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Prevalence and Molecular Characterization of ESBL Producing *Enterobacteriaceae* from Highly Polluted Stretch of River Yamuna, India

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The rapid increase in number and diversity of Extended Spectrum β-Lactamases (ESBLs) producing *Enterobacteriaceae* in natural aquatic environment is a major health concern worldwide. This study investigates abundance and distribution of ESBL producing multidrug resistant *Enterobacteriaceae* and molecular characterization of ESBL genes among isolates from highly polluted stretch of river Yamuna, India. Water samples were collected from ten different sites distributed across Delhi stretch of river Yamuna, during 2014-15. A total of 506 non duplicate *Enterobacteriaceae* isolates were obtained. Phenotypic detection of ESBL production and antibiotic sensitivity for 15 different antibiotics were performed according to CLSI guidelines (Clinical and Laboratory Standard Institute, 2015). A subset of ESBL positive *Enterobacteriaceae* isolates were identified by 16S rRNA gene and screened for ESBL genes, such as $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{OXA}. Out of 506 non-duplicate bacterial isolates obtained, 175 (34.58%) were positive for ESBL production. Susceptibility pattern for fifteen antibiotics used in this study revealed higher resistance to cefazolin, rifampicin and ampicillin. A high proportion (76.57%) of ESBL positive isolates showed multidrug resistance phenotype, with MAR index of 0.39 at Buddha Vihar and Old Delhi Railway bridge sampling site. Identification and PCR based characterization of ESBL genes revealed the prevalence of $bla_{\text{CTX-M}}$ and bla_{TEM} genes to be 88.33% and 61.66%, respectively. Co-occurrence of $bla_{\text{CTX-M}}$ and bla_{TEM} genes was detected in 58.33% of the resistant bacteria. The bla_{OXA} gene was not detected in any isolates. This study highlights deteriorating condition of urban aquatic environment due to rising level of ESBL producing *Enterobacteriaceae* with multidrug resistance phenotype.

Keywords: River Yamuna, *Enterobacteriaceae*, multidrug resistance, ESBL

Introduction

The relentless rise of multidrug resistant pathogens that threaten the successful treatment of infectious diseases is a global public health concern. It has been estimated that more than 700,000 people die each year from infectious diseases caused by antibiotic resistant bacteria [1]. β-lactams constitute the important group of antimicrobial agents for treatment of bacterial infections [2]. To counter the defense, production of Extended Spectrum β-Lactamases (ESBLs) is the predominant cause of resistance to this class of drug among gram-negative as well as gram-positive bacteria. ESBLs encoded by different families of *bla* genes, more common among them being CTX-M, TEM and SHV are either chromosomally localized or plasmid based genetic elements [3, 4]. $bla_{\text{CTX-M}}$ identified as the most predominantly reported ESBLs worldwide during the last few decades over-count the classical bla_{TEM} and bla_{SHV} type of ESBL genes [5]. ESBL producing bacteria have also been reported to carry resistance genes for other class of antibiotics like aminoglycosides, quinolone and tetracycline [6]. The

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resistance dissemination represents serious concern as the emerging rate of multidrug resistance in bacteria would leave us with no treatment options, dragging the humanity to pre-antibiotic era.

The escalating problem of ESBL producing bacteria is no longer confined to clinical settings. Aquatic systems such as rivers and lakes are constantly impacted by anthropogenic activities, receiving effluents from industrial, hospital, urban and agricultural origins and constitute important antibiotic resistance reservoirs [7, 8]. The resistance patterns of bacteria influenced by the selective pressure generated by the pollutants vary in different geographical areas and even may vary over time [9]. These anthropogenically influenced aquatic systems provide ideal conditions for the transfer and selection of antibiotic resistance upholding their spread [10]. The assessment of antibiotic resistance and investigation of the molecular mechanisms are invaluable assets to clinical studies for better understanding of the development and spread of resistance.

The presence of ESBL producing members exhibiting MDR phenotype in river water is a cause of concern because human populations are exposed to these resistant pathogens. Delhi stretch of river Yamuna in India is a significant source of water for irrigation, industrial and domestic purposes. Simultaneously, with intensive urbanization and increasing population, it receives massive discharge of domestic and industrial waste contributing antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs). This study investigates abundance and distribution of ESBL producing multidrug resistant *Enterobacteriaceae* and molecular characterization of ESBL genes among isolates from highly polluted stretch of river Yamuna in the capital city of India.

