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Genomic characterization of carbapenemase-producing Enterobacterales from Dhaka food markets unveils the spread of high-risk antimicrobial-resistant clones and plasmids co-carrying *bla*$_{NDM}$ and *mcr-1.1*

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Background: The transmission of carbapenemase-producing Enterobacterales (CPE) in the external environment, especially through food, presents a significant public health risk.

Objectives: To investigate the prevalence and genetic characteristics of CPE in food markets of Dhaka, Bangladesh, using WGS.

Methods: CPE isolates were obtained from different food and water samples collected from food markets in the southern part of Dhaka, Bangladesh. The isolates subsequently underwent molecular typing, WGS employing both short- and long-read sequencers, and plasmid analysis.

Results: This study unveiled an extensive spread of CPE, with no significant difference in contamination rates observed in samples ($N=136$), including meat ($n=8$), fish ($n=5$), vegetables ($n=36$) or various food-washed water ($n=65$) from markets near hospitals or residential areas. Thirty-eight Enterobacterales from 33 samples carried carbapenemase genes (*bla*$_{NDM-1,\ -4,\ -7}$, *bla*$_{KPC-2}$, *bla*$_{OXA-181}$ or *bla*$_{IMI-1}$). Among these, the high-risk *Escherichia coli* ST410 clone was the most prevalent and distributed across various locations. Furthermore, the identification of IncHI2 plasmids co-harbouring resistance genes like *bla*$_{NDM-5}$ and *mcr-1.1*, without discernible epidemiological connections, is a unique finding, suggesting their widespread dissemination.

Conclusions: The analysis unveils a dynamic landscape of CPE dissemination in food markets, underscored by the proliferation of novel IncHI2 hybrid plasmids carrying both colistin- and carbapenem-resistance genes. This illuminates the ever-evolving landscape of antimicrobial resistance in Dhaka, urging us to confront its emergent challenges.

Introduction

Carbapenems have long been used as a last line of defence against severe bacterial infections due to their high potency and broad spectrum.[1] However, we are now witnessing an escalating challenge with the rapid rise of carbapenem-resistant Enterobacterales.[2] The primary mechanism behind carbapenem resistance involves the production of carbapenemase, an enzyme that breaks down this potent antibiotic. Genes encoding carbapenem resistance, such as bla_{NDM}, bla_{KPC} and $bla_{OXA\text{-}48\text{-}like}$, are often found on transmissible plasmids, facilitating rapid interspecies spread of this resistance.[3] Additionally, successful clonal lineages of carbapenemase-producing Enterobacterales (CPE) contribute significantly to the global dissemination of carbapenem resistance.[3] The proportion of carbapenem resistance in clinically isolated Enterobacterales is on the rise worldwide.[4] Furthermore, the spread of CPE extends beyond clinical settings, as evidenced by their presence in non-clinical samples such as sewage, livestock, retail foods and faecal matter from healthy individuals circulating within communities in specific regions.[5–7] The spread of carbapenem resistance has necessitated a reconsideration of an older drug, colistin, as a last-resort therapy; however, the emergence and spread of the mobile colistin-resistance gene *mcr* has further complicated the treatment of antimicrobial-resistant bacteria.[8] Of particular concern is the sporadic occurrence of Enterobacterales isolates harbouring both the carbapenemase and *mcr* genes.[9–11]

Despite the potential threat of CPE dissemination, our understanding of their genomic characteristics and transmission routes in Dhaka, a densely populated region in Bangladesh, remains limited. Although previous reports have noted the isolation of CPE from Dhaka's wastewater,[12] the extent and specifics remain largely unknown. We therefore designed a study to assess the extent and severity of CPE dissemination in the external environment of Dhaka, particularly in the local food markets, and to evaluate the gene acquisition mechanism to track their mode of spread. In our study, WGS revealed a diverse range of plasmids carrying various carbapenemase genes, predominantly bla_{NDM}. Moreover, the sequenced results illuminated a unique association between carbapenemase and *mcr* genes, both coexisting on a specific plasmid.

Materials and method

Samples

A total of 208 environmental samples were aseptically collected from 11 food markets in the Dhaka south region between 28 November and 28 December 2021. In this study, markets within 1 km of any hospital were categorized as 'near a hospital', whereas those located further away were considered residential areas. Of the 208 samples, 168 were from markets near hospitals, and 40 were from residential areas. These samples included approximately 100 to 200 g of edible parts of food items such as vegetables, meat, fish and eggs, as well as water samples from public drinking water faucets, food washing containers and drainage canals in the market area. All samples were promptly transported in ice boxes to the Department of Microbiology at BRAC University in Dhaka, where they were processed on the same day of receipt.

Phenotypic and genetic characterization

For each food sample, 25 g was mixed with 25 mL of normal saline and transferred into flasks containing 225 mL of sterile buffered peptone water and incubated at 37°C ± 1°C for 18 ± 2 h. From each water sample, 25 mL was aseptically mixed with 225 mL of sterile buffered peptone water and incubated in the same manner. To obtain carbapenem-resistant isolates, a loop full of enrichment culture for each sample was streaked onto CHROMagar ECC bacteriological medium (CHROMagar, Paris, France) supplemented with 0.25 mg/L of meropenem and 70 mg/L of $ZnSO_4$ and incubated overnight. All the colonies with different morphologies and colours were stored and subjected to further analysis. Species identification was carried out using MALDI-TOF MS (MALDI Biotyper; Bruker Daltonics GmbH & Co. KG, Bremen, Germany). Drug susceptibility testing was performed using the MicroScan WalkAway plus System and Neg MIC EN 2J panel, and then classified according to the CLSI guidelines (M100-S24). The MIC of colistin was determined using colistin Etest® (bioMérieux, Inc., NC, USA) according to the manufacturer's instructions. Bacteria grown overnight on brain heart infusion (BHI) agar plates (Nippon Becton Dickinson Company, Ltd, Tokyo, Japan) were suspended in PBS (Merck, Darmstadt, Germany). Bacterial suspensions were prepared to a density equivalent to a McFarland standard of 0.5. The adjusted bacterial solution was spread on BHI agar plates and colistin Etest® strips were placed on the plates. These plates were incubated overnight at 37°C and the MIC of colistin was evaluated.

