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Lentic and effluent water of Delhi-NCR: a reservoir of multidrug-resistant bacteria harbouring *bla*CTX-M, *bla*TEM and *bla*SHV type ESBL genes

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ABSTRACT

Antimicrobial resistance is not restricted to clinics but also spreading fast in the aquatic environment. This study focused on the prevalence and diversity of extended-spectrum β-lactamase (ESBL) genes among bacteria from lentic and effluent water in Delhi-NCR, India. Phenotypic screening of 436 morphologically distinct bacterial isolates collected from diverse sites revealed that 106 (~24%) isolates were ESBL positive. Antibiotic profiling showed that 42, 60, 78 and 59% ESBL producing isolates collected from Ghazipur slaughterhouse, Lodhi garden pond, Hauz Khas lake and Jasola wastewater treatment plant, respectively, were multidrug-resistant (MDR). The multiple antibiotic resistance (MAR) index varied from 0.20 to 0.32 among selected locations. The prevalence of ESBL gene variants *bla*SHV, *bla*TEM and *bla*CTX-M were found to be 17.64, 35.29 and 64%, respectively. Furthermore, the analysis of obtained gene sequences showed three variants of *bla*CTX-M (15, 152 and 205) and two variants of *bla*TEM (TEM-1 and TEM-116) among ESBL producers. The co-existence of 2–3 gene variants was recorded among 48% ESBL positive isolates. New reports from this study include the *bla*CTX-M gene in *Acinetobacter lwoffii*, *Enterobacter ludwigii, Exiguobacterium mexicanum* and *Aeromonas caviae*. Furthermore, the identification of *bla*TEM and *bla*SHV in an environmental isolate of *A. caviae* is a new report from India.

Key words: AMR, aquatic environment, *bla*CTX-M, *bla*SHV, *bla*TEM, ESBLs

HIGHLIGHTS

- Occurrence of ESBL genes from *Exiguobacterium mexicanum* was first reported.
- *bla*CTX-M gene from environmental isolate of *Aeromonas caviae* is the first report.
- Co-resistance of β-lactam and non-β-lactam classes of antibiotics was observed among a high proportion of ESBL positive bacterial isolates.
- This study emphasizes the need for more comprehensive genetic research of diverse microorganisms from environmental settings.

INTRODUCTION

Extensive use of antibiotics in developed as well as in developing countries had led to a rapid increase in their concentration in the aquatic environment, as a significant fraction of antibiotics is unutilized and released into wastewater or effluent from treated wastewater (Zhou *et al.* 2011; Sultan *et al.* 2020). Due to the frequent use of third-generation cephalosporins for the treatment of infections caused by members of Enterobacteriaceae, the upshot has been recorded in resistance to these antibiotics. Extended-spectrum β-lactamases (ESBLs) are recognized to provide resistance to most of the β-lactam antibiotics along with the other type of antibiotics like aminoglycosides, monobactam, carbapenem, ansamycin, tetracycline and polymyxins (Taco *et al.* 2014; Gogry *et al.* 2019; Siddiqui *et al.* 2020). The presence of various classes of antimicrobials in the wastewater contributes as an important factor towards the emergence of resistance and their dissemination (Aminov & Mackie 2007). There are numerous reports of antibiotic-resistant bacteria (ARB) in environmental settings such as wastewater treatment plants (WWTPs), rivers, hospital effluents, lakes and ponds (Falodun & Ikusika 2019; Bhattacharyya *et al.* 2020; Smyth *et al.* 2020). The emergence and distribution of antibiotic resistance genes (ARGs) among pathogenic organisms can impair human and animal fitness. In the environment, ARG transmission occurs through the horizontal gene transfer (HGT) mechanism (Zhang *et al.* 2011). Reuse of wastewater for different domestic as well as agricultural purposes favours the uptake

of ARGs by plants, humans and animals (Fatta-Kassinos *et al.* 2011). Wastewater and treated effluents have contributed towards an increase in antibiotic-resistant microorganisms in the environment and thereby enhanced the ARG load into the water bodies (Karthikeyan & Meyer 2006). The WWTPs are measured as significant reservoirs for ARGs since activated sludge facilitates the richness of microbial community and also supports the HGT using mobile genetic elements (MGEs) of ARGs (Zhang *et al.* 2011; Moura *et al.* 2012).

To prevent the probable health risks originating from ARB in the aquatic environment, the evaluation of such infectious and harmful bacteria is of high importance. Therefore, the present study was carried out to determine the prevalence and diversity of ESBL genes among bacterial isolates from lentic and effluent water in Delhi-NCR, India.

MATERIALS AND METHODS

Inspection of sites and sample collection

In this study, four different sites were selected to investigate the occurrence and distribution of ARGs among bacterial isolates from lentic and effluent water in Delhi-NCR, one of the largest metropolitan cities of India with a population size of approximately 24 million. Sampling site Hauz Khas lake is a 125 acres 14th-century water body in New Delhi, now shrunk in size to 15 acres and has a maximum depth of 1–2.5 m. The lake is a site of recreation for people, and its water is used for irrigation, animal use in a nearby park (Deer Park). The second site, the Lodhi garden pond, present in Lodhi estate – a tourist site in Delhi, is a duck-fish integrated aquaculture pond. The pond is land-locked and does not receive any sewage water. The third site, Ghazipur slaughterhouse, has a capacity of 1,000 head of cattle to be slaughtered per day. A bio-methanation plant converts digested and undigested stomach contents of ruminants into gas and slurry. The blood released during the slaughtering process is treated before being released into the river Yamuna. The samples were collected from effluent water of the wastewater treatment plant. The fourth site, Jasola wastewater treatment plant, is the biggest treatment plant in South Delhi having the potential to impact the environment to a large extent. Out of four sites, two sites were effluent treated water, viz. Ghazipur slaughterhouse, Ghazipur U.P. India (28°62′N, 77°32′E) and Jasola wastewater treatment plant, Jasola Vihar, New Delhi (28°54′N, 77°28′E), and the other two sites were lentic water bodies, viz. Hauz Khas lake, Hauz Khas, New Delhi, Delhi (28°55′N, 77°19′E) and Lodhi garden pond, Lodhi Estate, New Delhi, Delhi (28°59′N, 77°21′E). Water samples were aseptically collected in a 100 mL sterile glass bottle, stored in an icebox (~4 °C) and transported within 4 h from the sampling sites to the Microbiology Research Laboratory. Before transportation, temperature and pH were recorded for each sampling site. The samples were collected during the year 2015–2016.

