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Screening of Extended Spectrum Beta-Lactamase Producing Bacteria in Clinical Liquid Waste

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Abstract

Admixture of Clinical Liquid Waste (CLW) discharging into Ecological Water Bodies (EWB) causes significant pollution with resistant bacteria. The issue is significant in Bangladesh where CLW management is at early stage of development. Extended-Spectrum β-Lactamase (ESBL) producing bacteria confer resistance to Broad Spectrum β-Lactam Antibiotics (BSBLA) which has become a global concern now. The aim of this study was to characterise and assess the occurrence of ESBL producing bacteria in CLW of Bangladesh. Three CLW samples were collected from two leading hospitals in Dhaka City, Bangladesh in the year 2012. A total 166 isolates were retrieved and screened for ESBL production by the Double Disk Diffusion Synergy Test (DDST). Isolates with ESBL phenotype were further characterized by antibiotic susceptibility testing, PCR and sequencing of β-lactamase genes. A total of 30 ESBL producers with Multi Drug Resistant (MDR) properties were isolated. Gene specific PCR against bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{SHV} detected CTX-M as major ESBL genotype (83%) followed by occurrence of TEM-genotypes (40%). Prevalence of SHV was low in CLW samples (3%). More than 20% of CLW -ESBL isolates possessed both the TEM and CTX-M genotypes. The predominant ESBL isolates were phylogenetically identified as *Escherichia* spp. Seventy seven percent followed by *Citrobacter* spp. (17%), *Klebsiella* spp. (3%), and *Yokenella* spp. (3%). This investigation demonstrates that CLW possesses ESBL producing MDR bacteria which might act as potential threat to disseminate resistant determinants in the surrounding environment.

Keywords: ESBL, Multidrug Resistant Bacteria, Clinical Liquid Wastewater, Ecological Water Bodies.

Introduction

Clinical Liquid Waste (CLW) includes hazardous waste materials excreted from inpatient and outpatient departments, laboratories (microbiology, biochemistry, histopathology, blood bank, etc.), operation theatres, radiology and other departments of different hospitals and clinics. The majority of antibiotics used in medicine are only partially metabolized by the patients.[1,2] Thus CLW acts as the storehouse of different types of antibiotics and harmful infectious agents such as the pathogens and microorganisms possessing multidrug resistant (MDR) genes. If these hazardous CLW remains untreated and discharged directly into the ecological water bodies (EWB); it results in an increase in the active antibiotic concentration and MDR bacteria in EWB and subsequently introduces resistant gene pool to the environment.[3] In recent years, the emergence of antibiotic resistant microorganisms (particularly Extended Spectrum β-Lactamase (ESBL) producing and MDR bacteria) in untreated CLW has been evidenced in Bangladesh, where CLW management is at early stage of development.[3-5] This may pose a public health risk, which demands the investigation on this issue in a large extent for future evaluation and control.

Antibiotics of β-lactam group have been most widely used in infection management due to its effectiveness on a broad range of microorganisms. In Bangladesh, β-lactam antibiotics are one of the most frequently used antibiotic group but unfortunately these drugs are often sold over the

counter without a physician's prescription.[6] This indiscriminate and inappropriate use of antibiotics results in an alarming situation like the progressive loss of susceptibility towards β-lactam antibiotics in the treatment of a wide range of clinical conditions.[7] The production of β-lactamases is an important mechanism of resistance to β-lactam antibiotics especially in gram negative bacteria.