The presence of carbapenemase genes (bla_{NDM}, bla_{KPC}, $bla_{OXA\text{-}48}$, bla_{GES}, $bla_{IMP\text{-}1}$, $bla_{IMP\text{-}6}$, bla_{VIM}) in obtained isolates was determined by multiplex PCR using the Cica Geneus® Carbapenemase Genotype Detection KIT2 (Kanto Chemical, Tokyo, Japan) according to the manufacturer's instructions. The carbapenem-inactivation method (CIM) was conducted following the procedure outlined by van der Zwaluw *et al.*,[13] and adhered to the CLSI guidelines (M100-S24).

WGS and plasmid analysis

Thirty-eight isolates positive for carbapenemase activity were subjected to WGS analysis using the Illumina and Oxford Nanopore sequencers. Genomic DNA was extracted by DNeasy PowerSoil Pro Kit (Qiagen). For Illumina sequencing, library preparation was performed using Illumina DNA PCR-Free Prep (Illumina), and sequencing was conducted with the NovaSeq 6000 platform. A Nanopore sequencing library was prepared using a Ligation Sequencing Kit (SQK-LSK109, Oxford Nanopore Technologies) and run on a FLO-MIN106 flowcell using MinION. Hybrid assembly was done by Unicycler (v0.5.0).[14,15] For the isolates EC13, EC24 and EC33, a Nanopore sequencing library was prepared using a Rapid Barcoding Kit 96 (SQK-RBK110.96, Oxford Nanopore Technologies) and run on a FLO-MIN106 flowcell using GridION. Raw sequencing reads were assembled using Flye v. 2.9,[16] followed by polishing with Illumina reads using Pilon v. 1.22.[17] Obtained sequence data were deposited to the DNA Data Bank of Japan/European Nucleotide Archive/GenBank under the BioProject accession number PRJDB15848. Detection of antimicrobial resistance (AMR) genes and plasmid incompatibility (Inc) typing was performed by abricate (https://github.com/tseemann/abricate) using the ResFinder[15,18] and PlasmidFinder[18,19]

databases, respectively. MLST and plasmid MLST was done by mlst 2.19.0 (https://github.com/tseemann/mlst). Complete genome sequences of *Escherichia coli* ST410 strains were downloaded from the NCBI RefSeq database. A phylogenetic tree was constructed by CSI phylogeny[19,20] using the AMA1167 strain as a reference[20,21] and drawn on iTOL.[21,22] Plasmid sequences were compared and depicted using Easyfig.[11]

Results

Prevalence of bla$_{NDM}$-harbouring E. coli and Klebsiella pneumoniae isolates in Dhaka food markets

In this study, we obtained isolates that grew on a plate containing meropenem from a total of 136 samples among 208 samples from 11 markets, of which 33 (24.3%) were found to contain CPE. The prevalence of CPE in markets near hospitals (25/108, 23.1%) closely mirrors that in residential areas (8/28, 28.6%). Within various food categories, meat samples—such as mutton, chicken and chicken-washed water—showed the highest percentage of CPE-positive isolates, with 50% (8/16) testing positive. Following closely were fish samples, including fish and fish-washed water, with a CPE-positive rate of 27.3% (3/11), whereas all vegetable and vegetable-washed water samples combined had a CPE-positive rate of 23.4% (18/77). Individually, however, the vegetable-washed water samples had the highest CPE-positive rate (7/50, 14%), yielding nine different isolates. In total, 38 CPE strains were collected from 33 samples, with 30 strains obtained from samples near hospitals and 8 from the residential areas (Tables 1 and 2).

Most of the CPE (30) yielded *bla*$_{NDM}$, including 26 *E. coli* and 4 *K. pneumoniae*. Also, the gene was carried by one *Citrobacter* isolate, which was closest to *C. arsenitis* (average nucleotide identity value, 91.1%). All but two *E. coli*, along with the *Citrobacter* sp., carried *bla*$_{NDM-5}$; the others had either *bla*$_{NDM-1}$ or *bla*$_{NDM-7}$. Two *E. coli* in this study were found to co-carry both *bla*$_{NDM-5}$ and *bla*$_{OXA-181}$. Of the four *K. pneumoniae*, two carried *bla*$_{NDM-7}$, one carried *bla*$_{NDM-1}$, and the last one had both *bla*$_{NDM-1}$ and *bla*$_{KPC-2}$ (Table 2).

Identification of IMI carbapenemase-producing Enterobacter spp.

Notably, seven (7/38) carbapenemase-producing *Enterobacter* isolates in our research were identified using CIM, and WGS revealed that all of them carried the *bla*$_{IMI}$ type of carbapenemase (Table 2). These isolates were further classified into four different species and seven different STs, including six newly assigned STs. The *bla*$_{IMI-1}$ gene was harboured by an integrative mobile element designated as EcloIMEX.[22,23] The genetic structure of EcloIMEX in these isolates was found to be similar to one of the four types of the mobile element available in the GenBank (with 100% coverage and >98% identity) except RA29: EcloIMEX-2, -3, -8 or -9. RA29 appeared to carry EcloIMEX-3 (96% coverage and 98% identity); however, the sequence upstream of the resolvase gene was similar to EcloIMEX-2 and therefore the entire structure was chimaeric (Figure S1a, available as Supplementary data at *JAC-AMR* Online). EcloIMEX types and species or sampling locations were not correlated (Figure S1b and Table 2).