Materials and Methods

Survey, sample collection and isolation of bacteria

The Yamuna river is an important source of water supply to the inhabitants of densely populated Delhi. Simultaneously, it is under extreme pollution pressure due to high load of pollutants discharged through drains from the nearby domestic, hospital, agricultural and industrial sources. In order to investigate prevalence of ESBL producing *Enterobacteriaceae* in the river Yamuna, sampling was carried out during 2014–2015. A total of ten sampling sites were selected from Wazirabad barrage to Okhla barrage covering the National Capital Territory (Fig. 1). Sampling sites were selected to observe the influence of important discharges of treated or untreated wastewater into the river (Table 1). Water samples were aseptically collected in 250 ml sterile glass bottles (*Schott Duran*) and immediately transported to the laboratory for further analysis. Physical parameters like temperature and pH were measured at the sampling sites. Collected water samples were serially diluted and 100 µl of each dilution was spread on MacConkey agar plates. After incubation at 37℃ for 14–18 h, colonies with distinct morphology were picked and purified by repeated sub-culturing on media plates.

Screening for ESBL producing bacteria

All non-duplicate bacterial isolates were screened for ESBL production initially by preliminary test using 3rd

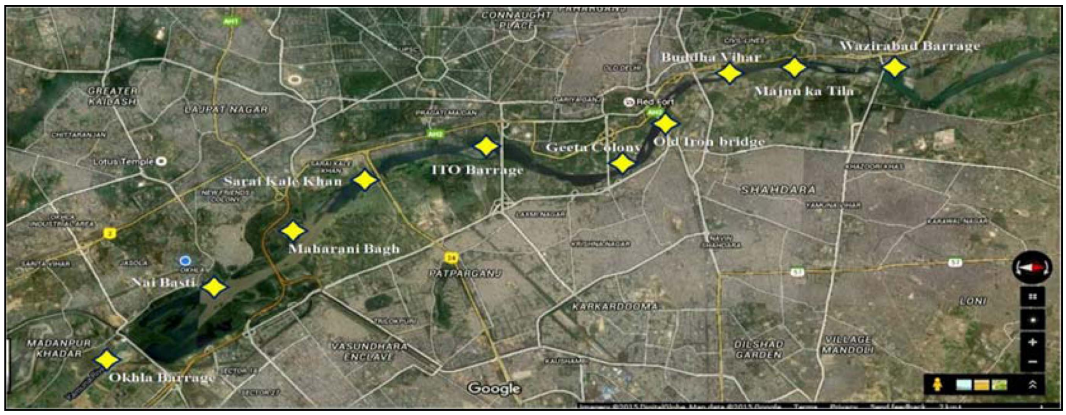

Fig. 1. Sampling sites selected along the Delhi stretch of river Yamuna shown on Google map.

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Table 1. Description of sampling sites along the Delhi stretch of river Yamuna.

S. No.	Site code	Sampling site	Major drain discharge	Latitude	Longitude
1	WB	Wazirabad Barrage	-	28°42′40.0212″N	77°13′51.2436″E
2	MT	Majnuka Teela	Najafgarh Drain	28°41′43.6308″N	77°13′44.7168″E
3	BV	Buddha Vihar	Kudsia Drain	28°40′28.4448″N	77°33′56.4888″E
4	RB	Old Delhi Railway Bridge	Sen Nursing Home Drain	28°39′50.0328″N	77°14′53.8872″E
5	GC	Geeta Colony	Delhi Gate Drain	28°38′59.5500″N	77°15′52.9380″E
6	IB	ITO Barrage	Drain NO. 12A	28°37′39.9523″N	77°15′10.1488″E
7	SR	Sarai Kale Khan	Barapulla Drain	28°35′21.9480″N	77°16′15.1572″E
8	MB	Maharani Bagh Substation	Maharani Bagh Drain	28°36′1.3464″N	77°15′39.5784″E
9	NB	Nai Basti	Shahdara Drain	28°33′57.3522″N	77°17′42.3204″E
10	OB	Okhla Barrage	-	28°32′38.2092″N	77°18′48.1752″E

generation cephalosporins (ceftazidime, ceftriaxone and cefotaxime) on Muller Hinton Agar (MHA) plates according to CLSI guidelines [11]. Isolates showing zone of inhibition diameter ≤ 22 mm for ceftazidime, ≤ 25 mm for ceftriaxone and/or ≤ 27 mm for cefotaxime were suspected as ESBL producer. All suspected ESBL producing isolates were further subjected to confirmation by phenotypic disc confirmatory test (PDCT). For this, discs containing cefotaxime (30 μg) and ceftazidime (30 μg) with and without clavulanic acid (10 μg) were placed on MHA plates. Isolate showing an increase in the zone diameter of ≥ 5 mm for the antibiotic + clavulanate discs as compared to antibiotic alone was considered as ESBL positive. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were included in each test as ESBL positive and negative control, respectively.