Extended-Spectrum β-Lactamases (ESBLs) have been contributing to the dramatic increase in resistance to new generation β-lactam agents throughout the world.[8-11] Public healthcare units like hospitals and clinics must safeguard the health of the community. But in countries like Bangladesh, healthcare liquid wastes particularly CLW were laden with ESBL-MDR bacteria and seemed to pose a huge public health threat in the transfer of such resistance to the bacterial pathogens causing community acquired infections, thereby limiting our antibiotic pool. To measure the extent of these threatening microbes in the environment and followed by taking proper initiatives, a detail assessment of ESBL-MDR bacteria is required. Previous studies have shown that *Escherichia coli* and *Klebsiella pneumoniae* are the major ESBL-producing organisms worldwide while CTX-M-type enzymes were the most prevalent ESBLs.[12-16] There are a few reports from Bangladesh where they found the presence of different ESBL producing gram negative bacteria in CLW.[17-20] A report claims that the direct relationship between the use of antibiotics and the antibiotic resistance properties of microorganisms.[3] In our previous investigation we detected the presence of ESBL within *Escherichia* spp. from CLW indicating that patients are spreading ESBL-MDR isolates in the environment, through hospital wastes.[4] However, still there is a lack of information on molecular characterisation of ESBL producing organisms isolated from CLW in Bangladesh. Therefore, the objectives of the present study were in depth screening and characterisation of ESBL producing bacteria from CLW in Bangladesh.

Materials and Methods

Study area and sample collection: Three CLW samples were collected from two renowned hospitals of Dhaka city: Dhaka Medical College Hospital (DMCH) and Sir Salimullah Medical College Hospital (SSMCH). From DMCH, one sample was collected at the sewage drain of the emergency unit in February, 2012 and another sample was collected from the outdoor sewage drain in April, 2012. In June 2012, one sample was collected from SSMCH CLW-outlet which was directly connected to ecological water body river Buriganga. Each of the CLW samples were collected in a clean sterile 500 ml Schott Duran's bottle (Schott, Germany). About 200 ml of liquid samples containing suspended particles were collected. Collected samples were directly brought to the laboratory of the Department of Microbiology, University of Dhaka in an insulated ice box with minimum delay and were bacteriologically examined immediately.

Bacterial enumeration and isolation: Collected CLW samples sample was serially diluted up to 10^{-4} with sterile normal saline (0.85%) and was used for microbiological analysis. Total viable bacterial count was determined for CLW samples using commercially available Nutrient Agar (NA) media (Oxoid, UK). MacConkey (MA) agar (Oxoid, No. 3) was used as a selective culture medium for the detection and isolation of Gram-negative bacteria from CLW samples. Amoxycillin (AM, 50 μg/mL), Ciprofloxacin (CIP, 0.16 μg/mL), and Cephalosporin (CEP, 5 μg/mL) antibiotics were supplemented separately to NA and MA media to determine Total Antibiotic Resistant Count (TARC), which included Total Viable Resistant Bacterial Count (TVRBC) and Total Viable Resistant Enterobacteriaceae Count (TVREC), respectively. For total Viable Bacterial Count (TVBC) no antibiotic was used in the NA and MA media. Following incubation

at 37°C for 18-24 hours, appearance of individual colony on each plate was enumerated and recorded in every case. Isolates from DMCH emergency unit outflow, outdoor sewage drain, and SSMCH outflow were designated as A, B, C respectively followed by numeric (ex: A1, B1, C1).

Phenotypic detection of ESBL: All of the selected isolates from CLW were tested for ESBL production by phenotypic Double Disk Diffusion Synergy Test (DDST).[21] Ceftazidime (CAZ) (30µg), Ceftriaxone (CRO) (30µg), Cefotaxime (CTX), (30µg) and Amoxycillin / Clavulanic acid, (AMC) (30µg) discs were used.

The test inoculum, turbidity matched to 0.5 Optical Density (OD) was spread on Muller-Hinton Agar (MHA) (Oxoid, UK) using a sterile cotton swab. A disc of augmenting (20 µg AM+ 10 µg CLA) was placed on the center of MHA; then discs of CAZ (30µg), CRO (30µg), CTX (30µg), and AZT (30µg) were kept around it in such a way that each disc was at distance ranging between 15 and 20 mm from the augmented disc (Centre to Centre). The plates were incubated at 37°C overnight. The organisms were considered to be ESBL positive when the zone of inhibition around any of the expanded-spectrum Cephalosporin discs (CAZ/CRO/CTX) showed a clear enhancement towards the augmented disc.