Phenotypic and genetic traits of microbiological resistance

Based on the antimicrobial susceptibility profile, all 38 isolates in this study were meropenem-resistant. The majority of the CPE with *bla*$_{NDM}$ demonstrated varied resistance to diverse groups of antibiotics. For instance, all *E. coli* exhibited resistance to fluoroquinolones, specifically ciprofloxacin and levofloxacin. However, only 34.6% (9/26) of the *E. coli* strains tested resistant to gentamicin. Additionally, based on the Etest results, three *E. coli* having *bla*$_{NDM}$ also showed resistance to colistin. The antimicrobial susceptibility pattern of *K. pneumoniae* varied among individual isolates; however, none of the *Klebsiella* strains showed resistance to amikacin or tigecycline. The only *Citrobacter* isolate identified in this study showed resistance to all antibiotics tested except tigecycline (Table 2).

Moreover, 55% (21/38) of the CPE in this study carried the ESBL gene *bla*$_{CTX-M-15}$, comprising 16 *E. coli*, 4 *K. pneumoniae* and the single *Citrobacter*. Of these CPE with *bla*$_{CTX-M-15}$, 42.8% (9/21) also carried various *bla*$_{CMY}$ genes, and 62% (13/21) had *bla*$_{TEM}$. Multiple genes encoding aminoglycoside modification enzymes and/or tetracycline, macrolide or sulphonamide resistance determinants were also found in numerous resistant strains. All *Klebsiella* and strains from the *Enterobacter cloacae* complex, as well as four *E. coli*, carried plasmid quinolone resistance determinants. In addition, we detected plasmid-mediated rifampicin resistance (ARR-3) in two *K. pneumoniae* resistant to quinolones (Table S1).

Spread of various types of plasmids harbouring bla$_{NDM}$ genes and the emergence of the plasmid co-harbouring bla$_{NDM}$ and bla$_{OXA-181}$

Long-read sequencing revealed that the *bla*$_{NDM}$ genes were carried by various types of plasmids. One *E. coli* and two *K. pneumoniae* isolates originating from different sampling sites carried *bla*$_{NDM-7}$ on a plasmid of IncX3 backbone. The plasmids of RA110 and RA123 were virtually the same, with only one- and two-nucleotide differences among 45 122 bp when compared with plasmids found in Bangladesh (p_dm378A_NDM7, CP096179.1)[24,25] and Myanmar (p110_X3, AP018141.1).[26] These plasmids had homology with an IncX3-type plasmid harbouring *bla*$_{NDM-5}$ from China (pKW4-2-NDM5, CP102905.1) with less than six-nucleotide variance including two substitutions on the *bla*$_{NDM}$ gene.[25,27] The IncFII-type plasmid harbouring either *bla*$_{NDM-1}$ or *bla*$_{NDM-5}$ was found in our isolates (Table 2). The *bla*$_{NDM-1}$-carrying plasmid from EC15 was almost identical (100% query coverage/99% identity) to an IncFII plasmid, pMC-NDM (HG003695.1), from Poland.[27,28] The *bla*$_{NDM-5}$-carrying plasmid from EC34 was similar (≥95% query coverage/≥99% identity) to a portion of the *bla*$_{NDM-5}$-carrying IncFII-type from various countries (GenBank accession numbers, CP054171.1 from India and AP018144.1 from Myanmar), in addition to Bangladesh.

The most prevalent plasmid types identified in this study were those carrying *bla*$_{NDM-5}$ and multiple F replicons, which were classified into eight different types according to a repertoire of carried replicons. These plasmids commonly possessed AMR genes against sulphonamide (*sul1*), trimethoprim (*dfrA12*), aminoglycoside

Table 1. Prevalence of carbapenemase-producing Enterobacterales in Dhaka food market samples, Bangladesh

Sample type	Location of the market	No. of samples positive for meropenem non-susceptible isolates	No. of CPE-positive samples (%)	No. of isolates
All samples	Total	136	33 (24.3)	38
	Near hospital	108	25 (23.1)	30
	Residential area	28	8 (28.6)	8
Chicken	Near hospital	4	2 (50.0)	2
	Residential area	1	1 (100)	1
Fish	Near hospital	3	1 (33.3)	1
	Residential area	2	1 (50)	1
Mutton	Near hospital	2	1 (50.0)	1
	Residential area	1	1 (100)	1
Coriander	Near hospital	5	2 (40.0)	2
	Residential area	3	1 (33.3)	1
Betel leaf	Near hospital	7	3 (42.9)	4
	Residential area	2	0 (0)	0
Red spinach	Near hospital	3	2 (66.7)	2
	Residential area	0	0 (0)	0
Carrot	Near hospital	2	1 (50.0)	1
	Residential area	0	0 (0)	0
Cabbage	Near hospital	6	1 (16.7)	1
	Residential area	0	0 (0)	0
Lemon	Near hospital	2	1 (50.0)	1
	Residential area	1	1 (100)	1
Okra	Near hospital	4	1 (25.0)	1
	Residential area	0	0 (0)	0
Yard-long bean	Near hospital	1	1 (100)	1
	Residential area	0	0 (0)	0
Chicken-washed water	Near hospital	7	2 (28.6)	3
	Residential area	1	1 (100)	1
Fish-washed water	Near hospital	5	0 (0)	0
	Residential area	1	1 (100)	1
Vegetable-washed water	Near hospital	38	6 (15.8)	8
	Residential area	12	1 (8.3)	1
Drain water	Near hospital	1	1 (100)	2
	Residential area	0	0 (0)	0
Other market foods	Near hospital	18	0 (0)	0
	Residential area	4	0 (0)	0

