surleac *et al.* 2020). The increased rate of multidrug-resistant pathogens carrying the co-existence of the resistant gene of different classes of β-lactamase and carbapenemase, results in a significant challenge in disease treatment.

ESBL characteristics were observed in 20.81% of isolates tested ($n = 41$), confirmed by double-disk synergy testing in our study. Amongst these isolates, *E. coli* showed a higher phenotype for ESBL production at 63.64%, while *K. pneumoniae* showed a phenotype distribution of 50%. MBL phenotypes were determined by β-lactam resistance to either imipenem or meropenem in all the colonies showed 63.60 and 36.40% for the two species as seen in Figure 4 in this study. In Bangladesh, a study found a notably high percentage (90%) of ESBL-producing *E. coli* in wastewater samples (Asaduzzaman *et al.* 2022). Similarly, in Nigeria, ESBL characteristics were observed in 63.15% of *E. coli* isolates from wastewater (Adekanmbi *et al.* 2020). Studies in Burkina Faso also presented varying rates, with one study (Kagambèga *et al.* 2024) reporting 28% of *E. coli* and 36% of *K. pneumoniae* isolates exhibiting ESBL characteristics, while another study reported much higher rates of 93.5% for *E. coli* and 95% for *K. pneumoniae* (Garba 2023). In Nepal, ESBL isolates were prevalent in 57.1% of *E. coli* and 33.3% of *Klebsiella* spp. (Mahato *et al.* 2019). Another study conducted in Poland identified ESBL-positive strains

predominantly in *E. coli* (82.9%) and to a lesser extent in *Klebsiella* (3.9%) from wastewater samples (Korzeniewska & Harnisz 2013). Regarding MBL phenotypes, Burkina Faso studies revealed 15.74% of *E. coli* isolates and 8.19% of *K. pneumoniae* isolates exhibiting MBL characteristics in one study (Garba 2023), while another reported a higher prevalence of 76.6% MBL phenotype in *K. pneumoniae* isolates (Okafor & Nwodo 2023). Furthermore, in Romania, a significant proportion (82.35%) of *K. pneumoniae* isolates from wastewater displayed MBL phenotype (Surleac *et al.* 2020).

In our study, we investigated the presence of various β-lactamase genes, namely bla_{SHV}, bla_{NDM-1}, bla_{CTX-M}, bla_{IMP}, and bla_{TEM}, in both *E. coli* and *K. pneumoniae* isolates. We observed distinct patterns in the distribution of these genes among the isolates. For the bla_{SHV} gene, it was found to be present in 20% of *E. coli* isolates and 6.89% of *K. pneumoniae* isolates. In contrast, the bla_{NDM-1} gene showed higher prevalence rates, with 12.72% of *E. coli* isolates and 20.68% of *K. pneumoniae* isolates testing positive. The bla_{CTX-M} gene was also detected, albeit at lower rates, in 10.9% of *E. coli* isolates and 8% of *K. pneumoniae* isolates. Notably, our study did not detect the presence of bla_{IMP} and bla_{TEM} genes in any of the isolates examined (Supplementary material, Table S6). Comparing with other studies, a study in Bangladesh noted high prevalence rates of bla_{CTX-M}-1 and bla_{NDM-1}. Specifically, from wastewater of rural households, 60% of isolates possessed the bla_{CTX-M}-1 gene and 35% harbored the bla_{NDM-1} gene. In comparison, *E. coli* isolated from wastewater of poultry farms showed lower percentages, with 43% carrying bla_{CTX-M}-1 and 33% possessing bla_{NDM-1}. *E. coli* from the wastewater of urban food markets exhibited higher prevalence rates, with 83% positive for bla_{CTX-M}-1 and 58% positive for bla_{NDM-1} (Asaduzzaman *et al.* 2022). In Burkina Faso, 9.5% of *E. coli* and 58.9% of *K. pneumoniae* isolates harbored the bla_{SHV} gene, while bla_{CTX-M} genes were detected in 38.7% of *E. coli* and 26.3% of *K. pneumoniae* isolates (Kagambèga *et al.* 2024). In Nigeria, $bla_{CTX-M-1}$ and $bla_{CTX-M-9}$ genes were prevalent in 63.15% of *E. coli* isolates, with 36.84% possessing $bla_{CTX-M-2}$, $bla_{CTX-M-8}$, and $bla_{CTX-M-25}$ genes. Additionally, half of the isolates carried the bla_{TEM} gene, while bla_{NDM-1} and bla_{SHV} genes were absent (Adekanmbi *et al.* 2020). Studies in South Africa revealed varying prevalence rates, with bla_{TEM} detected in 56.4% (Adefisoye & Okoh 2016) and 84.2% (Adegoke *et al.* 2020) of *E. coli* isolates from wastewater. Moreover, bla_{CTX-M} was present in 52.6% of *E. coli* isolates, bla_{CTX-M} was found alongside bla_{TEM} (84.2%) and bla_{NDM-1} (15.8%) in *E. coli* isolates, with no bla_{SHV} detected (Adegoke *et al.* 2020). In another study conducted in South Africa, *K. pneumoniae* isolates from wastewater exhibited bla_{TEM} and bla_{SHV} in 17 and 13% of β-lactam resistant isolates, respectively, and bla_{IMP} in 9.8% of isolates (Okafor & Nwodo 2023). In Pakistan, 40.62% of *E. coli* and 25% of *K. pneumoniae* isolates from wastewater were found to possess the bla_{CTX-M} gene (Rizwan *et al.* 2022). These findings underscore the diverse distribution of β-lactamase genes in wastewater isolates, emphasizing the importance of regional surveillance for effective antimicrobial management.