Antibiotic susceptibility tests: The antimicrobial susceptibility pattern of the DDST test positive isolates that were morphologically distinct on NA was determined in vitro by using the standardized agar-disc-diffusion method known as the Kirbey-Bauer.[22] It is a modification of Bauer's method.[23] Sixteen different commercially available antibiotic discs belonging to 11 individual groups of antibiotics (table II) and MHA were used for the test.

PCR detection of ESBL specific gene and sequence analysis: All the isolates phenotypically positive for ESBL production were selected for detailed molecular analysis. Total DNA from the isolates were prepared as boiled DNA to use as template DNA for PCR. PCRs using primers specific for antibiotic resistance genes such as bla_{TEM} and $bla_{CTX\text{-}M}$ were carried out using a thermal cycler (Biometra, Germany). The PCR reactions followed the protocol of Arlet et al. 1995[24] and Pagani et al 2003[25] for bla_{TEM} and $bla_{CTX\text{-}M}$ respectively. The sequence of the primers used for the PCR reactions were-bla_{TEM}F: ATGAGTATTCAACATTTCCG and bla_{TEM} R:CCAATGCTTAATCAGTGAGC; $bla_{CTX\text{-}M}$F: ATGTGCAGYACCAGTAARGT and $bla_{CTX\text{-}M}$R: TGGGTRAARTARGTSACCAGA, with the annealing temperature 55°C and 50°C respectively. Full 1ength sequences (858 bp for bla_{TEM}, and 593 bp for $bla_{CTX\text{-}M}$) were assembled into using the SeqMan Genome Assembler (DNAstar, USA) and compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov /GenBank) by means of the Basic Local Alignment Search Tool (BLAST) to identify close phylogenetic relatives. Multiple sequence alignment of the retrieved reference sequences from NCBI was performed with the ClustalW software[26] and was exported to the Molecular Evolutionary Genetics Analysis (MEGA) program[27] for phylogenetic tree construction using the Neighbor joining algorithm selecting 1000 bootstrap replication.

Plasmid DNA analysis: Plasmid DNA of ESBL specific gene positive isolates was extracted using Wizard ®Plus Minipreps plasmid DNA Purification kit (Promega, USA). Isolated plasmid DNA was separated in 0.8% agarose gel (ethidium bromide added) in a Tris-Acetate-EDTA (TAE) buffer and photograph was taken using GelDoc (AlphaImager, USA). *Escherichia coli* V_{517} plasmid was used as control marker plasmid DNA.

16S rRNA Gene sequencing and identification: The 16S rRNA gene of representative ESBL isolates belonging to each morphological group was amplified using primers 27F and 1492R. The

purified products were further used for sequencing and phylogenetic analysis as same as the protocol used in case of ESBL genes.

Results

Bacteriological enumeration of clinical liquid wastewater: Total Viable Bacterial Count)TVBC(, Total Viable Resistant Bacterial Count (TVRBC), and Total Viable Resistant Enterobacterial Count (TVREC) were enumerated in CLW collected from DMCH in duplicate (table I). Among TVRBC, total Cirpofloxacin (CIP) resistant count was approximately 10 times lower than total Ampicillin (AMP) or Cephalosporin (CEP) resistant viable count (table I).Metcalf *et al.*, 1991reported that the expected viable bacterial count in domestic sewage system is $1x10^8$cfu/mL.[28] Values of Predicted Probable Number (PPN, the ratio of total expected viable count to total viable bacterial count) higher than one (ratio >1) indicated the presence of active antimicrobial agents in the effluents. The findings revealed that all CLW samples analyzed during this investigation had a PPN >1, indicating the presence of significant levels of antibiotics and other toxic compounds in the CLW.

Table I: Total and resistant bacteria count in Clinical Liquid Waste (CLW).