(*aadA2*) and macrolide (*mphA*) with a few exceptions (Figure S2). These genes are typically found in an IncFII plasmid, pM214_FII.[28,29] Some plasmids additionally harboured AMR genes: pEC27-NDM-5 carried 15 AMR genes in addition to bla_{NDM-5} (Figure S2). This plasmid had homology with pAMA1167-NDM-5 (97% query coverage/99% identity), a typical plasmid carried by an emerging extraintestinal *E. coli* clone ST410-B3 (see below). Of note, an *E. coli* ST167 isolate co-carrying both bla_{NDM-5} and $bla_{OXA-181}$ on an IncFIA-FIB(AP001918)-FII-ColKP3 plasmid (pEC04-NDM-5_OXA-18), showed similarity to a portion of pNDM_P30_L1_05.20 (87% query coverage/99% identity), which carried bla_{NDM-5} in an *E. coli* ST167 isolate originating from England (Figure 1a).[29] The genetic cluster containing $bla_{OXA-181}$ and a partial ColKP3 replicon was homologous to a plasmid identified in a Bangladeshi hospital, p_dm378A_OXA-181 (Figure 1a). Isolate RA111 carried a plasmid harbouring bla_{KPC-2} besides a bla_{NDM-5} plasmid. The bla_{KPC-2} plasmid was similar to a portion of pBA33875_KPC2 (CP035181.1) (85% query coverage/99% identity), the one isolated in a hospital in India (Figure 1b).[30,31]

Phylogeny and epidemiology of E. coli ST410 isolates

The results of the MLST analysis revealed that 26 *E. coli* isolates belonged to 14 different STs, and each of the four *K. pneumoniae* isolates was assigned to a different ST. Some of the isolates in this study were identified as endemic clones that have been reported globally, such as *E. coli* ST167 carrying bla_{NDM-5} and *K. pneumoniae* ST147 carrying bla_{NDM-1}.[31,32] Among the 38 isolates in our study, the most prevalent was *E. coli* ST410 carrying bla_{NDM-5} (9/38), which was isolated from four different sampling locations. Cluster analysis, including other ST410 isolates globally reported, showed that the Bangladeshi isolates belonged to one of three

Table 2. Phenotypic and genetic characteristics of CPE isolates

Isolates	Sample type[a]	Markets name and location	Species	ST	Carbapenemase type (bla)	Colistin resistance genes	Plasmid/EcloIMEX (% identity) harbouring carbapenemase genes	Antimicrobial susceptibility (MIC, mg/L)[b]							
								Meropenem	Ceftazidime	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	Tigecycline	Colistin
EC02	Yard-long bean	A[c]	*E. coli*	1702	NDM-5		IncFIA-FIC(FII)	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.25
EC04	Coriander	A[c]	*E. coli*	167	NDM-5, OXA-181		IncFIA-FIB (pB171)-FII-ColKP3	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.25
EC05	Fish	A[c]	*E. coli*	410	NDM-5		IncFIA-FII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.5
EC07	Mutton	A[c]	*E. coli*	46	NDM-5		IncFIA-FIB-FII (pRSB107)	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.5
EC08	Red spinach	B[c]	*E. coli*	10	NDM-5		IncFIA-FIB-FII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.5
EC09	Chicken	C[c]	*E. coli*	361	NDM-5	mcr.1.1	IncHI2-HI2A-pKPC-CAV1312	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	**2**
EC11	Red spinach	C[c]	*E. coli*	448	NDM-7		IncX3	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.25
EC12	Betel leaf	D[c]	*E. coli*	2659	NDM-5		Not determined	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.19
EC13	Cabbage	E[c]	*E. coli*	617	NDM-5		FIA-FII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.75
EC14	Betel leaf◈	F[c]	*E. coli*	410	NDM-5		IncFIA-FIB-FII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.38
EC15	Betel leaf◈	F[c]	*E. coli*	410	NDM-1		IncFII	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.25
EC17	Chicken	G[c]	*E. coli*	6335	NDM-5	mcr.1.1	IncHI2-HI2A-pKPC-CAV1312	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	1	**2**
EC18	Coriander	H[d]	*E. coli*	410	NDM-5		IncFIA-FIB-FII (pRSB107)-Col156	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.38
EC19	Fish	H[d]	*E. coli*	410	NDM-5		IncFIA-FIB-FII(pRSB107)-Col156	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.25
EC20	Mutton	H[d]	*E. coli*	410	NDM-5		IncFIA-FIB-FIC(FII)	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.5
EC21	Chicken	I[d]	*E. coli*	8318	NDM-5		IncHI2-HI2A-pKPC-CAV1312	**>2**	**>8**	**>8**	≤8	**>2**	**>4**	≤0.5	0.5
EC23	Vegetable-washed water•	A[c]	*Citrobacter* sp.	39	NDM-5		IncFIA(HI1)-R	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.5
EC24	Vegetable-washed water•	A[c]	*E. coli*	226	NDM-5	mcr.1.1	IncHI2-HI2A-pKPC-CAV1321- N-X2	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	**4**
EC25	Drain water◆	A[c]	*E. coli*	167	NDM-5		IncFIA-FIB-FIC(FII)	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.5
EC26	Vegetable-washed water	B[c]	*E. coli*	156	NDM-5		IncFIA-FIB-FIC(FII)	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.38
EC27	Chicken-washed water◈	C[c]	*E. coli*	410	NDM-5		IncFIA-FIB-IncFII(pAMA1167-NDM-5)	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.19
EC28	Chicken-washed water	C[c]	*E. coli*	410	NDM-5		IncFIA-FII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.125
EC30	Vegetable-washed water	G[c]	*E. coli*	156	NDM-5		IncFIA-FIB-FIC(FII)	**>2**	**>8**	8	≤8	**>2**	**>4**	≤0.5	0.125
EC33	Chicken-washed water	H[d]	*E. coli*	167	NDM-5, OXA-181		Not determined	**>2**	**>8**	**>8**	**>32**	**>2**	**>4**	≤0.5	0.5
EC34	Fish-washed water	H[d]	*E. coli*	2851	NDM-5		IncFII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.125
EC36	Vegetable-washed water	H[d]	*E. coli*	410	NDM-5		IncFIA-FIB-IncFII (pAMA1167-NDM-5)	**>2**	**>8**	**>8**	32	**>2**	**>4**	≤0.5	0.125
RA03	Carrot	J[c]	*Enterobacter cloacae*	477	IMI-1		EcloIMEX-3 (99.93)	**>2**	≤1	≤2	≤8	≤0.5	≤0.12	≤0.5	0.19

