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Characterization of Extended Spectrum Beta-Lactamases (ESBL) Producing *Escherichia coli* Isolates from Surface Water Adjacent to Pharmaceutical Industries in Bangladesh: Antimicrobial Resistance and Virulence Pattern

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The pharmaceutical industry in Bangladesh produces a diverse range of antibiotics for human and animal use, however, waste disposal management is inadequate. This results in substantial quantities of antibiotics being discharged into water bodies, which provide suitable environment for the growth of antibiotic-resistant bacteria, capable of spreading resistance genes. This study intended for exploring the bacterial antibiotic resistance profile in adjoining aquatic environmental sources of pharmaceutical manufacturing facilities in Bangladesh. Seven surface water samples were collected from the vicinity of two pharmaceutical industries located in the Savar area and 51 *Escherichia coli* isolates were identified using both phenotypic and genotypic methods. Antibiotic susceptibility tests revealed the highest percentage of resistance against ampicillin, azithromycin, and nalidixic acid (100%) and the lowest resistance against meropenem (1.96%) out of sixteen different antibiotics tested. 100% of the study *E. coli* isolates were observed with Multidrug resistance phenotypes, with the Multiple Antibiotic Resistance (MAR) value ranging from 0.6-1.0. Furthermore, 69% of the isolates were Extended Spectrum Beta-Lactamases (ESBL) positive as per the Double Disk Diffusion Synergy Test (DDST). ESBL resistance genes bla_{TEM}, $bla_{\text{CTX-M-13}}$, $bla_{\text{CTX-M-15}}$, and bla_{SHV} were detected in 70.6% (n = 36), 60.8% (n = 32), 54.9% (n = 28), and 1.96% (n = 1) of the isolates, respectively, by Polymerase Chain Reaction (PCR). Additionally, 15.68% (n = 8) of the isolates were positive for *E. coli* specific virulence genes in PCR. These findings suggest that pharmaceutical wastewater, if not properly treated, could be a formidable source of antibiotic resistance spread in the surrounding aquatic environment. Therefore, continued surveillance for drug resistance among bacterial populations around drug manufacturing facilities in Bangladesh is necessary, along with proper waste disposal management.

Keywords: **Surface water sample, pharmaceutical industries, multi drug resistance, *Escherichia coli,* ESBL resistance genes, virulence genes**

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Introduction

Antibiotic pollution in the environment is a growing global concern due to its adverse impact on human, animal and environmental health [1]. Prolonged exposure of environmental microbes to antibiotics nurture the development and transmission of antibiotic resistance [2], posing a significant challenge to medical practice and public health [3]. Therefore, from the One Health perspective, it is urgent to uncover the role of environment in the evolution and spread of antibiotic resistance [1]. Anthropogenic activities, including those associated with medical facilities, pharmaceutical industries, municipalities, agriculture, and aquaculture, contribute to the transmission of antibiotic-resistant bacteria and their determinants in the environment [4, 5].

Pharmaceutical waste and wastewater contaminated with antibiotics and other compounds has become a topic of concern due to their potential to impose selective pressure on the microbiota in the environment, even at low concentrations [6, 7]. A study reported high levels of antibiotics from multiple groups in pharmaceutical wastewater and receiving waters in South Asian countries, used for both human and animal infection [8]. Frequent use and misuse of antibiotics, inadequate wastewater treatment system and meteorological conditions are some of the factors contributing to the occurrence of antibiotic pollution in these regions.

Escherichia coli, the facultative flora of human and animal gastrointestinal tract, is often used as an indicator of water quality from microbiological aspect [9]. However, this bacteria can rapidly acquire antibiotic resistance from environmental residues and turn into a potent reservoir and vector of resistant determinants to other pathogens [10]. ESBL-producing *E. coli* are of particular concern owing to their resistance against most beta-lactam antibiotics, including cephalosporins, and co-resistance to other group of antibiotics such as fluoroquinolones, aminoglycosides, and trimethoprim [11]. Their presence have been reported in diverse ecological niches including surface water, industrial wastewater, hospital wastewater, agricultural, recreational waters and others [12, 13]. Multiple genetic determinants, including $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{SHV}, and bla_{TEM} promote the development of ESBL resistance, and these genes can be transferred to other species through genetic exchange mechanisms [14]. Therefore, increased surveillance of ESBL-producing *E. coli* in environmental habitats is crucial to counteract the dissemination of antibiotic resistance. Moreover, there are six pathotypes of *E. coli* based on their virulence determinants named as - Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC) and Diffusely Adhering *E. coli* (DAEC). Besides ESBL, it is essential to have insight into the virulence prospects of *E. coli* to monitor the public health risks from environmental exposure to this opportunistic commensal bacteria [15].