Bacterial count	Count (cfu/mL) (an average of duplicate plate count)		
	Sample 1	Sample 2	Sample 3
1. Total Viable Bacterial Count (TVBC)	4.475x10^4	2.05x10^6	2.05x10^5
2. Total Enterobacteriaceae Count (TEC)	3.90x10^4	4.45x10^5	6.5 x10^4
3. Total Antibiotic Resistant Count (TARC)			
A. Total Viable Resistant Bacterial Count (TVRBC)			
i) Total Ampicillin Resistant Viable Bacterial Count (TARVB)	ND	3.15 x10^5	1.70 x10^5
ii) Total Ciprofloxacin Resistant Viable Bacterial Count (TCRVB)	ND	5.5 x10^4	5.0 x10^4
iii) Total Cephalosporin Resistant Viable Bacterial Count (TCERVB)	ND	3.0x10^5	6.0x10^4
B. Total Viable Resistant Enterobacterial Count (TVREC)			
Total Ampicillin Resistant Enterobacterial Count (TAREC)	ND	1.50 x10^4	6.5 x10^3
Predicted Probable Number (PPN)	>1	>1	>1

ND: Not detected

PPN is the quotient of Total Expected Count to Total Count that was determined using the expected count as 1x 10^8cfu/mL

Isolation of ESBL producing bacteria: A total of 166 isolates from NA and MA plates were selected and tested for detection of ESBL producers by DDST. Among them, 30 isolates showed three distinguished phenotypic ESBL producing pattern (figure 1).

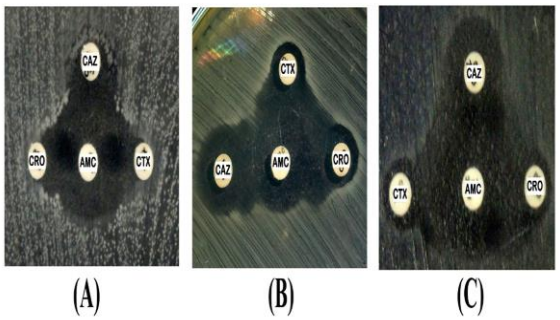

Figure 1: Distinguished patterns of Double Disk Diffusion Synergy Test (DDST) of CLW-ESBL isolates.

Representative of isolates with respective patterns are: Pattern A: C32; Pattern B: A18 and Pattern C: C49; Elaboration: CTX for Cefotaxime, CAZ for Ceftazidime, CRO for Ceftriaxone, AMC for Amoxycillin/Clavulanic acid.

These 30 ESBL positive isolates were selected for further investigation.

ESBL morphological groups and their antibiotic susceptibility pattern: According to their colony characteristics on MA media, 30 CLW-ESBL isolates were classified into four morphogroups. The four morphogroups were: dark pink and flat; pale pink and gummy; colorless; and dark pink with depression in the middle figure 2.

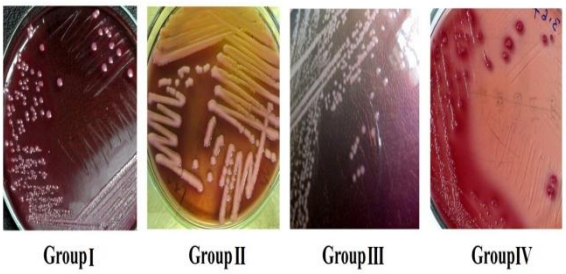

Figure 2: Four distinguished colony morphology of the ESBL positive isolates on MacConkey agar plates.
Plate 1: Group I- dark pink and flat colonies
Plate 2: Group II- pale pink and gummy colonies
Plate 3: Group III- colorless small colonies
Plate 4: Group IV- dark pink colonies

Their antibiogram were analyzed against 11 different groups of antibiotics. Isolates showed maximum resistance against β-lactam group of antibiotics (Cefotaxime-CTX), followed by Penicillin group (Ampicillin-AMP and Amoxycillin-AM) whereas, most of the isolates were susceptible to Imipenem-IMP and Levofloxacin-LEV.