Antibiotic susceptibility test

All ESBL positive isolates were tested for their susceptibility towards different class of antibiotics on MHA plates by disk diffusion method (Kirby-Bauer method) as per CLSI guidelines (2015). Fifteen antibiotics representative of eleven different classes used in the study includes: Aminoglycosides: amikacin (AK; 30 μg), Penicillin: ampicillin (AMP; 10 μg), Penicillin combinations: ampicillin/sulbactam (A/S; 10/10 μg), piperacillin/tazobactam (P/T; 100/10 μg), Cephalosporins: cefazolin (CZ; 30 μg), cefoxitin (CX; 30 μg); Quinolones: ciprofloxacin (CIP; 5 μg), levofloxacin (LE; 5 μg), Carbapenem: imipenem (IPM; 10 μg), Polypeptide: polymixin (PB; 300 units), colistin (CL; 10 μg), Folate pathway inhibitor: trimethoprim (TR; 5 μg), Tetracycline: tetracycline (TE; 30 μg), Phenicol: chloramphenicol (C; 30 μg) and, Ansamycin: rifampicin (RIF; 30 μg). Plates incubated overnight at 37℃ were used to determine the zone of inhibition (ZOI) and the isolates were categorized as susceptible, intermediate or resistant. Isolates showing resistance to three or more classes of antibiotics were regarded as multidrug resistant (MDR) and extensively drug resistant (XDR) if they were resistant to all except one or two classes of antibiotics [12]. Multiple antibiotic resistance (MAR) index of each sampling site was calculated by the formula $a/(b \times c)$, where "a" represents the total antibiotic resistance score of all isolates, "b" represents the number of antibiotics tested and "c' represents the number of isolates from each sample [13]. Minimum inhibitory concentration (MIC) of six different antibiotics i.e. CAZ, CTX, CIP, C, TR and RIF were determined against ESBL positive isolates by the CLSI recommended broth micro-dilution method using 96 well micro-titer plates. Plates were incubated at 37℃ for 12–14 h and optical density recorded by spectrophotometer (Labtronics LT-2800) at 595 nm.

Identification of bacterial isolates

Phenotypically ESBL positive bacterial isolates were identified by 16S rRNA gene analysis. For this, gene specific primer set was used to amplify 1250 bp 16S rRNA gene. PCR amplicons were purified and sequenced with corresponding primers in SciGenome Lab, Cochin, India. Sequences were analyzed using FinchTV (Geospiza, Inc.) and BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Homology analysis of 16S rRNA gene sequence was carried out by the BLAST

Table 2. Details of primer sequences used for molecular studies.

Target gene	Primer pair	Primer sequences	Amplicon size (bp)	Reference
16S rRNA	RR-F RR-R	5'-GGCGGACGGGTGAGTAATG-3' 5'-GAAGTCGGAATCGCTAGTAATCG-3'	1250	14
bla_{TEM}	TEM-F TEM-R	5'-ATGAGTATTCAACATTTYCGTGTCGCC-3' 5'-TTACCAATGCTTAATCAGTGAGGCACC-3'	861	This study
bla_{CTX-M}	CTX-F CTX-R	5'-SCVATGTGCAGYACCAGTAA-3' 5'-GCTGCCGGTYTTATCVCC-3'	480	14
bla_{OXA}	OXA-F OXA-R	5'-ATGCGTGTATTAGCCTTATCGGC- 3' 5'-GAGCACTTCTTTTGTGATGGCTTGG-3'	775	This study

algorithm available at NCBI website (http://www.ncbi.nlm.nih.gov/).

Characterization of ESBL(s) genes

Phenotypically ESBL positive isolates were screened for the presence of bla_{CTX-M}, bla_{TEM} and bla_{OXA} genes. For this, genomic DNA was isolated and used as template to amplify the genes with designed primers (Table 2). PCR amplification was carried out in a 50 µl reaction volume, consisting of 5 µl of 10× buffer, 4 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µM of each forward and reverse primer, 2U of Taq-polymerase (Thermo Scientific) and 2.0 µl of template DNA. The cycling parameters were adjusted as follows: initial denaturing (95℃ for 5 min), 30 cycles of denaturation (94℃ for 1 min), annealing (respective Tm) and extension (72℃ for 1 min) followed by a final extension of 10 min at 72℃. PCR amplicons were purified and sequenced (SciGenome Lab, Cochin, India). Sequenced data was analyzed and gene variants of bla_{CTX-M} and bla_{TEM} were determined using NCBI nucleotide database.