The pharmaceutical industry in Bangladesh primarily focuses on drug formulation and manufacturing of finished products. As per the Bangladesh Association of Pharmaceutical Industries (BAPI) and Directorate General of Drug Administration (DGDA), there are currently 257 licensed pharmaceutical companies in the country. Unfortunately, the waste disposal and wastewater treatment practices in Bangladesh are inadequate [16]. As a result, the water bodies surrounding drug manufacturing facilities receive untreated wastewater, leading to a plethora of drug resistant bacteria and related genes in the environment [17]. However, still there is no study reported on the presence of antibiotic-resistant and ESBL-producing *E. coli* in surface water surrounding pharmaceutical industries in Bangladesh. Therefore, this study intended to evaluate the prevalence of antibiotic-resistant and ESBL-producing *E. coli* in surface water surrounding pharmaceutical industries in Bangladesh.

Materials and Methods

Study setting, sampling location, and physicochemical analysis

In the current study, seven surface water samples were collected from different locations near and around two pharmaceutical industries located in the Savar area of Bangladesh, which is a hub for the pharmaceutical industry. Sample collection period was June 2020 to December 2020. Sterile 500 ml Schott Duran's bottles (Germany) were used to collect the samples, sealed tightly and transported immediately to the laboratory in an insulated ice box. Temperature and pH of the sam-

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ples were measured using a mercury thermometer graduated from 0℃ to 100℃ and a glass electrode pH meter (SCHOTT instrument), respectively.

Bacteriological analysis of the samples

The bacteriological quality of the collected water samples was assessed using standard conventional culture methods. Briefly, using sterile normal saline (0.85%) the samples were serially diluted up to 10^{-4} and 100 µl from each dilution was inoculated onto non-selective nutrient agar (NA) (Oxoid, UK) for the total bacterial count enumeration. To determine the total gram-negative bacteria, selective MacConkey agar (HIMEDIA) was used. Additionally, ESBL-producing bacteria were counted using ESBL Chrom-agar supplemented with ceftriaxone antibiotic (stock solution prepared at 0.57 mg/ml), where the incubation was at 37℃ for 24 h. The growth of individual colonies on each plate was recorded and enumerated.

Phenotypic and molecular screening of *E. coli*

The presumptively identified *E. coli* isolates obtained from MaC Conkey and ESBL chromogenic agar were further confirmed phenotypically by patching them onto Eosin Methylene Blue (EMB) agar. Isolates displaying a green metallic sheen on EMB agar were selected for Polymerase Chain Reaction (PCR) amplification of the *E. coli*-specific *uidA* gene [18]. Genomic DNA of the bacterial isolates was extracted using a modified boiling method described elsewhere [19]. Briefly, pure colony of the isolates were cultured overnight in 5 ml of nutrient broth at 37℃. A volume of 1 ml of culture was collected in a 1.5-ml eppendorf tube and centrifuged for 10 min at 12,000 rpm. The cell pellets were washed with distilled water, re-centrifuged, and finally suspended in 200 µl of PCR water. The Eppendorf tubes were then subjected to boiling at 100℃ for 10 min. After boiling, each eppendorf tube was immediately placed on ice for 10 min. Then centrifugation was done at 10,000 ×*g* for 10 min, and 100 µl of the supernatant was collected into a fresh eppendorf tube.

Two sets of primer UAL-754 and UAR-900 were used to amplify the uidA gene by polymerase chain reaction (PCR) [18, 20]. The PCR reaction was carried out using a commercial kit (Promega, USA) and a PCR thermocycler (Biometra, Germany). The reaction conditions included an initial denaturation step at 94℃ for 2 min, followed by 25 cycles of denaturation at 94℃ for 1 min, annealing at 58℃ for 1.5 min, extension at 72℃ for 2 min, and a final extension step at 72℃ for 5 min. The PCR products (5 µl) were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide, visualized under UV light and digitalized using the AlphaImager HP System Versatile Gel Imaging (USA).

Antimicrobial susceptibility assay and phenotypic screening of ESBL production

Antibiotic sensitivity of the isolates was evaluated by the standard Kirby-Bauer disk diffusion method employing Mueller-Hinton agar medium (Oxoid Limited, England), in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (2020) [21]. The study utilized 16 antibiotic disks from eight different groups- Ampicillin (AM), Amoxycillin+Clavunic acid (AMC), Azithromycin (AZM), Amikacin (AK), Gentamycin (GN), Chloramphenicol (C), Cefixime (CFM), Cefuroxime (CXM), Ceftriaxone (CRO), Ceftazidime (CAZ), Cefotaxime (CTX), Ciprofloxacin (CIP), Levofloxacin (LEV), Nalidixic acid (NA), Meropenem (MEM), and Tetracycline (TE). These antibiotics are frequently used and produced in Bangladesh.

To test for ESBL production, the Double Disk Diffusion Synergy Test (DDST) was performed. The test inoculum was spread onto Mueller-Hinton agar (MHA) after matching the turbidity to 0.5 McFarland. An augmenting disk (20 µg amoxicillin + 10 µg clavulanic acid) was placed on the surface of MHA, and then disks of ceftriaxone (30 µg), ceftazidime (30 µg), and cefotaxime (30 µg) were positioned around it, ensuring that each disk was 15–20 mm away from the augmenting disk (center to center). The spacing between the disks was adjusted as needed for each strain to accurately detect synergy.