Isolation and identification of ESBL positive bacteria

For isolation, samples were serially diluted up to 1×10^{-4}, after dilution of the samples 100 μL of each dilution were spread on Luria Agar (LA) plates and kept for 16–18 h incubation at 37 °C (Siddiqui *et al.* 2019). After incubation, colonies with different morphology (based on size, appearance, colour, texture, etc.) were picked and streaked on LA plates for further detection of ESBL producing isolates. Morphologically distinct isolates from all four sites were screened by a preliminary test. It was performed using third-generation antibiotics cephalosporins and monobactam by the disk diffusion method. Suspected ESBL producers in the preliminary test were further confirmed by the phenotypic disc confirmatory test (PDCT) which was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2016) for ESBL production. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as ESBL negative and positive control, respectively.

Antibiotic profiling and multiple antibiotic resistance index

Antibiotic profiling of ESBL positive bacterial isolates was performed by the disk diffusion method on Muller Hinton Agar (MHA) plate using 12 different antibiotic classes as per CLSI guidelines (CLSI 2016). Twenty different antibiotics associated with 12 classes include Aminoglycosides: amikacin; Penicillin: ampicillin; Penicillin + inhibitor: piperacillin/tazobactam; ampicillin/sulbactam, Cephalosporins: ceftriaxone, cefotaxime, ceftazidime, and cefazolin, cefoxitin; Quinolone: ciprofloxacin, levofloxacin, Carbapenem: imipenem, ertapenem; Polypeptide: polymyxin B and colistin, Folate pathway inhibitor: trimethoprim, tetracyclin: tetracycline, Phenicol: chloramphenicol, ansamycin: rifampicin, Monobactam: aztreonam (Himedia India) included in the study. After spreading of inoculum on MHA, plates were incubated overnight at 37 °C and the susceptibility pattern was determined by the zone inhibition test. Isolates showing resistant phenotype against three (equal or more than three) different classes of antibiotics were categorized as multidrug-resistant (MDR) (Magiorakos *et al.*

2012). The multiple antibiotic resistance (MAR) index of isolates against different classes of antibiotics was calculated. MAR index is calculated by formula *a*/*b*, where '*a*' signifies the number of antibiotics to which the isolate was resistant and '*b*' represents the number of antibiotics to which the isolate was subjected. MAR index was inferred for each location and specific isolate as detailed by Krumperman (1983). Minimum inhibitory concentration (MIC) of eight antibiotics, i.e., ceftazidime (CAZ), ciprofloxacin (CIP), ampicillin (AMP), rifampicin (RIF), cefotaxime (CTX), chloramphenicol (C), colistin (CL) and trimethoprim (TR), against ESBL positive isolates was determined by the broth microdilution method as per CLSI guidelines. Luria Broth (LB) was used for the culture of bacterium and as a reference. 10.24 mg/mL stock solution of antibiotics were prepared and inoculated into the first well of each column onto the polystyrene plate. The progressive serial broth dilution was performed to obtain the required concentrations of 1,024, 512, 256, 128, 64, 32, 16, 8, 4 and 2 μg/mL in 96 well microtitre plate, and medium was inoculated with 10 μL of 1,000 times diluted 0.1 OD600 culture and incubated overnight at 37 °C. The optical density (OD) was measured at 600 nm using a microplate reader (Thermo Scientific MultiscanGo).

Isolation of genomic and plasmid DNA

For isolation of genomic and plasmid DNA, a pure bacterial colony was suspended into sterile LB media and incubated overnight to obtain mid to late log phase of the bacterial culture. The cells were pelleted by centrifugation. Further, the genomic DNA from $ESBL^+$ samples was extracted by Phenol:Chloroform:Isoamyl alcohol (PCI) procedure and plasmid DNA isolated using alkaline lysis methods. Extracted DNA was visualized over the ultraviolet (UV) transilluminator (GeNei-India). These genomic and plasmid DNA were used as a template in a polymerase chain reaction (PCR) for amplification of various ESBL genes. For amplification of genes, 50 μL master mix were prepared that comprises of 39.5 μL PCR grade water, 5 μL of 10× buffer (with 25 mM $MgCl_2$), 1 μL of deoxynucleotide triphosphate mixture (10 mM dNTP), 1 μL of forward primer, 1 μL of reverse primer, 0.5 μL Taq polymerase (5 U/μL, GeNei-India) and 2 μL of extracted DNA template. PCR conditions were kept as follows: Initial denaturation for 5 min at 95 °C, followed by 30 cycles of cyclic denaturation for 1 min, annealing for 45 s at respective annealing temperature (Tm) of different genes, an extension for 1 min at 72 °C and a final extension for 10 min at 72 °C. After that amplified PCR products were loaded in well (1% agarose gel). After running of gel, DNA bands were visualized over UV transilluminator (GeNei-India). Primers used in PCR amplification for various genes are mentioned in Table 1.

Table 1 | Primer sequences used for amplification of different genes in this study