Continued

Table 2. *Continued*

Isolates	Sample type[a]	Markets name and location	Species	ST	Carbapenemase type (bla)	Colistin resistance genes	Plasmid/EcloIMEX (% identity) harbouring carbapenemase genes	Antimicrobial susceptibility (MIC, mg/L)[b]							
								Meropenem	Ceftazidime	Gentamicin	Amikacin	Ciprofloxacin	Levofloxacin	Tigecycline	Colistin
RA110	Vegetable-washed water	J[c]	*K. pneumoniae*	3113	NDM-7		IncX3	**>2**	**>8**	≤2	≤8	≤0.5	1	≤0.5	0.19
RA111	Vegetable-washed water	J[c]	*K. pneumoniae*	48	NDM-1, KPC-2		IncFIB (pNDM-Mar)-IncHI1B(pNDM-MAR)	**>2**	**>8**	>8	≤8	**>2**	4	1	0.125
RA117	Vegetable-washed water[•]	A[c]	*E. coli*	361	NDM-5		IncFIA-FII	**>2**	**>8**	≤2	≤8	**>2**	**>4**	≤0.5	0.19
RA123	Drain water[◆]	A[c]	*K. pneumoniae*	403	NDM-7		IncX3	**>2**	**>8**	≤2	≤8	2	1	≤0.5	0.25
RA143	Chicken-washed water[◇]	C[c]	*Enterobacter sichuanensis*	1592	IMI-1		EcloIMEX-2 (98.98)	**>2**	**>8**	≤2	≤8	≤0.5	≤0.12	≤0.5	0.19
RA157	Vegetable-washed water	E[c]	*K. pneumoniae*	147	NDM-1		IncFIB (pQil)	**>2**	**>8**	≤2	16	**>2**	**>4**	≤0.5	0.125
RA29	Lemon	B[c]	*Enterobacter vonholyi*	2038	IMI-1		EcloIMEX-3 (97.98)	**>2**	≤1	≤2	≤8	≤0.5	≤0.12	≤0.5	0.25
RA30	Betel leaf	G[c]	*Enterobacter sichuanensis*	2039	IMI-1		EcloIMEX-8 (98.25)	**>2**	≤1	≤2	≤8	≤0.5	≤0.12	≤0.5	0.125
RA46	Okra	C[c]	*Enterobacter cloacae*	2040	IMI-1		EcloIMEX-9 (98.91)	**>2**	4	≤2	≤8	≤0.5	≤0.12	≤0.5	0.38
RA85	Coriander	G[c]	*Enterobacter bugandensis*	2041	IMI-1		EcloIMEX-2 (99.68)	**>2**	≤1	≤2	≤8	≤0.5	≤0.12	≤0.5	0.125
RA86	Lemon	K[d]	*Enterobacter vonholyi*	2042	IMI-1		EcloIMEX-2 (99.96)	**>2**	≤1	≤2	≤8	≤0.5	≤0.12	≤0.5	0.38

[a]◈,•,◆,◇Denote isolates originating from the same sample.
[b]Bold values denote resistance to that specific antibiotic.
[c]Near a hospital.
[d]Residential area.