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences and bla_{CTX-M} gene sequences of test isolates were deposited in GenBank database under the accession numbers KY458511 -KY466932 and KY466971-KY467023, respectively. Complete gene sequences of bla_{TEM} gene were deposited under the accession numbers KY466933-KY466970.

Results

A total of 506 non-duplicate bacterial isolates were obtained from the ten different sampling sites. Analysis of preliminary and phenotypic disc confirmatory tests revealed 175 (34.58%) isolates to be ESBL producers. Percentage of ESBL producing isolates obtained from sampling sites ranged from 18.6% at sampling site GC to 58.33% at IB as shown in Fig. 2.

Antibiotic susceptibility pattern of ESBL producing isolates

All phenotypically ESBL positive isolates showed variation in their susceptibility pattern towards wide range of antibiotics tested. Antibiotic profiling data (Table 3) showed that majority of ESBL producing isolates were resistant to cefazolin (74.28%), rifampicin (70.28%) and ampicillin (66.28%). Varying degree of resistance for cefoxitin (46.28%), piperacillin/tazobaactam (39.42%), trimethoprim (36%), ampicillin/sulbactam (29.71%), ciprofloxacin (27.42%), polymixin B (26.28%), colistin (20%), amikacin (14.85%), tetracycline (14.85%), levofloxacin (13.71%), imipenem (7.42%) and chloramphenicol (7.42%) were observed among isolates. MDR and XDR phenotypes were observed in 76.57% and 6.85% isolates respectively. Resistance pattern of isolates from differ-

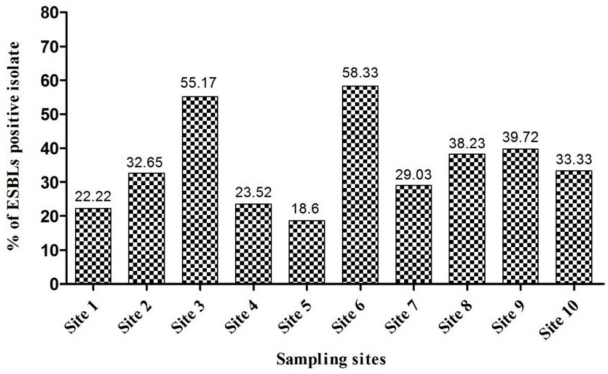

Fig. 2. Prevalence of ESBL positive isolates among samples from different site.

Table 3. Percentage (%) of isolates showing resistance against different antibiotics.

Antibiotic	Site 1 (*n* = 6)	Site 2 (*n* = 16)	Site 3 (*n* = 16)	Site 4 (*n* = 8)	Site 5 (*n* = 8)	Site 6 (*n* = 21)	Site 7 (*n* = 27)	Site 8 (*n* = 26)	Site 9 (*n* = 29)	Site 10 (*n* = 18)	Total (*n* = 175)
Rifampicin	50	87.5	93.75	62.5	75	85.71	62.96	57.69	58..62	72.22	70.28
Cefazolin	33.33	93.75	75	75	100	95.23	81.48	65.38	58.62	61.11	74.28
Ampicillin/Sulbactam	0	37.5	37.5	37.5	50	28.57	40.74	26.92	31.03	0	29.71
Piperacillin/Tazobactam	33.33	43.75	31.25	50	37.5	38.09	44.44	38.46	37.93	38.88	39.42
Ampicillin	50	87.5	56.25	75	75	71.4	66.66	65.38	58.62	66.66	66.28
Imipenem	0	6.25	0	25	0	4.76	11.11	7.69	13.79	0	7.42
Cefoxitin	0	43.75	56.25	37.5	50	61.90	59.25	38.46	44.82	33.33	46.28
Chloramphenicol	0	6.25	18.75	0	25	4.76	18.51	0	4.34	0	7.42
Colistin	0	12.5	50	25	0	19.04	7.40	26.92	24.13	5.55	20
PolymyxinB	0	18.75	43.75	50	25	19.04	33.33	19.23	34.48	11.11	26.28
Tetracycline	0	12.5	18.75	12.5	37.5	14.28	22.22	11.53	17.24	5.55	14.85
Amikacin	0	12.5	25	0	0	9.52	11.11	15.38	34.48	5.55	14.85
Ciprofloxacin	0	37.5	25	50	37.5	38.09	37.03	23.07	20.68	0	27.42
Levofloxacin	0	6.25	18.75	25	12.5	28.57	11.11	15.38	13.79	0	13.71
Trimethoprim	0	37.5	37.5	62.5	50	38.09	44.44	30.76	37.93	16.66	36
MAR index	0.11	0.36	0.39	0.39	0.38	0.36	0.37	0.29	0.32	0.21	-

ent sites are shown in Fig. 3. The highest MAR index of 0.39 was found for site 3 (BV) and 4 (RB), followed by 0.38, 0.37, 0.36, 0.36, 0.32, 0.29, 0.21 and 0.11 for site 5 (GC), 7 (SR), 2 (MT), 6 (IB), 9 (NB), 8 (MB), 10 (OB) and 1 (WB) respectively.