Targeted gene	Primer	Sequences	Amplicon length	References
16S rRNA	RRF RRR	5′-GGCGGACGGGTGAGTAATGT-3′ 5′-CGATTACTAGCGATTCCGACTTCA-3′	1,252	Azam *et al.* (2016)
*bla*CTX-M	CTX-F CTX-R	5′-TGTGCAGYACCAGTAAAGTKAT -3′ 5′-TARGTCACCAGAACVAGCG -3′	610	This study
*bla*CTX-M-1	C F -1 C R -1	5′-AGGAAGTGTGCCGCTGTATG -3′ 5′-GGTTGAGGCTGGGTGAAGTAA -3′	750	This study
*bla*CTX-M-2	CM2F CM2R	5′-ATGATGACTCAGAGCATTCGCC-3′ 5′-TCGTTGGTGGTGCCATAATCTCC-3′	742	Azam *et al.* (2016)
*bla*CTX-M-8	CM8F CM8R	5′-AACGCACAGACGCTCTACC-3′ 5′-GGGTAGCCCAGCCTGAAT-3′	517	Azam *et al.* (2016)
*bla*CTX-M-9	CT-9 F CT-9 R	5′-ATGGTGACAAAGAGAGTGCA-3′ 5′-GTTCTGTTGCGGCTGGGTAA-3′	811	Siddiqui *et al.* (2019)
*bla*CTX-M-25	CTX-25F CTX-25R	5′-ATGATGAGAAAAAGCGTAAGGCGGGCG-3′ 5′-TTAATAACCGTCGGTGACAATTCTGGCGG-3′	876	This study
*bla*TEM	TEMF TEMR	5′-ATGAGTATTMAACATTTYCGTGTCGCC-3′ 5′-TTACCAATGCTTAATCAGTGAGGCACCTATC-3′	861	Azam *et al.* (2016)
*bla*SHV	S-F S-R	5′-ATGCGTTATATTCSCCTGTGTAT-3′ 5′-GCGTTGCCASTGCTCGATCAG-3′	861	Siddiqui *et al.* (2019)

Molecular characterization of CTX-M type ESBL genes

Phenotypically ESBL positive isolates were screened for the presence of different ESBL genes using specific primers (Table 1). PCR products of various *bla*CTX-M, *bla*TEM and *bla*SHV genes were purified and sequenced with corresponding primers. Sequencing was carried out by Agrigenome, India. The obtained gene sequence data were examined using FinchTV and Bio-Edit. Nucleotide sequences were subject to Basic Local Alignment Search Tool (BLAST) to identify different gene variants.

Bacterial identification using 16S rRNA approach

All the CTX-M positive samples were characterized by 16S rRNA sequencing. To perform it, 16S rRNA-specific primers were used for PCR amplification of 16S rRNA gene and the obtained products were sequenced. The obtained gene sequence data were analyzed, and homology was searched using BLAST at NCBI.

Conjugation transfer experiment

Conjugation was performed to learn about the capacity of ESBL positive bacteria to move their resistant factors (*bla*CTX-M, *bla*TEM and *bla*SHV) to recipient bacteria. Four isolates, i.e., JST71, HK106, SH52 and LG1, from different sampling sites harbouring ESBL genes and having MDR phenotype were selected as a donor, and the *E. coli* J^{53} (Sodium Azide resistant) was selected as recipient. The primary culture of donors was grown in cefotaxime (CTX 2 μg/mL) supplemented LB media and the recipient grown in azide (100 μg/mL) supplemented LB medium. Secondary culture of both donor and recipient were mixed in equal volume in LB and kept at 37 °C for 24 h (HiMedia, India). After that, serial dilutions were prepared in LB medium and spread on LA plates containing NaN_3 (100 μg/mL) and CTX (2 μg/mL). Plasmid DNA was isolated from trans-conjugant and used as a template for PCR amplification to detect the presence of ESBL genes.

RESULTS

Screening of ESBL producers

A total of 436 non-duplicate bacterial isolates were isolated from Ghazipur slaughterhouse (103), Lodhi garden pond (98), Hauz Khas lake (140) and Jasola wastewater treatment plant (95). Based on the disc diffusion method, the preliminary and PDCT depicts that 106 isolates were ESBL positive. Thus, the prevalence of ESBLs producing bacteria in Ghazipur slaughterhouse, Lodhi garden pond, Hauz Khas lake and Jasola wastewater treatment plant was found to be 32, 23, 20 and 23%, respectively (Table 2).

Antibiotic profiling

Non-*β*-lactam

Among non-*β*-lactam, polymyxins class of antibiotics exhibited less effective towards phenotypically ESBL positive isolates. 39.62 and 33.01% ESBL positive isolates were resistant towards colistin and polymyxin B, respectively (Figure 1(a)). Resistance to rifampicin was found in 26.41% of ESBL positive isolates, followed by amikacin (19.81%), trimethoprim (18.86%) and chloramphenicol (6.60%). 1.88% of ESBL positive isolates were found to be resistant towards ciprofloxacin, levofloxacin and tetracycline (Table 3).

Table 2 | ESBL producers from different locations

S. no.	Sampling sites	Site code	Location		Number of isolates	ESBL producers (%)
			Latitude	Longitude		
1	Ghazipur slaughterhouse	SH	28°62′N	77°32′E	103	32
2	Lodhi garden pond	LG	28°59′N	77°21′E	98	23
3	Hauz Khas lake	HK	28°55′N	77°19′E	140	20
4	Jasola wastewater treatment plant	JST	28°54′N	77°28′E	95	23

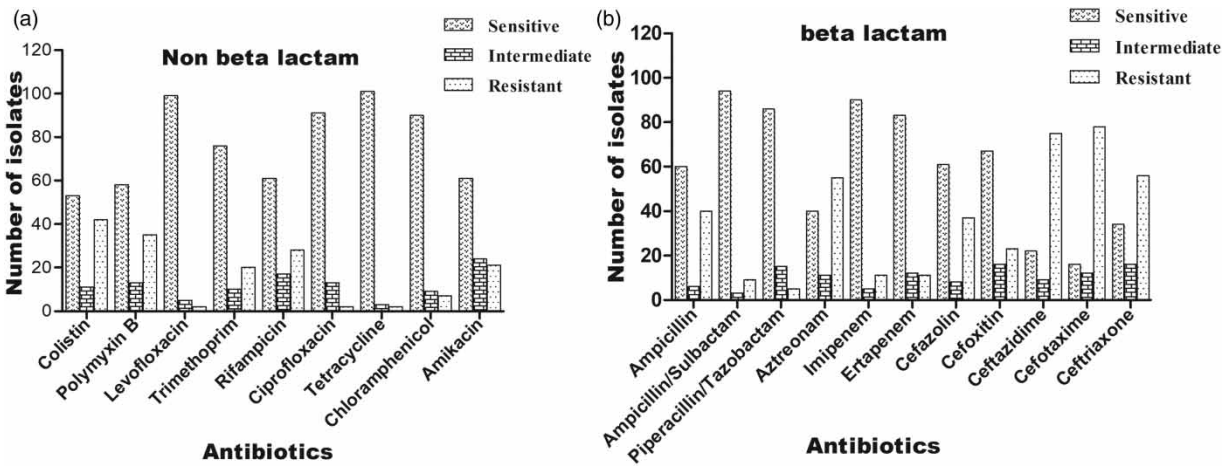

Figure 1 | Resistance pattern of ESBL positive isolates: (a) non-β-lactam antibiotics and (b) β-lactam antibiotics.