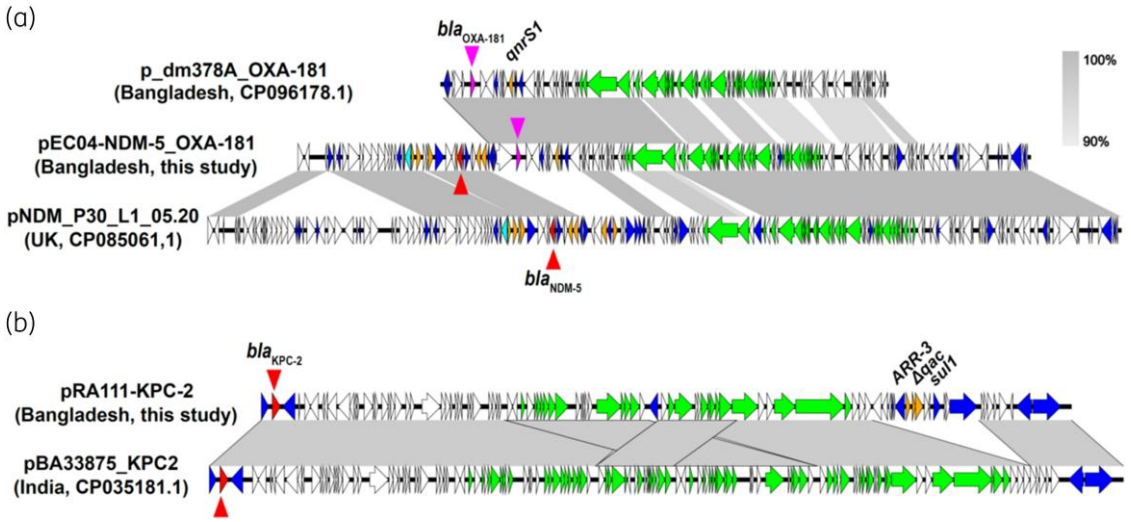

Figure 1. Plasmids carrying bla_{NDM-5} and $bla_{OXA-181}$ or bla_{KPC-2} identified in this study are structurally related to those identified in clinical settings. (a) Genetic structure of pEC04-NDM-5, a plasmid co-carrying bla_{NDM-5} and $bla_{OXA-181}$, and structural comparison with similar plasmids. Genes are depicted by arrows: bla_{NDM}, red; $bla_{OXA-181}$, magenta; other AMR genes, orange; *IntI1*, cyan; other mobile genetic elements, blue; conjugal transfer genes, green; other genes, white. Homologous regions are shaded in grey. (b) Genetic structure of pRA111-KPC-2 and a similar plasmid pBA33875. bla_{KPC-2} is coloured red and other genes are coloured as described in (a). Homologous regions (>99% identity) are shaded in grey.

major clusters in the phylogenetic tree (clusters A, B and C in Figure 2). Isolates belonging to clusters B and C commonly possessed hallmarks of the previously defined B3/*H*24RxC or 410-B3 subclade of ST410[33–36]: carriage of the F1:A1:B49 plasmid with bla_{NDM-5}, chromosomal carriage of bla_{CMY-2}, and YRIN insertion in FtsI (alias penicillin-binding protein 3, PBP3) after position P333, which is reported to confer reduced susceptibilities to several β-lactams[33] (Figure 2). The majority of our ST410 isolates belonged to a cluster that apparently corresponded to B3/*H*24Rx or ST410-B2 (cluster A in Figure 2). They showed a different repertoire of β-lactamase genes and were found to carry three different plasmids carrying bla_{NDM-5}, indicating independent acquisition of these genes and plasmids (Figure 2). Most of the isolates included in this cluster also possessed a four amino acid insertion in FtsI as was found in ST410-B3 isolates; however, the inserted sequence was not identical and was either of the four sequences, YRIN, YRIK or YRIP after position P333, or TIPY after position Y334, the latter three of which also are known to confer reduced susceptibility to β-lactams (Figure 2).[35,37] It is possible that these isolates acquired these insertions independently in addition to bla_{NDM-5}-carrying plasmids. Notably, the isolates closely related to these Bangladeshi isolates in this clade were of animal origin. For instance, A1_181 was isolated from a wild gull,[36] whereas CARB35[37] and AR24.2b[38] were from dogs originating in different European countries. In addition, the isolate 042 was isolated from a clinician in a veterinary clinic.[39]

Genomic structure of the plasmid co-harbouring bla_{NDM-5} and mcr-1.1

In this study, three *E. coli* isolates, EC09, EC17 and EC24 from different sampling locations, were found to carry the plasmid-mediated colistin resistance gene *mcr-1.1* on a plasmid of IncHI2/HI2A backbone (Table 2). The plasmid co-harbouring bla_{NDM-5} and *mcr-1.1* from the EC09 isolate, pEC09-NDM-5, co-harbours several other resistance genes, apparently conferring resistance to all antibiotics tested, except for fosfomycin and tigecycline (Table 2, Table S1). It is reported that the IncHI2 plasmid is a major carrier of *mcr* genes, whereas that carrying bla_{NDM} is rarely reported.[40] Among the top 100 hit plasmids identified by NCBI BLAST analysis (as of February 2023), 24 were *mcr-1.1*-harbouring plasmids, whereas 2 were bla_{NDM}-positive. The plasmid with greatest identity to pEC09-NDM-5 was pRS571-MCR1.1, an *mcr-1.1*-carrying IncHI2 plasmid originating from a clinical specimen in Bangladesh. Comparative analysis showed that the genetic structure of these plasmids is almost identical (>99% identity) except for the resistance gene region, in which pEC09-NDM-5 harbours bla_{NDM-5} (Figure 3). Meanwhile, the resistance gene region of pEC09-NDM-5 was identical to those from typical IncFII plasmids carrying bla_{NDM-5}. An IncHI2 plasmid pJNQH497-1 carrying bla_{NDM} was also identified by BLAST analysis using pEC09-NDM-5 as a query; however, the genetic structure surrounding bla_{NDM} differed between these two plasmids, suggesting independent acquisition of bla_{NDM-5} by these plasmids. These lines of evidence suggest that pEC09-NDM-5 has emerged from a pRS571-MCR-1.1-like ancestral plasmid by acquisition of a resistance gene cluster containing bla_{NDM-5}. The genetic cluster of pEC09-NDM-5 was bracketed by two IS*26* sequences, implying that IS*26* was involved in the gene acquisition event.

Discussion

The results of our study underscore the widespread presence of CPE in both local foods and environments in Dhaka, aligning with previous findings from South Asia. The similar contamination rates observed in food markets near hospitals and community areas suggest that the transportation of fresh produce or other food items from their origins may significantly contribute to the dissemination of CPE, irrespective of the proximity to hospitals.