MIC of CAZ, CTX, C, CIP, RIF and TR were determined in 60 ESBL positive isolates. The tested isolates showed a high tolerance to CAZ (8->512 µg/ml), CTX (4->512 µg/ml), C (2-128 µg/ml), CIP (2-256 µg/ml), RIF (4-256 µg/ml) and TR (4-512 µg/ml) as shown in Table 4.

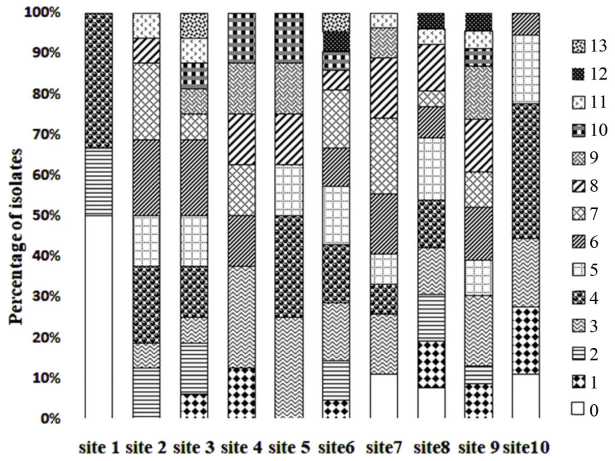

Fig. 3. Percentage of isolates from sampling sites exhibiting resistance to different numbers of antibiotics.

Identification and characterization of ESBL positive isolates

Selected on random basis a total of 60 ESBL positive isolates from different sites were identified by 16S rRNA gene sequencing. Out of these, 32 isolates were found to be *E. coli* followed by *Shigella* (8), *Enterobacter cloacae* (8), *Escherichia fergusonii* (4), *Acinetobacter* sp. (2), and only one isolate each of *Acinetobacter variabilis, Acinetobacter baumannii, Acinetobacter junii, Aeromonas caviae, Citrobacter freundii* and *Kluyvera georgiana* were obtained from selected samples. When analyzed for the presence of resistance determinants imparting ESBL phenotype, $bla_{\text{CTX-M}}$ was found to be the most prevalent ESBL gene (88.33%) with a higher proportion of $bla_{\text{CTX-M-15}}$ variant (65%), followed by $bla_{\text{CTX-M-55}}$ (11.66%) and $bla_{\text{CTX-M-152}}$ (11.66%). Genes coding for $bla_{\text{TEM-116}}$ were present in 31.66% of the isolates. Nearly 30% of the isolates possessed non-ESBL $bla_{\text{TEM-1}}$. Co-occurrence of $bla_{\text{CTX-M}}$ and bla_{TEM} genes was detected in 58.33% of the resistant bacteria (Table 4). The bla_{OXA} gene was not detected in any of the isolates.

Discussion

Urban aquatic environment in particular those receiving contaminants of anthropogenic origin like domestic,

Table 4. Presence of β-lactamases genes among ESBL producing isolates.