Table 3 | Showing site-wise resistance pattern

		Ghazipur slaughterhouse (n = 33)			Lodhi garden pond (n = 23)			Hauz Khas lake (n = 28)			Jasola wastewater treatment plant (n = 22)		
Antibiotics		**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
Non β-lactam	Amikacin	24	8	1	16	4	3	11	6	11	10	6	6
	Colistin	17	4	12	16	2	5	12	2	14	8	3	11
	Polymyxin B	15	10	8	19	1	3	13	2	13	11	0	11
	Trimethoprim	25	3	5	22	0	1	15	6	7	14	1	7
	Rifampicin	28	3	2	10	4	9	14	6	8	9	4	9
	Ciprofloxacin	30	2	1	23	0	0	20	7	1	18	4	0
	Tetracycline	33	0	0	23	0	0	27	0	1	18	3	1
	Chloramphenicol	30	2	1	20	3	0	21	2	5	19	2	1
	Levofloxacin	32	1	0	20	3	0	26	1	1	21	0	1
β-lactam	Ampicillin	24	2	7	14	1	8	12	2	14	10	1	11
	Ampicillin/Sulbactam	33	0	0	21	1	1	25	0	3	15	2	5
	Piperacillin/Tazobactam	30	3	0	21	1	1	20	7	1	15	4	3
	Aztreonam	7	5	21	9	1	13	13	2	13	11	3	8
	Imipenem	33	0	0	18	3	2	22	0	6	17	2	3
	Ertapenem	29	3	1	20	3	0	20	2	6	14	4	4
	Cefazolin	23	4	6	14	4	5	15	0	13	9	0	13
	Cefoxitin	25	5	3	16	2	5	16	4	8	10	5	7
	Ceftazidime	3	1	29	5	2	16	5	3	20	9	3	10
	Cefotaxime	0	4	29	4	3	16	8	3	17	4	2	16
	Ceftriaxone	20	4	9	6	3	14	7	2	19	1	7	14

n, number of ESBL positive isolates; S, sensitive; I, intermediate and R, resistant.

β-lactam

Among ESBL positive isolates, highest resistance (73.85%) was observed for cefotaxime, followed ceftazidime (70.75%), ceftriaxone (52.83%), monobactam (aztreonam) (51.88%), ampicillin (37.73%), cefazolin (34.90%) and cefoxitin (21.69%). Furthermore, some of these isolates showed resistance to the combination of drugs, ampicillin/Sulbactam (8.94%) and piperacillin/tazobactam (4.71%). 10.38% of isolates were resistant towards carbapenem class of antibiotics, viz. ertapenem and imipenem (Figure 1(b)). Site-wise resistance pattern of ESBL positive isolates against different beta-lactam antibiotics is shown in Table 3.

MAR index and MIC

42, 60, 78 and 59% isolates had MDR phenotype from Ghazipur slaughterhouse, Lodhi garden pond, Hauz Khas lake and Jasola wastewater treatment plant, respectively, among all tested ESBLs producing isolates. The MAR index value of Jasola wastewater treatment plant and Hauz Khas lake was found similar 0.32, followed by Lodhi garden pond 0.22 and Ghazipur slaughterhouse 0.2 water. MIC (μg/mL) values for tested antimicrobials of ESBL positive isolates were ascertained as CTX (<2–1,024), AMP (<2– > 1,024), CAZ(2– > 1,024), RIF (<2–16), C (<2–256), CIP (<2–32), CL (<2– > 1,024) and TR (<2– > 1,024) (Table 4).

Identification of ESBL determinant

Among 106 ESBLs producers, 68 (64%) isolates were harbouring the *bla*CTX-M gene. Further, site-wise 77.27, 73.91, 66 and 57.14% isolates from Jasola wastewater treatment plant, Lodhi garden pond, Ghazipur slaughterhouse and Hauz Khas lake harbouring *bla*CTX-M gene, respectively. Analysis based on particular groups showed that *bla*CTX-M-group-1 was the predominant type of ESBL gene (52/68) and *bla*CTX-M-group-25 (22/68) was the second most predominant type. Three different variants of *bla*CTX-M viz. *bla*CTX-M-15 associated with group-1, *bla*CTX-M-205 and *bla*CTX-M-152 from group-25 were identified among isolates. Among the *bla*CTX-M gene, the most prevalent was *bla*CTX-M-15 (70.5%) followed by *bla*CTX-M-205 (22.05%) and *bla*CTX-M-152 (4.41%) (Table 4). The most prevalent *bla*TEM type was *bla*TEM-116 and detected in 62% of isolates, whereas 33% of isolates possessed non-ESBL *bla*TEM-1 (Table 4). Moreover, the *bla*SHV gene was successfully amplified from 17.64% of phenotypically ESBL positive isolates. 35.29% of the isolates possessed both *bla*TEM and *bla*CTX-M genes among the ESBLs producing isolates (Table 4).

Molecular characterization of ESBL producing isolates

16S rRNA gene-based characterization of bacterial isolates showed diversity (21 different types) and belonged to six different families (Enterobacteriaceae, Bacillaceae, Moraxellaceae, Aeromonadaceae, Brucellaceae and Pseudomonadaceae). The isolates were identified as *Enterobacter* sp. (15), *E. coli* (13), *Acinetobacter* sp. (9), *Citrobacter* sp. (7), *Bacillus* sp. (3), *Enterobacter cloacae* (3), *Acinetobacter lwoffii* (2), *Serratia marcescens* (2), *Acinetobacter calcoaceticus* (2) and one isolates each of *Aeromonas caviae, Acinetobacter beijerinckii, Citrobacter werkmanii, Ochrobactrum anthropi, Exiguobacterium mexicanum, Ralstonia* sp., *Kluyvera georgiana, K. pneumoniae, Pantoea agglomerans, Enterobacter ludwigii, Citrobacter freundii* and *Pantoea* sp. (Table 4).