Several studies have shown that β-lactamase genes, particularly those that code for ESBLs (TEM, SHV, CTX-M) and KPC, NDM, and OXA carbapenemases cause the most serious health concerns (Waśko *et al.* 2022). The co-existence of multiple antibiotic resistance genes, particularly ESBLs (like bla_{CTX-M}, bla_{TEM}) and carbapenemase genes (bla_{KPC}, bla_{NDM}), increases the bacterial ability to resist not just one but several classes of antibiotics. Furthermore, interactions between these genes can create a synergistic effect, further enhancing resistance. The presence of both ESBLs and carbapenemases in a single isolate can confer resistance to almost all *β*-lactam antibiotics, including advanced ones like cephalosporins and carbapenems (Chaturvedi *et al.* 2020). Our study has shown evidence of the co-existence of other MBL genes and ESBL genes. The presence of both the MBL and ESBL genes in confirmed isolates demonstrates the advent of different lactamase variants. Among *E. coli* isolates, 3.63% showed the co-existence of two genes, bla_{NDM-1} and bla_{CTX-M}, while 0.9% of *E. coli* isolates exhibited the co-existence of three genes: bla_{NDM-1}, bla_{CTX-M}, and bla_{SHV}. Additionally, 5.74% of *K. pneumoniae* isolates demonstrated the co-existence of different gene types. Specifically, bla_{NDM-1} and bla_{CTX-M} were found in 3.44% of *K. pneumoniae* isolates, while bla_{CTX-M} and bla_{SHV} were found in 2.29% of them. Overall, 5.07% of isolates exhibited co-existence with resistant genes. Notably, resistant genes such as bla_{TEM} and bla_{IMP} were not found to be co-expressed by any of the isolates. The presence of the resistant genes for the MBL and ESBL variants in our isolates suggests that various β-lactamase variants among pathogens in our healthcare centers emerged simultaneously. This result shows the possession of a threat of the expansion of microbial populations with high resistance through environmental reservoirs. The co-occurrence pattern of ESBLs and MBLs in our study was 5.01% (Table 4) of isolates which is less than the study of Salvia *et al.* (11.5%) (Salvia *et al.* 2022) and greater than the study of Mirza *et al.* (1.7%) (Mirza *et al.* 2019). Previous research in Burkina Faso noted co-occurrence rates of bla_{CTX-M}-bla_{TEM}-bla_{SHV} at 2.7 and 10.5% for *E. coli* and *K. pneumoniae*, respectively. Again, the co-occurrence of bla_{CTX-M}-bla_{SHV} was observed at 4 and 37.9% for *E. coli* and *K. pneumoniae*, respectively, while bla_{CTX-M}-bla_{TEM} co-occurred at rates of 36 and 11.6%, respectively. Additionally, bla_{TEM}-bla_{SHV} co-occurred at rates of 1.33 and 1.1% for *E. coli* and *K. pneumoniae*, respectively (Kagambèga *et al.* 2024). In a South African study, *E. coli* isolated from wastewater showed

co-occurrence of $bla_{CTX\text{-}M} + bla_{NDM\text{-}1}$ in 10.5% and $bla_{TEM} + bla_{NDM\text{-}1}$ in 15.8% (Adegoke *et al.* 2020). In another South African study *K. pneumoniae* isolated from wastewater exhibited co-occurrence of $bla_{CTX\text{-}M}$-1–bla_{TEM} (3%), $bla_{CTX\text{-}M}$-1–bla_{SHV} (3%), and bla_{IMP}–$bla_{NDM\text{-}1}$ (2%) (Okafor & Nwodo 2023). The presence of the resistance genes for the MBL and ESBL variants in our isolates suggests that various β-lactamase variants among pathogens in our healthcare centers emerged simultaneously.

CONCLUSIONS

Dhaka, one of the most densely populated cities in the world, faces a high risk of water contamination due to the potential merging of its wastewater and water distribution systems. This poses serious public health risks, including contamination of the food supply chain. In this study conducted across 11 areas of Dhaka, 36 wastewater samples were collected and cultured. Out of 197 isolates identified via PCR, *E. coli* accounted for 55.8% and *K. pneumoniae* for 44.2%. Antibiotic susceptibility tests showed both bacteria had the highest resistance to amoxicillin–clavulanate (95% for *E. coli* and 89.86% for *K. pneumoniae*) and the lowest resistance to gentamicin (8.05 and 8.87%, respectively). MDR was observed in 30% of *E. coli* and 35.56% of *K. pneumoniae isolates*. Among *E. coli*, the prevalence of antibiotic-resistance genes included $bla_{NDM\text{-}1}$ (8.9%), bla_{SHV} (13.9%), and $bla_{CTX\text{-}M}$ (7.6%). In *K. pneumoniae*, the percentages were $bla_{NDM\text{-}1}$ (12.8%), bla_{SHV} (4.3%), and $bla_{CTX\text{-}M}$ (5.0%). Co-existence of multiple antibiotic-resistance genes was observed in 4.54% of *E. coli* isolates and 5.74% of *K. pneumoniae* isolates. The city's open drainage system increases the risk of community water contamination, potentially facilitating the transfer of these resistance genes. Further research is needed to confirm this connection. Immediate action by the Water Supply and Sewerage Authority (WASA) is needed to prevent wastewater from merging with the water supply, which could lead to widespread health risks.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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