Four morphological groups of CLW-ESBLs were distinctly different in their antibiotic resistance pattern. Group I has 23 isolates, among which 20 isolates were resistant to CTX and 16 isolates were resistant to AMP. Group II had one isolate A20, which was resistant to AMP, TET, CIP, FEP, and CTX. Group III had one isolate A16, which showed resistance against AMP and CTX, and sensitive to all other antibiotics tested. Group IV has five isolates and all of them were resistant to AMP, CTX, and CAZ.

Table II: Four morphogroups of ESBL isolates showing variations in their drug resistance pattern and genotyping of β-lactamases genes.

Source Morphological groups of CLW ESBLs	Isolate ID	Antibiotic Resistance Pattern	*bla*$_{tem}$ (853 bp)	*bla*$_{ctx-M}$ (593 bp)	*bla*$_{shv}$ (827 bp)
Gr. I; Dark pink and flat	A12	AMP, TET, N, CIP, AZM, AZT, FEP, CTX, CAZ	+	+	-
	A18	AMP, TET, ATM, FEP, CTX, CAZ	-	+	-
	A19	AMP, N, CTX	-	+	-
	A22	AMP, N, AZM, FEP, CTX	-	+	-
	B2	IMP, AZT, FEP, CTX, CAZ	-	+	-
	B4	AMP	+	-	-
	B7	AMP, TET, CIP, AZM, IMP, AZT, FEP, CTX, CAZ	-	+	-
	B15	AZT, CTX, CAZ	-	+	-
	B18	AZT, CTX, CAZ	-	+	-
	B24	AMP, AZT, CRO	+	-	-
	B29	AZT, FEP, CAZ	-	+	-
	B57	CTX, CAZ	+	+	-
	C6	AMP, TET, N, CIP, AZM, AK, AZT, C, FEP, CTX, CAZ	+	+	-
	C13	AMP, N, AZT, FEP, CTX, CAZ, CRO	+	-	-
	C32	AMP, AZT, FEP, CTX, CAZ, CRO	-	+	-
	C39	AMP, TET, AZM, AZT, FEP, CTX, CAZ, CRO	-	+	-
	C47	IMP, FEP, CTX	+	+	-
	C49	AMP, TET, N, CIP, AZM, CTX	+	+	-
	C57	ATM, FEP, CTX, CAZ	-	+	-
	C72	AMP, CIP, AZM, ATM, CTX, CAZ	+	-	-
	C79	AMP, CIP, CTX	-	+	-
	C84	AMP, CIP, AZM, ATM, FEP, CTX	-	+	-
	C100	AMP, CTX	+	+	-
Gr. II:Pale pink and gummy	A20	AMP, TET, CIP, FEP, CTX	+	+	-
Gr. III:Colorless	A16	AMP, CTX	-	-	-
Gr. IV:Dark pink with a depression in the middle	A3	AMP, TET, N, CIP, AK, IMP, AZT, FEP, CTX, CAZ	+	+	-
	C18	AMP, AZT, FEP, CTX, CAZ	-	+	+
	C67	AMP, TET, CIP, AZM, AZT, FEP, CTX, CAZ, CRO	-	+	-
	C70	AMP, TET, AK, AZT, FEP, CTX, CAZ, CRO	-	+	-
	C92	AMP, CTX, CAZ	-	+	-

Amoxycillin (AM), Ampicillin (AMP), Amikacin (AK), Azithromycin (AZM), Aztreonam (AZT), Cirpofloxacin (CIP), Levofloxacin (LEV), Chloramphenicle (C), Cefepime (FEP), Cefotaxime (CTX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefixime (CFM), Imipenem (IMP), Nitrofuran (N), Tetracycline (TET)

Analysis of extended spectrum β-Lactamase (ESBL) Genes: The prevalent ESBL genotype was *bla*$_{CTX-M}$ type, 83% of among the total isolates (table II, figure 3). The frequency of *bla*$_{TEM}$genotype was 40% (12/30) and only 3% (1/30) were positive for *bla*$_{SHV}$gene (table II). On

the other hand, 23% of the isolates had both *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes.