Conjugation experiment

The conjugation assay revealed that donor bacterial isolates including LG1, HK 106, JST 71 and SH52 from different sites were able to transfer their resistance genes to the recipient bacteria (Figure 2). The *bla*CTX-M genes were successfully amplified in all four tarns-conjugants and *bla*TEM from HK106 and SH52. None of the four transconjugant exhibited amplification of the *bla*SHV gene.

Accession numbers of the analyzed nucleotide sequence

Accession numbers of 16S rRNA genes (partial sequences) deposited in GenBank database are MN093322-MN093328, MT672728-MT672743, MN327103, MN327105, MN327106, MN327109, MN327111, MN327112, MT581480-MT581484, MT577527-MT581484, MT577527-MT577533, MT579677, MT577960-MT577363, MT576960-MT577367, MT576700, MN267572, MT267573, MT267576, MT267577, MT267579, MN267554-MN267556, MT576690-MT576693 and MT576698. The partial sequences of *bla*CTX-M genes were deposited in GenBank database under the accession number of MT636756-MT636759, MT636769-MT636782 and MT640044-MT640050. *bla*CTX-M gene complete gene sequences deposited under the accession numbers MT636760 and MT636783-MT636801. Complete gene sequences of *bla*TEM were deposited under the accession numbers MT636802-MT636822.

DISCUSSION

Our study established the substantial occurrence of ARB in the surface water of Hauz Khas lake and Lodhi garden pond along with effluent water from WWTPs, i.e., Ghazipur slaughterhouse and Jasola from Delhi-NCR India. There are several reports of the prevalence of ARB in many freshwater sources from India (Siddiqui *et al.* 2019; Sultan *et al.* 2020), and this study thoroughly documented the prevalence and diversity of ESBL genes among ESBL producers in lentic and effluent water from Delhi-NCR, India. In both lentic as well as lotic water bodies, antimicrobial resistance has significantly increased due to the heavy load of

Table 4 | Antibiotic resistance phenotype and genotype along with MAR index and MIC of the different isolates

Isolates	16S rRNA sequence-based Identification	β-lactamase gene/s	Minimum Inhibitory concentration (μg/mL)								Antibiotic resistance	MAR Index
			TR	CL	C	RIF	AMP	CAZ	CIP	CTX		
HK1	*Enterobacter sp.*	*bla*CTX-M-205, *bla*TEM-116	1024	8	2	< 2	256	128	< 2	128	CL, PB, TR, RIF, AMP, A/S, AT, CZ, CX, CAZ, CTX, CTR	0.6
HK4	*Enterobacter sp.*	*bla*CTX-M	< 2	256	< 2	< 2	< 2	1024	< 2	< 2	RIF, CAZ	0.1
HK8	*Ochrobactrum anthropi*	*bla*CTX-M	< 2	< 2	< 2	4	16	8	< 2	< 2	RIF, AT, CAZ, CTX, CTR	0.25
HK24	*Acinetobacter calcoaceticus*	*bla*CTX-M-15, *bla*CTX-M-205, *bla*TEM, *bla*SHV	1024	< 2	64	16	1024	8	< 2	8	PB, IPM, ETP, CZ, CX, CTR	0.3
HK32	*Enterobacter cloacae*	*bla*CTX-M-205, *bla*TEM-116	1024	256	2	32	1024	128	< 2	256	CL, PB, TR, RIF, AMP, A/S, AT, CZ, CX, CAZ, CTX, CTR	0.6
HK41	*Enterobacter cloacae*	*bla*CTX-M-205, *bla*TEM-116	1024	16	16	< 2	256	128	< 2	32	AK, CL, TR, AMP, CZ, CX, CTX, CTR	0.4
HK43	*Exiguobacterium mexicanum*	*bla*CTX-M-15, *bla*CTX-M-205, *bla*TEM-116, *bla*SHV	> 1024	64	64	8	1024	256	< 2	1024	AK, CL, PB, AMP, AT, ETP, CZ, CX, CAZ, CTR	0.5
HK48	*Citrobacter sp.*	*bla*CTX-M-205	256	< 2	8	< 2	< 2	1024	< 2	256	AK, CL, PB, AMP, IPM, ETP, CZ	0.35
HK52	*Acinetobacter sp.*	*bla*CTX-M-15, *bla*CTX-M-205, *bla*TEM-116, *bla*SHV	< 2	256	< 2	< 2	< 2	1024	< 2	< 2	AK, CL, PB, AMP. AT, IPM, ETP, CZ, CX, CAZ, CTR	0.55
HK91	*Ralstonia sp.*	*bla*CTX-M, *bla*TEM-116	2	128	4	< 2	8	4	< 2	8	AK, C, RIF, AMP, AT, CZ, CAZ, CTX, CTR	0.45
HK106	*Citrobacter sp.*	*bla*CTX-M-15, *bla*CTX-M-205, *bla*TEM-116, *bla*SHV	< 2	> 1024	4	< 2	< 2	> 1024	32	512	AK, CL, PB, CIP, TR, AMP, P/T, AT, IPM, ETP, CZ, CX, CAZ, CTX, CTR	0.75
HK108	*Citrobacter freundii*	*bla*CTX-M-205, *bla*TEM-116	< 2	< 2	< 2	< 2	< 2	4	< 2	< 2	AK, CL, PB, C, TE, AMP, CAZ, CTX, CTR	0.45
HK110	*Enterobacter sp.*	*bla*CTX-M-205, *bla*TEM-116	128	4	4	< 2	< 2	16	< 2	4	AK, CL, C, CAZ	0.2
HK111	*Pantoea sp.*	*bla*CTX-M-205, *bla*SHV	8	4	2	< 2	8	16	< 2	16	CL, LE, AT, CAZ, CTX, CTR	0.3
HK117	*Enterobacter sp.*	*bla*CTX-M-205	4	1024	16	4	< 2	1024	< 2	128	AK, PB, C, RIF, AMP, CAZ, CTX, CTR	0.35
HK132	*Acinetobacter calcoaceticus*	*bla*CTX-M-205, *bla*TEM-116	< 2	1024	128	4	128	16	< 2	< 2	AK, TR, CZ, CX, CAZ	0.25
SH1	*Serratia marcescens*	*bla*CTX-M-15	2	64	4	< 2	4	8	< 2	8	CAZ, CTX, CTR	0.15
SH14	*Kluyvera georgiana*	*bla*CTX-M-15	< 2	128	4	< 2	8	4	2	8	CAZ, CTX, CTR	0.15
SH17	*Acinetobacter sp.*	*bla*CTX-M-15	< 2	256	< 2	< 2	< 2	1024	< 2	< 2	CZ, CAZ, CTR	0.15
SH23	*Citrobacter sp.*	*bla*CTX-M-15	2	128	4	< 2	8	4	< 2	8	PB, CTX, CTR	0.15
SH25	*Bacillus sp.*	*bla*CTX-M-15	< 2	16	8	< 2	< 2	8	< 2	< 2	CL, PB, AT, CAZ, CTX	0.50