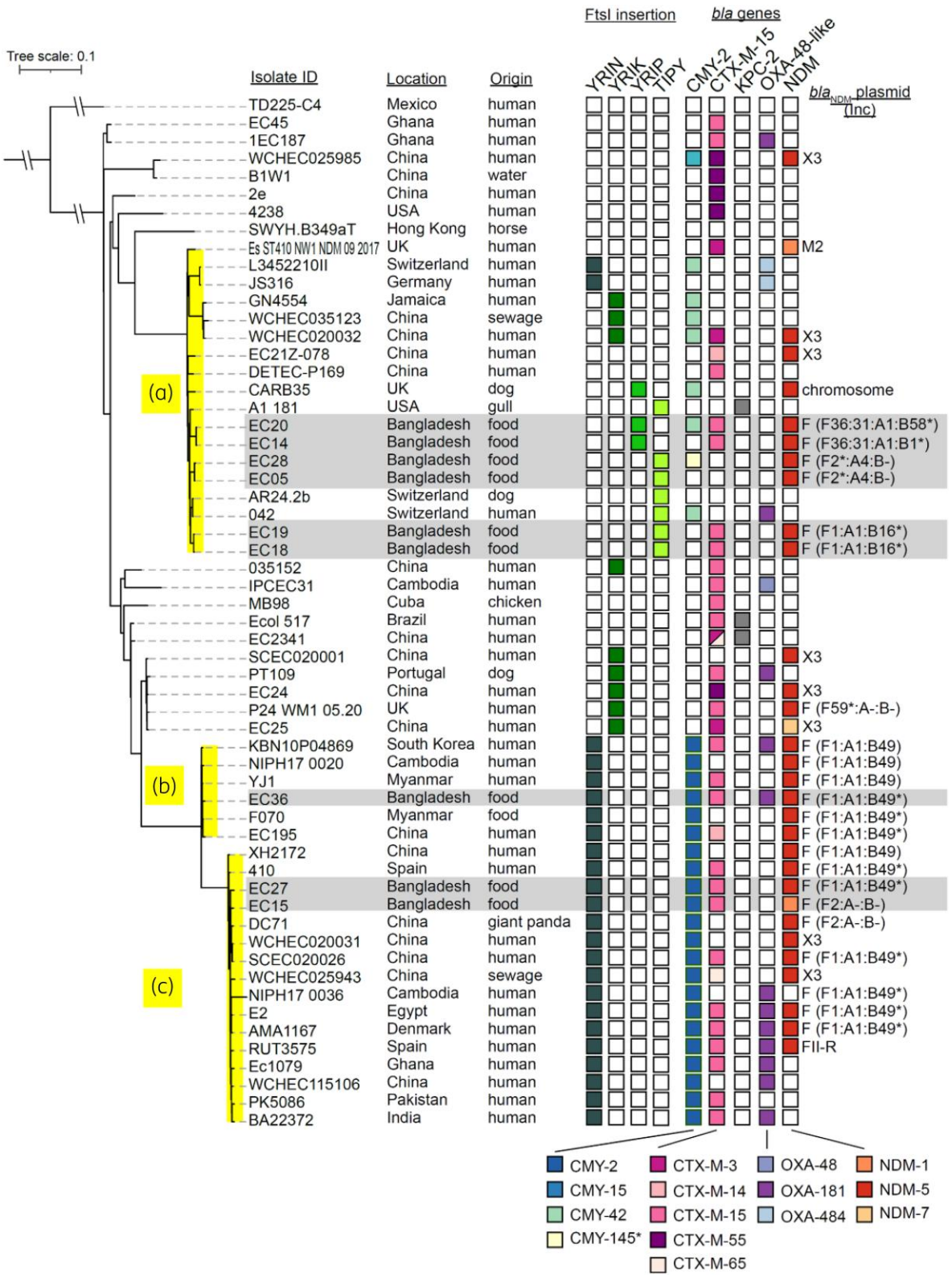

Figure 2. Bangladeshi *E. coli* ST410 isolates are classified into three clusters. Phylogenetic analysis was performed with *E. coli* ST410 genome sequences reported from other countries. Bangladeshi isolates are shaded grey. Clusters including these isolates are marked yellow. *Indicates completely matched sequences were not found in the database. bla_{NDM} was carried by chromosome in CARB35.

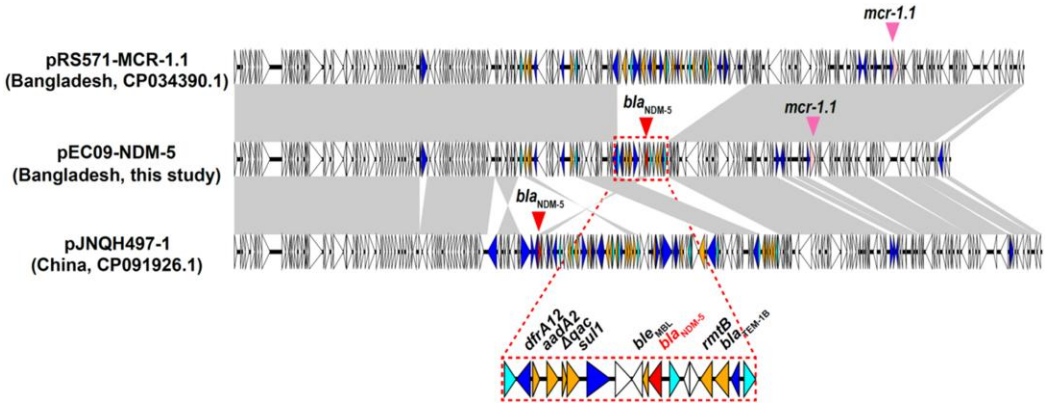

Figure 3. Genetic structure of the IncHI2 plasmid co-carrying *bla*$_{NDM-5}$ and *mcr-1.1*. Related plasmids carrying *bla*$_{NDM-5}$ or *mcr-1.1* are included for comparison. *bla*$_{NDM}$, red; *bla*$_{OXA-181}$, pink; other AMR genes, orange; IS*26* transposase gene, cyan; other mobile genetic elements, blue; other genes, white. Homologous regions (>99% identity) are shaded in grey.