Strain	Species	Identity %	MIC (µg/ml)						ESBL gene	Non-ESBL gene
			CAZ	CTX	C	CIP	RIF	TR		
WB4	*Escherichia coli*	100%	>512	>512	4	4	4	>512	$bla_{CTX-M-15}$	bla_{TEM-1}
WB5	*Escherichia coli*	100%	>512	>512	2	8	8	>512	$bla_{CTX-M-15}$	bla_{TEM-1}
WB6	*Escherichia coli*	100%	>512	>512	128	4	4	256	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
MT3	*Escherichia coli*	99%	>512	>512	4	4	8	>512	$bla_{CTX-M-15}$	-
MT25	*Escherichia coli*	99%	>512	>512	4	2	4	>512	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
BV5	*Escherichia coli*	99%	8	256	2	2	4	64	$bla_{CTX-M-15}$	-
BV8	*Escherichia coli*	99%	>512	64	4	4	256	256	$bla_{CTX-M-15}$	-
BV12	*Escherichia coli*	100%	>512	512	8	128	8	>512	$bla_{CTX-M-55}$	bla_{TEM-1}
BV20	*Escherichia coli*	100%	64	>512	4	4	64	>512	$bla_{CTX-M-55}$	bla_{TEM-1}
RB1	*Escherichia coli*	100%	32	>512	4	256	8	>512	$bla_{CTX-M-15}$	-
RB20	*Escherichia coli*	100%	16	512	4	64	8	32	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
GC5	*Escherichia coli*	99%	512	512	2	2	256	>512	$bla_{CTX-M-15}$	-
IB7	*Shigella* sp.	99%	256	64	2	4	8	256	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
IB11	*Acinetobacter baumannii*	99%	>512	128	2	2	4	>512	$bla_{CTX-M-152}$	-
IB12	*Escherichia fergusonii*	100%	>512	256	16	4	16	4	$bla_{CTX-M-15}$	-
IB13	*Escherichia fergusonii*	100%	>512	>512	8	64	256	>512	$bla_{CTX-M-15}$	-
IB14	*Shigella* sp.	99%	>512	>512	2	4	8	512	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
IB22	*Escherichia coli*	100%	512	128	4	4	128	>512	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
IB24	*Enterobacter cloacae*	99%	>512	>512	4	64	4	4	$bla_{CTX-M-15}$	bla_{TEM-1}
SR2	*Enterobacter cloacae*	99%	64	8	8	4	4	>512	$bla_{CTX-M-15}$	-
SR9	*Aeromonas caviae*	100%	16	8	4	4	4	128	-	-
SR10	*Escherichia coli*	99%	128	256	2	4	4	256	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
SR11	*Acinetobacter* sp.	99%	>512	256	4	4	4	64	$bla_{CTX-M-15}$	-
SR13	*Shigella* sp.	100%	>512	>512	4	4	8	>512	$bla_{CTX-M-55}$	bla_{TEM-1}
SR22	*Escherichia coli*	99%	>512	256	16	4	4	4	$bla_{CTX-M-55}$	bla_{TEM-1}
SR33	*Escherichia coli*	100%	>512	128	8	128	4	512	-	bla_{TEM-1}
SR37	*Enterobacter cloacae*	100%	16	4	32	8	64	4	$bla_{CTX-M-15}$	-
SR38	*Enterobacter cloacae*	100%	512	512	8	256	8	>512	$bla_{CTX-M-15}$	bla_{TEM-1}
SR39	*Escherichia coli*	99%	>512	16	32	4	8	8	-	-
SR41	*Escherichia coli*	100%	256	512	4	64	4	4	$bla_{CTX-M-15}$	-
SR45	*Escherichia coli*	100%	128	64	2	4	4	32	-	-
SR46	*Escherichia coli*	100%	64	32	4	8	128	>512	$bla_{CTX-M-15}$	-
SR47	*Acinetobacter variabilis*	100%	>512	256	4	16	4	64	$bla_{TEM-116}$, $bla_{CTX-M-152}$	-
MB16	*Escherichia coli*	99%	512	>512	2	8	4	>512	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
MB27	*Escherichia coli*	100%	512	512	8	64	16	>512	$bla_{TEM-116}$	-
MB29	*Shigella* sp.	100%	>512	512	8	128	4	4	$bla_{CTX-M-15}$	bla_{TEM-1}

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Table 4. Continued.