SH26	*Acinetobacter lwoffii*	*bla*CTX-M-15	< 2	8	4	< 2	2	8	2	< 2	AMP	0.05
SH28	*Aeromonas caviae*	*bla*CTX-M-15	> 1024	128	64	< 2	16	16	< 2	< 2	CL, C, TR, AMP, AT, CAZ, CTX, CTR	0.40
SH34	*Serratia marcescens*	*bla*CTX-M-15	4	128	4	< 2	8	4	< 2	8	CL, AT, CAZ, CTX	0.20
SH36	*Acinetobacter beijerinckii*	*bla*CTX-M-15	< 2	128	4	< 2	8	8	< 2	8	TR, AMP, CAZ, CTX	0.20
SH42	*Acinetobacter sp.*	*bla*CTX-M	< 2	256	< 2	< 2	< 2	1024	< 2	< 2	CL, AT, CAZ, CTX	0.20
SH51	*Acinetobacter sp.*	*bla*CTX-M	< 2	16	8	< 2	< 2	8	< 2	< 2	AT, CAZ, CTX	0.15
SH52	*Citrobacter werkmanii*	*bla*CTX-M-15, *bla*TEM-1, *bla*SHV	< 2	256	2	< 2	2	> 1024	< 2	1024	CL, PB, TR, RIF, AT, ETP, CZ, CX, CAZ, CTX	0.50
SH60	*Citrobacter sp.*	*bla*CTX-M	2	128	4	< 2	8	4	< 2	8	CL, PB, AT, CAZ, CTX	0.25
SH68	*Citrobacter sp.*	*bla*CTX-M	8	4	2	< 2	8	16	< 2	16	AT, CAZ, CTX	0.15
SH71	*Citrobacter sp.*	*bla*CTX-M	4	64	4	< 2	8	8	< 2	8	AT, CZ, CAZ, CTX	0.20
SH74	*Acinetobacter sp.*	*bla*CTX-M	2	256	4	< 2	8	8	< 2	16	AMP, CTX	0.10
SH77	*Citrobacter sp.*	*bla*CTX-M	4	1024	4	< 2	< 2	512	16	256	AT, CAZ, CTX	0.15
SH78	*Acinetobacter sp.*	*bla*CTX-M	8	4	2	< 2	8	16	< 2	16	CL, AT, CAZ, CTX, CTR	0.25
SH80	*Acinetobacter sp.*	*bla*CTX-M	4	64	4	< 2	8	4	< 2	8	CL, PB, AT, CAZ, CTX	0.25
SH85	*Acinetobacter sp.*	*bla*CTX-M, *bla*TEM	2	256	4	< 2	8	8	< 2	16	CL, PB, CIP, AT, CX, CAZ, CTX, CTR	0.40
SH86	*Acinetobacter lwoffii*	*bla*CTX-M	4	> 1024	4	< 2	< 2	> 1024	4	256	AK, CL, AT, CZ, CAZ, CTX	0.30
SH98	*Acinetobacter sp.*	*bla*CTX-M-152, *bla*TEM	< 2	16	8	< 2	< 2	8	< 2	< 2	CL, PB, TR, CX, CAZ, CTX, CTR	0.35
LG1	*Bacillus sp.*	*bla*CTX-M-15, *bla*TEM, *bla*SHV	1024	512	2	< 2	128	64	< 2	16	TR, RIF, AMP, A/S, AT, CZ, CX, CAZ, CTX	0.45
LG6	*Escherichia coli*	*bla*CTX-M-15	16	64	2	8	16	16	< 2	4	CL, PB, RIF, AT, CAZ, CTX	0.30
LG11	*Enterobacter sp.*	*bla*CTX-M	2	8	4	< 2	< 2	2	< 2	< 2	AK, CL, RIF, P/T, CAZ, CTX, CTR	0.35
LG20	*Enterobacter sp.*	*bla*CTX-M, *bla*TEM	< 2	512	8	< 2	32	1024	< 2	32	RIF, AMP, CZ, CX, CAZ, CTR	0.30
LG24	*Escherichia coli*	*bla*CTX-M-15	< 2	64	8	< 2	< 2	512	< 2	64	AMP, AT, CAZ, CTX, CTR	0.25
LG29	*Enterobacter sp.*	*bla*CTX-M	2	128	2	< 2	6	4	< 2	8	CL, AT, CAZ, CTX, CTR	0.25
LG32	*Enterobacter sp.*	*bla*CTX-M	2	8	4	< 2	8	4	< 2	8	AK, CL, PB, AT, CAZ, CTX, CTR	0.35
LG33	*Enterobacter sp.*	*bla*CTX-M	< 2	128	4	< 2	8	8	< 2	8	AMP, AT, CAZ, CTX, CTR	0.25
LG45	*Escherichia coli*	*bla*CTX-M	2	128	4	< 2	8	4	< 2	8	CL, RIF, AT, CAZ, CTX, CTR	0.30
LG51	*Escherichia coli*	*bla*CTX-M, *bla*TEM,	64	4	256	2	1024	64	< 2	64	RIF, AMP, AT, IPM, CAZ, CTX, CTR	0.35
LG55	*Escherichia coli*	*bla*CTX-M	2	8	2	< 2	8	4	< 2	8	CTX	0.05
LG71	*Enterobacter cloacae*	*bla*CTX-M-15	1024	< 2	16	4	512	> 1024	< 2	1024	AK, RIF, AMP, CZ, CX	0.25