Sequencing and homology search using Gen Bank database of the ESBL specific *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes showed that all TEM positive isolates were 100% similar to TEM-1 enzyme of *Escherichia coli* strain AS713010 (JN037848.1). All CTX positive isolates showed 100% similarity with *Escherichia coli* strain BLSE2012CF1 CTX-M-15 (figure 2).

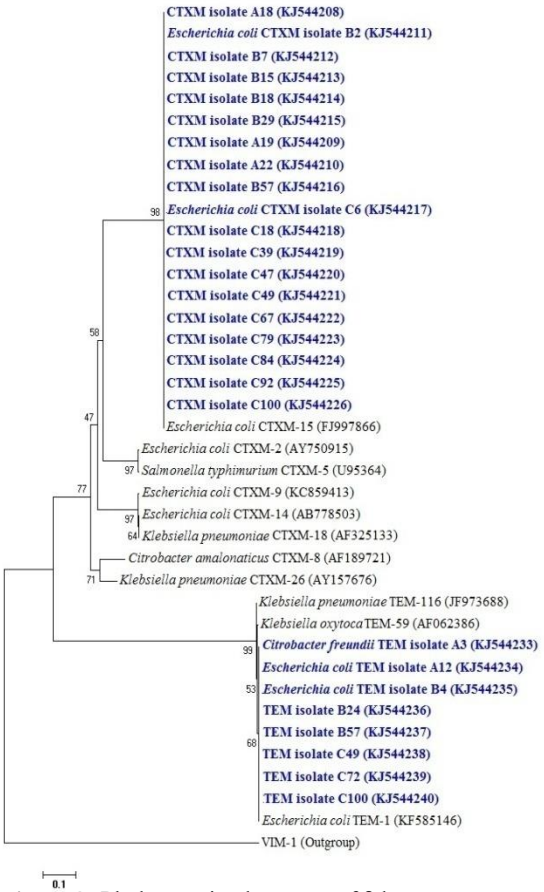

Figure 3: Phylogenetic placement of β lactamase genes (*bla*$_{TEM}$ and *bla*$_{CTX-M}$).

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.48188406 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA5.

Accession number of NCBI submitted ESBL-TEM positive isolates are KJ544233-40 and ESBL- CTX positive isolates are KJ544208-26.

Plasmid Profile of ESBL Isolates: Among 30 ESBL positive MDR isolates seven isolates were plasmid free. They included: B4, B24, C32, C79, C84, and C100 (morphogroup I) and A20 (morphogroup III). Large plasmids above the chromosomal range were found in seven isolates. Among them, isolate A18 contains only a large size plasmid, whereas, others contain three or more small size plasmids at varying size ranges.

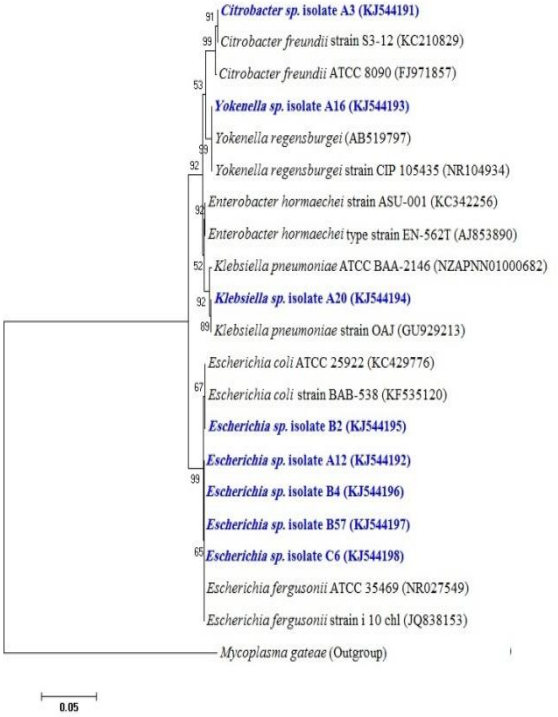

Figure 4: Phylogenetic placement of 16S rRNA gene sequences of ESBL positive isolates.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.48188406 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA5.