Strain	Species	Identity %	MIC (μg/ml)						ESBL gene	Non-ESBL gene
			CAZ	CTX	C	CIP	RIF	TR		
MB30	*Escherichia coli*	99%	>512	256	64	4	256	512	$bla_{CTX-M-15}$	bla_{TEM-1}
MB32	*Escherichia fergusonii*	100%	>512	>512	2	4	4	16	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
MB35	*Escherichia coli*	100%	>512	>512	2	2	64	4	$bla_{CTX-M-15}$	-
MB37	*Enterobacter cloacae*	100%	128	>512	4	16	4	>512	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
MB39	*Escherichia coli*	100%	512	512	8	128	4	64	$bla_{CTX-M-15}$	-
MB41	*Shigella* sp.	100%	512	512	4	64	4	4	-	-
MB42	*Enterobacter cloacae*	99%	>512	256	2	128	4	8	$bla_{CTX-M-15}$	bla_{TEM-1}
NB7	*Escherichia coli*	100%	>512	512	8	128	64	512	$bla_{CTX-M-15}$	bla_{TEM-1}
NB13	*Enterobacter cloacae*	100%	>512	128	8	64	8	>512	$bla_{CTX-M-55}$	bla_{TEM-1}
NB14	*Escherichia coli*	99%	16	>512	16	4	4	4	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
NB22	*Escherichia coli*	99%	>512	16	4	8	4	>512	$bla_{CTX-M-15}$	-
NB27	*Escherichia coli*	100%	512	128	4	4	8	4	$bla_{TEM-116}$, $bla_{CTX-M-152}$	-
NB30	*Enterobacter cloacae*	100%	512	>512	8	64	8	64	$bla_{CTX-M-15}$	bla_{TEM-1}
NB32	*Escherichia coli*	99%	>512	512	4	8	128	32	-	-
NB34	*Escherichia coli*	99%	>512	>512	4	4	8	8	$bla_{CTX-M-15}$	-
NB39	*Citrobacter freundii*	100%	128	64	8	64	16	512	$bla_{CTX-M-55}$	-
NB42	*Shigella* sp.	100%	>512	64	2	2	8	>512	$bla_{CTX-M-15}$	bla_{TEM-1}
NB43	*Escherichia coli*	99%	>512	64	8	2	4	4	$bla_{TEM-116}$, $bla_{CTX-M-152}$	-
NB45	*Escherichia fergusonii*	99%	>512	64	4	4	16	512	$bla_{TEM-116}$, $bla_{CTX-M-15}$	-
NB50	*Shigella* sp.	100%	>512	>512	4	64	4	>512	$bla_{CTX-M-55}$	bla_{TEM-1}
BO1	*Shigella* sp.	100%	32	>512	4	4	8	>512	$bla_{CTX-M-152}$	bla_{TEM-1}
BO11	*Acinetobacter junii*	99%	>512	>512	2	256	64	>512	$bla_{TEM-116}$, $bla_{CTX-M-152}$	-
BO16	*Acinetobacter* sp.	100%	>512	32	4	4	4	16	$bla_{CTX-M-15}$	bla_{TEM-1}
BO27	*Kluyvera georgiana*	99%	512	>512	8	64	8	32	$bla_{TEM-116}$, $bla_{CTX-M-152}$	-

hospital, industrial and agricultural waste constitute important antibiotic resistance reservoirs. In developing countries, occurrence of multidrug resistant bacteria in urban rivers, lakes and streams are well documented [14–18]. The microbiological counts and their resistance profiles are of significant importance as they are a direct indicator of threat to community health. In the present study, 34.58% of bacterial isolates from heavily polluted Delhi stretch of river Yamuna, India were found to be ESBL producers conferring resistance to cephalosporins. This high prevalence of ESBL positive *Enterobacteriaceae* was more than previous reports on *E. coli* and other gram negative bacteria from river Yamuna [14, 15]. Moreover, studies from rivers (n = 40) and lakes (n = 18) of Switzerland and rivers (n = 15) of Taiwan reported more or less similar prevalence (36.2% *Enterobacteriaceae* and 30% *E. coli*, respectively) of ESBL producing isolates [18, 19]. Whereas, a low prevalence of ESBL producing isolates have been reported from different rivers of Italy (13.55% *Enterobacteriaceae*) [20], Poland (11.73% *E. coli*) [21], Korea (2.5% *E. coli*) [22] and Tunisia (1.7% *Enterobacteriaceae*) [23].

Anthropogenic-driven selective pressures contributing to the persistence and dissemination of multidrug resistant gram-positive and gram-negative bacteria are of critical relevance to clinical settings [17]. Among 60 tested isolates *E. coli* was found to be most abundant (32) followed by *Shigella* (8), *Enterobacter cloacae* (8), *Escherichia fergusonii* (4), *Acinetobacter* sp. (2) and only one isolate each of *Acinetobacter variabilis, Acinetobacter baumannii, Acinetobacter junii, Aeromonas caviae, Citrobacter freundii* and *Kluyvera georgiana*. This is in accordance with previous studies showing the predominance of *E. coli* in environmental studies [17, 23, 24]. Most of these ESBL producing isolates showed resistance for β-lactam antibiotics i.e. ampicillin and cefazolin, similar to the resistance pattern observed from an urban river sediment habitat of China [24]. In line with previous reports [25–27], imipenem from the carbapenem class and chloramphenicol & amikacin from non-β-lactam class were found to be effective against ESBL producing isolates. Colistin resistance was found to be 20%, higher than previous reports from both clinical and environmental isolates [25, 28]. This increased resistance to colistin, one of the last resort antibiotics to treat infections caused by MDR pathogens is worrisome. MDR phenotype was observed among 76.57% ESBL positive isolates, in accordance with the report from surface water and wastewater (77%) in Netherland [29]. High MIC value (in the range of 2 - >512 μg/ml) for ceftazidime, cefotaxime and trimethoprim demonstrated high tolerance to β-lactam and folate pathway inhibitor among ESBL positive isolates.