(*Continued*)

Table 4 | Continued

Isolates	16S rRNA sequence-based Identification	β-lactamase gene/s	Minimum Inhibitory concentration (μg/mL)								Antibiotic resistance	MAR Index
			TR	CL	C	RIF	AMP	CAZ	CIP	CTX		
LG80	*Escherichia coli*	*bla*CTX-M	2	8	4	< 2	4	4	< 2	8	AT, CAZ, CTX, CTR	0.20
JST14	*Escherichia coli*	*bla*CTX-M	< 2	2	4	< 2	8	4	< 2	8	CL, AT, CAZ	0.15
JST15	*Escherichia coli*	*bla*CTX-M	2	8	4	< 2	8	4	< 2	8	CZ	0.05
JST21	*Enterobacter ludwigii*	*bla*CTX-M-152	8	4	2	< 2	8	16	< 2	16	AT, CAZ, CTR	0.15
JST28	*Escherichia coli*	*bla*CTX-M-15, *bla*SHV	1024	< 2	2	8	1024	2	2	8	AK, TR, RIF, AMP, AT, CZ, CAZ, CTX, CTR	0.45
JST33	*Klebsiella pneumoniae*	*bla*CTX-M-15, *bla*TEM-1, *bla*SHV	256	64	4	< 2	< 2	256	< 2	256	AK, CL, PB, AMP, AT, IPM, CZ, CAZ, CTX, CTR	0.50
JST36	*Enterobacter sp.*	*bla*CTX-M, *bla*TEM-1, *bla*SHV	512	64	4	< 2	< 2	1024	< 2	256	AK, CL, PB, RIF, AMP, AT, CZ, CAZ, CTX, CTR	0.50
JST61	*Pantoea agglomerans*	*bla*CTX-M-15, *bla*CTX-M-205, *bla*TEM-1	1024	512	256	< 2	128	256	< 2	256	CL, PB, TR, RIF. AMP. A/S, CZ, CX	0.40
JST62	*Enterobacter sp.*	*bla*CTX-M, *bla*TEM	> 1024	< 2	64	16	> 1024	256	< 2	512	RIF, AMP, P/T, AT, IPM, ETP, CZ, CX, CAZ, CTX, CTR	0.55
JST63	*Bacillus sp.*	*bla*CTX-M-15	1024	1024	< 2	< 2	128	128	< 2	128	CL, PB, TR, RIF, AMP, A/S, CZ, CX, CTX	0.45
JST68	*Escherichia coli*	*bla*CTX-M-15, *bla*CTX-M-205, *bla*TEM-1, *bla*SHV	1024	1024	< 2	< 2	64	256	< 2	512	CL, PB, TR, RIF, AMP, A/S, CZ, CX, CAZ, CTX, CTR	0.55
JST69	*Enterobacter sp.*	*bla*CTX-M-205	2	8	4	< 2	8	4	< 2	8	CTX, CTR	0.10
JST70	*Escherichia coli*	*bla*CTX-M-205	8	4	2	< 2	8	16	< 2	16	CL, PB, CZ, CTX	0.20
JST71	*Enterobacter sp.*	*bla*CTX-M-152, *bla*TEM, *bla*SHV	256	< 2	< 2	< 2	1024	128	< 2	1024	AK, CL, PB, C, TE, RIF, AMP, A/S, ETP, CZ, CX, CTX, CTR	0.65
JST72	*Escherichia coli*	*bla*CTX-M	1024	4	4	< 2	8	16	< 2	32	AK, CL, TR, RIF, AMP, A/S, P/T, CZ, CTX, CTR	0.50
JST73	*Enterobacter sp.*	*bla*CTX-M	128	8	4	< 2	8	4	< 2	16	PB, AT, CTX, CTR	0.20
JST78	*Escherichia coli*	*bla*CTX-M-15 *bla*TEM	> 1024	8	4	8	> 1024	1024	< 2	128	AK, RIF, AMP, IPM, ETP, CZ, CX	0.35
JST79	*Enterobacter sp.*	*bla*CTX-M	256	128	4	< 2	8	4	< 2	8	CL, PB, LE, TR, RIF, AMP, P/T, AT, ETP, CZ, CX, CAZ, CTX, CTR	0.70

AMP, Ampicillin; A/S, Ampicillin/Sulbactam; CX, Cefoxitin; AT, Aztreonam; IPM, Imipenem; P/T, Piperacillin/Tazobactam; CZ, Cefazolin; CAZ, Ceftazidime; CTX, Cefotaxime; CTR, Ceftriaxone; ETP, Ertapenem; AK, Amikacin; CL, Colistin; PB, Polymyxin B; CIP, Ciprofloxacin; LE, Levofloxacin; C, Chloramphenicol; TE, Tetracycline; TR, Trimethoprim; RIF, Rifampicin.

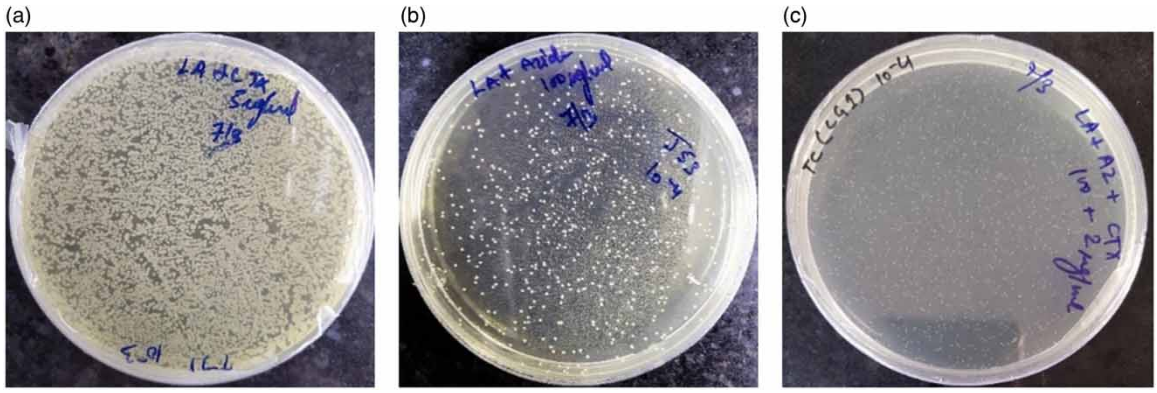

Figure 2 | Growth of donor and recipient (b) bacterial cells on LA plates supplemented with CTX and Sodium azide and with CTX + Sodium azide: (a) donor, (b) recipient and (c) transconjugant.