Previous research has also shown that AMR bacteria can be transmitted directly to humans through the food chain, whether by handling raw food, cross-contamination with other foods or indirectly through the environment.[41] This is particularly relevant in developing nations like Bangladesh, where limited biosecurity and food safety measures, coupled with frequent human-animal-environment interactions, are more common. Additionally, untreated wastewater discharge from poultry farms, animal husbandry and urban markets has been identified as a significant contributor to the spread of AMR bacteria and genes in Bangladesh.[42] Our surveillance revealed that vegetable-washed water contained the highest concentration of CPE. Inadequate treatment of this contaminated water in wastewater treatment plants may lead to its mixing with other environmental water sources, facilitating the dissemination of AMR bacteria and genes throughout Dhaka city and beyond.

This study identified global spread of high-risk clones, including *E. coli* ST167 and ST410, and *K. pneumoniae* ST147. Notably, *E. coli* ST410 isolates accounted for a quarter of the total isolates and were isolated from four different sampling locations. Phylogenetic analysis, incorporating sequences from a public database, revealed that most Bangladeshi ST410 isolates belong to a cluster also containing isolates from wild birds, companion animals, a clinician at a veterinary clinic, and sewage, implying potential circulation across sectors. Additionally, these isolates carry the *bla*$_{CTX-M-15}$ gene, alongside *bla*$_{NDM-5}$, and/or possess four-amino-acid insertions in FtsI, rendering them resistant to all β-lactams, including aztreonam, except for isolate EC05. However, tigecycline and colistin could be potential treatment options for infections caused by these organisms, at least to date.

In this research, *bla*$_{NDM}$ was the most prevalent gene in various plasmids, along with plasmids carrying resistance genes like *bla*$_{KPC}$, *bla*$_{OXA}$, *bla*$_{IMI}$ and various EcloIMEX types. Notably, our study revealed a novel occurrence: three instances of co-carriage of the *bla*$_{NDM-5}$ and *mcr-1.1* genes on the same IncHI2 plasmids in *E. coli* originating from food. Co-carriage of the *mcr-1.1* gene with any carbapenem resistance gene on the same plasmid is rare and concerning, with limited prior reports.[9,10] Strikingly, these *E. coli* strains were isolated from three separate food markets without any direct epidemiological linkage, including two from chicken samples and one from vegetable-washed water. The widespread use of colistin in broiler farms in Bangladesh[43] and recent detection of Enterobacterales carrying the *mcr-1* gene in poultry and the environment[44,45] further underscore the significance of our study. The identified IncHI2 plasmids may acquire a genetic cassette containing *bla*$_{NDM-5}$ to yield the pEC09-NDM-MCR plasmids, as they show some similarity to the pRS571-MCR1 plasmid found in an *E. coli* strain from a hospitalized patient.[46] Interestingly, these *E. coli* isolates co-carrying resistance genes like *bla*$_{NDM-5}$ and *mcr-1.1* appeared to be susceptible to at least two antibiotics, implying available treatment options for the time being. However, co-transfer of resistance to both carbapenem and colistin may substantially jeopardize the efficacy of clinical therapy, considering colistin is one of the last-resort antibiotic for CPE infections. Furthermore, these strains may become sporadically drug-resistant by acquiring MDR plasmids from the local environment.

Although this study offers vital insights into the presence of CPE with various genes in food markets in Dhaka, it has limitations. The limited number of survey locations suggests the need for more extensive studies to comprehensively understand the dissemination of CPE in Dhaka city and beyond. Additionally, our findings may not be generalizable to other regions or countries with different environmental, demographic or epidemiological characteristics, necessitating large-scale global studies. Furthermore, this study solely examines environmental CPE presence and lacks clinical data, limiting our ability to link environmental CPE to clinical cases. Future studies should consider clinical data for this connection. Lastly, this research provides only a snapshot of CPE in local food markets, emphasizing the importance of continuous monitoring and long-term follow-up studies to track changes.

Conclusions

This study provides empirical evidence of diverse CPE dissemination in food markets with numerous additional AMR genes. Additionally, the identification of IncHI2 hybrid plasmids

co-localizing colistin and carbapenem resistance genes exemplifies the abundance of novel plasmids in Dhaka's environment. This study unequivocally highlights the significance of using WGS technologies to monitor antimicrobial resistance, as they enable the precise representation of mobilizable components that confer resistance.

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Transparency declarations

None to declare.

Author contributions

T.T.N., D.N. and Y.A. designed this study. N.H., M.M.H. and M.H.S. collected samples and isolated bacteria. T.T.N., Y.S., S.H., D.T., R.A., E.K., M.M., H.Z., A.U. and I.N. performed the analysis. T.T.N. and Y.S. wrote the paper, and all the authors reviewed the manuscript. D.N. and K.N. supervised this study.

Supplementary data

Figures S1 and S2 and Table S1 are available as Supplementary data at *JAC-AMR* Online.

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