16S rRNA gene identification of ESBL positive CLW: 16S rRNA gene sequences of isolates A12, B2, B4, B57, and C6 from the dominant morphogroup, Group I of CLW-ESBL (figure 4, table II) clustered with *Escherichia* spp. specifically with *Escherichia fergusonii* except isolate B2 which was more closely related to *Escherichia coli* (figure 5). Isolate A20 of group II, A16 of group III and A3 of group IV were closely related to *Klebsiella sp*, *Yokenella sp*. and *Citrobacter sp*., respectively (figure 3). Accession number of

NCBI submitted 16s rRNA gene sequences of CLW-ESBL isolates are KJ544191-98.

Table III: Number and size of plasmids from ESBL isolates of CLW samples

Isolate ID	Group	Number of plasmid	Plasmid size (Kb) Number of plasmid						Large Plasmid
A12		3	4.2	1.4					+
A18		1							+
A19	1	ND							
A22		3	5.3	4.8	2.6				
B2		3	5.3	3.5	<1				
B4		No plasmid							
B7		4	5.5	3.8	2.6	1.2			
B15		4	6.1	5.5	4.6				+
B18		4	6.1	5.5	4.6				+
B24		No plasmid							
B29		1	6.3						
B57		3	5.5	3.3					+
C6		5	6.1	5.3	4.4	3.5	<1		
C13		3	5.7	4.6					+
C32		No plasmid							
C39		1	1.7						
C47		6	6.6	6.4	4.5	3.2	1.3	<1	
C49		3	1.7	<1	<1				
C57		1	5.1						
C72		4	8.1	6.3	3.8	2.9			
C79		No plasmid							
C84		No plasmid							
C100		No plasmid							
A20	2	No plasmid							
A16	3	2	5.8	2.0					
A3	4	5	4.6	4.0	3.1	1.7			+
C18		1	4.7						
C67		1	1.1						
C70		1	<1						
C92		2	6.0	4.6					

Discussion

In Bangladesh, mostly CLW is directly released into municipal sewage system and subsequently discharged into the ecological water bodies. CLW contains resistant pathogenic bacteria and non-metabolized antimicrobial agents. To understand the contribution of CLW in the pollution of ecological water bodies, this investigation demonstrated that i) Predicted Probable Number (PPN) microbial populations in CLW was lower than the expected value; ii) CLW contained MDR and ESBL producing bacteria with CTX-M and TEM specific dominant genotypes; iii) the predominant species were *Escherichia* spp., *Klebsiella* spp. and *Citrobacter* spp. ; and iv) Antibiotic resistance properties might be both plasmid mediated and chromosomal borne.

In Bangladesh, all CLW originated from hospitals, veterinaries, and other sources directly or via municipality drainage system are discharged into ecological water bodies resulting serious pollutions of environment with resistant bacteria and resistant gene pool pollutions. In the ecological water bodies, bacteria from different sources like CLW, agriculture, urban and industrial wastewater are mixed together and genetic exchange between the environmental species and allochthonous species may occur resulting new

pathogenic bacteria of clinical importance.[29] Recently, the presence of antimicrobial-resistant bacteria in different ecological niches in Bangladesh has been reported.[29] Therefore, this problem has been assessed in current study, and the samples consisted of pathogenic and antibiotic resistant bacteria (table I). In this study, bacterial concentration in the CLW was found to be lower than 10^8 cfu/100 mL (table I), indicating the presence of antimicrobial agents in CLW.[28] Our recent quantitative analysis of antibiotics concentrations in CLW clearly demonstrated that CLW contained different antibiotics at MIC_{50} or sub-MIC_{50} levels (unpublished). Furthermore, these resistant bacteria contained transferable resistant markers. As a result, there remains a potential risk of resistant bacteria and resistant gene pool pollution of the environment.[4] The admixing of CLW with ecological water bodies like river, ponds, cannel, lakes etc. may cause serious pollutions and become a subject of clinical importance because this pollution cycles through food-chain and water.[30]