industrialization, overpopulation and mismanagement of the metropolitan cities (Devarajan *et al.* 2015; Yang *et al.* 2017). ESBL phenotype was observed in 32, 23, 20 and 23% of bacterial isolates from Ghazipur slaughterhouse, Lodhi garden pond, Hauz Khas lake and Jasola wastewater treatment plant Delhi-NCR, respectively. Other studies from the aquatic environment of Switzerland and Taiwan have similar reports where the prevalence of ESBL positive Enterobacteriaceae was reported to be 36.2 and 30%, respectively (Zurfluh *et al.* 2013; Chen *et al.* 2016). There are few reports of a higher percentage of ESBL producers with MDR bacteria from freshwater bodies (Taco *et al.* 2014; Bajaj *et al.* 2015). The occurrence of the high percentage of ESBL producing bacteria worldwide is challenging and worrisome. In our study, the majority of ESBL positive isolates exhibited resistance towards cefotaxime, ceftazidime, ceftriaxone, aztreonam, ampicillin and cefazolin similar to report from China urban river sediment (Lu *et al.* 2010). Furthermore, resistance towards colistin was found at a higher scale than earlier reports of clinical and environmental samples (Ansari *et al.* 2015). Thereby, a heightened resistance towards colistin which is the last drug available in the current pipeline to treat infections caused by MDR bacteria is alarming. Among ESBLs producing isolates 42, 60, 78 and 59% from the Ghazipur slaughterhouse, Lodhi garden pond, Hauz Khas lake and Jasola wastewater treatment plant, respectively, were found to be MDR. From the site Hauz Khas lake, the resistance pattern was found similar to those reported from surface water and wastewater by Taco *et al.* (2014). The MAR index value for each site was >0.2 which suggests a deleterious situation and a high pollution load in a particular sampling area indicating that the antibiotic exposure of a specific area could be higher (Krumperman 1983). High MIC value (2– > 1,024 μg/mL) for ceftazidime, cefotaxime, ampicillin, colistin and trimethoprim showed elevated resistance to β-lactam and folate pathway inhibitors among ESBL producers. Our findings are in line with previous reports from the freshwater of Delhi-NCR (Bajaj *et al.* 2015; Azam *et al.* 2016).

The *bla*CTX-M gene was detected among 64% ESBL positive isolates which corroborates previous reports from India and Australia (Reinthaler *et al.* 2010; Bajaj *et al.* 2015). In this study, we observed that *bla*CTX-M-15 was the most prevalent type which is similar to other reports (Azam *et al.* 2016; Maravić *et al.* 2016). The present study also justifies that the *bla*CTX-M-15 is the frequently dispersed ESBL among Enterobacteriaceae (Bevan *et al.* 2017). The co-occurrence of ESBL variants (*bla*CTX-M, *bla*TEM and *bla*SHV) identified in this study is in line with findings from the river Yamuna in Delhi-NCR (Siddiqui *et al.* 2019). *bla*TEM-116 was the most prevalent type among *bla*TEM similar to earlier reports of environmental isolates of Enterobacteriaceae (Maravić *et al.* 2016). *bla*SHV was found to be the least prevalent type of ESBL. The *bla*SHV gene is mainly dominant in clinical isolates (Maravić *et al.* 2016); however, it has been reported in freshwater from Switzerland (Zurfluh *et al.* 2013). The test isolates predominantly include members of Enterobacteriaceae (70.5%) followed by Moraxellaceae (20.5%), Bacillaceae (4.4%) and others (4.5%, i.e., Aeromonadaceae, Brucellaceae and Pseudomonadaceae). Our data demonstrate a high percentage of *E. coli* similar to other samples of environmental origin followed by *Acinetobacter* spp., which is in line with other reports (Lu *et al.* 2010; Taco *et al.* 2014; Maravić *et al.* 2016). New reports from this study include the *bla*CTX-M gene in *A. lwoffii*, *E. ludwigii, E. mexicanum* and *A. caviae*. Furthermore, the identification of *bla*TEM and *bla*SHV in an environmental isolate of *A. caviae* is a new report from India.

Results of our conjugation experiment revealed the successful transfer of ESBL genes from donor to recipient. The results of conjugation assay are in line with previous reports where movement of TEM, CTX-M and SHV genes were possible through HGT like the phenomenon of conjugation (Siddiqui *et al.* 2020).

CONCLUSION

This research spotlights the high prevalence of ESBL producing bacteria in lentic and wastewater effluents of Delhi-NCR, India. With a low level of hygiene and discharge of antimicrobial compounds into the aquatic environment, leading to not only building up of resistance among inhabitants of water bodies but also the transfer of resistance genes to human pathogenic and non-pathogenic bacteria. 42–78% of ESBL positive isolates from different sampling sites showed MDR phenotype, which is a matter of concern. The ESBL positive isolates harboured *bla*CTX-M-15, *bla*CTX-M-152, *bla*CTX-M-205 and *bla*TEM-116 gene variants. *bla*CTX-M-15 was found to be the most prevalent type. Furthermore, this study reports occurrence of *bla*CTX-M gene in *A. lwoffii*, *E. ludwigii*, *E. mexicanum* and *A. caviae*. The findings of this study showed that the urban aquatic environment (both lentic and lotic) of Delhi-NCR is crammed with ESBL producing bacteria. This study highlights the need for strong measures for positive actions to control the emergence and dissemination of MDR in the urban aquatic environment. Detailed studies are required to understand the emergence and transmission of ESBL producing bacteria in anthropogenically influenced aquatic environments.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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DATA AVAILABILITY STATEMENT

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

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