Based on the criteria from Krumperman [13], a MAR index value of less than or equal to 0.2 suggests the environment, where antibiotics are seldom or never used. A MAR index value greater than 0.2 suggests their origin from a high risk source of contamination where antibiotics are often used. The Wazirabad barrage is the starting point of sampling where environmental bacterial isolates are not exposed to anthropogenic impacts and showed least MAR index (0.11). Just downstream of the Wazirabad barrage, major drains carrying domestic, hospital and industrial waste discharge into the river. This anthropogenically influenced stretch showed an increased MAR index in the range of 0.21 to 0.39 at sampling sites. Furthermore, 50% of bacterial isolates obtained from Wazirabad barrage were sensitive to all tested antibiotics. Whereas, isolates collected from downstream of Wazirabad barrage showed multidrug resistance phenotype ranging from 68.9 to 100%. In our study, $bla_{\text{CTX-M}}$ gene was present among 88% isolates dominating other ESBL gene i.e. bla_{TEM}, contrary to the findings of Bajaj *et al.* who reported TEM type to be most prevalent among *E. coli* isolates from river Yamuna [15]. High prevalence of CTX-M has also been reported by Diwan *et al.* and Reinthaler *et al.* in India and Australia [30, 31]. Three variants of $bla_{\text{CTX-M}}$ gene ($bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-152}}$) were detected in this study, with $bla_{\text{CTX-M-15}}$ as the most predominant type (65%). Previous studies also report $bla_{\text{CTX-M-15}}$ as the most commonly identified ESBL gene in *Enterobacteriaceae* and referred as pandemic due to its worldwide prevalence [5, 32]. The extensive use of ceftriaxone and cefotaxime has been suggested as a reason for the emergence and spread of CTX-M enzymes [33]. CTX-M-55, a variant of CTX-M-15 by a single substitution of Ala-77-Val that confers higher level of ceftazidime resistance, was first identified in Thailand [34], thereafter making several incidences worldwide [35–37]. CTX-M-152, a variant of CTX-M-group-25, first identified in India in *Kluvyera georgiana* was detected in bacterial isolates of *A. baumannii*, *A. variabilis, A. junii* and *Shigella* sp. [14].

With no previous reports of $bla_{\text{TEM-116}}$ in *Acinetobacter variabilis* and *Escherichia fergusonii* from India, results of this study found occurrence of $bla_{\text{TEM-116}}$ in 19 out of 60 isolates. These findings were in concordance of $bla_{\text{TEM-116}}$ in environmental isolates of *Acinetobacter* sp. *from* Croatia [38]. $bla_{\text{TEM-116}}$ has previously been identified in *Klebsiella pneumoniae* isolates from Korea [39], Netherlands [40], and Tunisia [41].

Narrow spectrum β-lactamase gene $bla_{\text{TEM-1}}$ was detected among 18 isolates. $bla_{\text{CTX-M}}$ and bla_{TEM} resistant determinants were co-harbored by 58.33% isolates. The co-existence of more than one β-lactamase gene within the same isolate is well documented in several studies [42, 43]. The combination of $bla_{\text{CTX-M-15}} + bla_{\text{TEM-116}}$ found in 12 isolates and $bla_{\text{CTX-M-15}} + bla_{\text{TEM-1}}$ in 11 isolates had also been previously reported in *E. coli* by Azam *et al.* and Al-Agamy *et al.*, respectively [14, 44].

To conclude with, this study highlights a significant increase of multidrug resistant ESBL producing *Enterobacteriaceae* in Delhi stretch of river Yamuna. $bla_{\text{CTX-M-15}}$ was found to be the most dominant type of ESBL among

Enterobacteriaceae. Occurrence of $bla_{\text{CTX M-152}}$ variants in *Acinetobacter baumannii*, *Acinetobacter variabilis* and *Acinetobacter junii* and *Shigella* sp. not reported earlier, indicates horizontal spread of ESBL in urban aquatic environment. Susceptibility to carbapenem class of antibiotic seems it the only left over option for the infection control as the high percentage of colistin resistance among environmental isolates is of grave concern. Further studies are needed to obtain an overall view of escalating multidrug resistance in inhabitants of anthropogenically influenced water bodies to understand the transmission dynamics and develop management strategies.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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