In the present study, three different ESBL phenotypes for each of 30 ESBL positive isolates were observed and some additional isolates susceptible to CAZ, CRO, CTX and AMC (A22, B4, B24 and B57) were confirmed as ESBLs through genotypic screening. ESBLs are typically recognized for their unusual antibiotic resistance to their hosts, but there are also reports suggesting intermediate or even susceptible phenotype.[31] Among the 61 ESBL isolates, 61% isolates were *Escherichia* spp. and 11% were *Klebsiella* spp. CLW samples predominantly contained *Escherichia* spp. (figure 4). Among 30 CLW-ESBLs, 77% *Escherichia* spp. And 3% *Klebsiella* spp. were found. *Yokenella* spp. (3%) and *Citrobacter* spp. (17%) were less prevalent and have barely been reported as ESBL producers. Although *Yokenella* spp. has been reported from the environment, their presence in clinical settings is very recent[32] and therefore underestimated as a clinically significant pathogen or even ESBLs. The present study is so far the first report of *Yokenella* spp. from Bangladesh as ESBL producer.

The dominant genotype of ESBL among the three ESBL coding genes (bla_{TEM}, bla_{CTX-M}, and bla_{SHV}), were detected to be CTX-M type followed by TEM genotype. Among the 61 ESBL isolates, 83%, 40%, and 13% of CLW isolates were bla_{CTX-M}, bla_{TEM}, and bla_{SHV} types, respectively (Table II). Simultaneous presence of $bla_{TEM}$and bla_{CTX-M} genes was detected in 27% of CLW isolates. Phylogenetically, it was observed that TEM1 and CTX-M-15 types were predominant in Bangladesh samples (figure 4). In India, CTX-M-15 genotype was reported to be dominant within *E. coli* (75%) and *Klebsiella* spp. (73%).[33] Simultaneous presence of bla_{CTX-M} and bla_{TEM} was reported from hospital waste water and clinical samples in Brazil.[34] The dominance or co-dominance of CTX-M gene within the isolates may be due to overuse of ceftriaxone or due to fecal carriage and transfer gene by horizontal transmission.[33-35]

ESBLs are reported as mutant enzymes originating from TEM or SHV enzymes and can be plasmid mediated.[36] Bacteria can resist β-lactam antibiotics with the help of hydrolyzing enzymes, β-lactamases, which can be chromosome mediated, too.[37-39] In the current study, many of the ESBL isolates harbored multiple plasmids, indicating that the multidrug resistance properties of the isolates might be plasmid borne. Seven of the phenotypically and genotypically confirmed ESBL isolates from CLW did not harbor plasmid under the experimental conditions but they had a multidrug resistance profile (table II). This indicated the possibility of chromosomal inheritance of ESBL enzyme and other antibiotic resistance genes. ESBL producing strains contain MDR plasmids that may easily be transmitted between members of Enterobacteriaceae, consequently ESBL producing organisms are resistant to a variety of classes of antibiotics. As a result of horizontal gene transfer (HGT) by mobile genetic elements such as plasmids, transposons, and by transduction, non-ESBL producing pathogens might become potent ESBL producing pathogens, which is of significant concern because this event may evolve new and clinically important pathogenic bacteria.[29]

Conclusion

The present investigation analyzes a comparative perspective of phenotypic and genotypic ESBL producing bacteria from CLW in Bangladesh. The dominant ESBL producers in CLW samples from Bangladesh were detected to be *Escherichia* spp. and the dominant genotype was of CTX-M-15 type. The ESBL properties as well as multi-

drug resistance phenomena can be either chromosome or plasmid mediated. The epidemiology of ESBL-producing bacteria is a growing concern in Bangladesh with increasingly blurred boundaries between hospitals and the community. So, there is an urgent need to monitor and control the spread of ESBLs in the environment.

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