Molecular characterization of the ESBL producing isolates

Each bacterial isolate was subjected to characterization of their ESBL-genotypes and the presence of virulence factors. Specific regions of the bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-13}}$ resistance genes were amplified using four different polymerase chain reactions. The PCR reaction mixture volume and concentration were used as previously described, with the only difference being the primer sets and thermal cycling conditions for

Table 1. Primer sequences used for detection of bacterial 16S rRNA gene, *E. coli* specific *uidA* gene, antibiotic resistance gene, and virulence genes.

Target gene	Primer Sequence 5'→3'	Amplicon Size (bp)	Reference
16S rRNA	F-AGT TTG ATC CTG GCT CAG R-ACC TTG TTA CGA CTT	1484	[19]
uidA	UAL-AAA ACG GCA AGA AAA AGC AG UAR-ACG CGT GGT TAC AGT CTT GCG	147	[20]
bla_{TEM}	F-TCG GGG AAA TGT GCG CG R-TGC TTA ATC AGT GAG GAC CC	971	[50]
$bla_{ctx\text{-}M\text{-}15}$	F-CAC ACG TGG AAT TTA GGG ACT R-GCC GTC TAA GGC GAT AAA CA	996	[51]
$bla_{ctx\text{-}M\text{-}13}$	F-GGT TAA AAA ATC ACT GCG TC R-TTG GTC ACG ATT TTA GCC GC	866	[52]
bla_{SHV}	F-CAC TCA AGG ATG TAT TGT G R-TTA GCG TTG CCA GTG CTC G	885	[53]
ipaH	F-CTC GGC ACG TTT TAA TAG TCT GG R-GTG GAG AGC TGA AGT TTC TCT GC	933	[20]
Lt	F-GCA CAC GGA GCT CCT CAG TC R-TCC TTC ATC CTT TCA ATG GCT TT	218	[54]
eae	F-TCA ATG CAG TTC CGT TAT CAG TT R-GTA AAG TCC GTT ACC CCA ACC TG	482	[20]
eagg	F-AGA CTC TGG CGA AAG ACT GTA TC R-ATG GCT GTC TGT AAT AGA TGA GAA C	194	[20]

amplification of each resistance gene. Four different primer pairs were used (as shown in Table 1). For bla_{TEM} gene, PCR reactions were as followed- initial denaturation at 94℃ for 2 min, 30 cycles of 94℃ for 1 min, annealing at 60.5℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 10 min. For $bla_{CTX\text{-}M\text{-}15}$ gene, the reaction conditions were 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 sec, 52℃ for 30 sec, 72℃ for 30 sec, and a final extension for 7 min at 72℃. In regard of $bla_{CTX\text{-}M\text{-}13}$ gene, following PCR reaction conditions were used- 95℃ for 3 min initial denaturation , 30 cycles of denaturation at 95℃ for 1 min, annealing at 55℃ for 1 min, extension at 72℃ for 1 min, and final extension at 72℃ for 5 min. While in case of bla_{SHV} gene, initial denaturation was at 94℃ for 5 min, followed by 30 cycles of 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min, and a final extension at 72℃ for 7 min. The PCR amplicons were visualized by 1% agarose gel electrophoresis, as described in previous section.

Multiplex polymerase chain reaction (PCR) was conducted using four primer sets to detect the virulence genes *eae, ipaH, eagg,* and *Lt*. The reaction was carried out using a thermal cycler (2720 Thermal Cycler Applied Biosystems, USA) and initiated with the denaturation step at 94℃ for 10 min. Then it continued to 35 cycles of amplification, consisting of 40 sec of denaturation at 94℃, 30 sec of annealing at 55℃, and 50 sec of extension at 72℃. Final extension was done at 72℃ for 7 min. The amplicons were resolved using 2% agarose gel stained with ethidium bromide and visualized under UV light.

Molecular identification, nucleotide sequencing and phylogenetic analysis

Bacterial isolates of this study were identified by 16S rRNA PCR using universal primers 8F (5-AGT TTG ATC CTG GCT CAG-3) and 1492R (5-ACC TTG TTA CGA CTT-3), following a previously established protocol [22]. Among the 16S rRNA-positive isolates, seven representative isolates (chosen randomly from each collected sample) were selected for further analysis of the 16S rRNA gene sequence. Besides the representative isolates, one isolate positive for three ESBL genes and high MAR index was chosen for the sequencing as well. Big-Dye Terminator cycle sequencing kit and an ABI

Prism 310 Genetic Analyzer (Applied Biosystems Inc., USA) were employed to determine the amplicon nucleotide sequences. Chromas 2.6.5 (Technelysium, Australia) was used to analyze the Chromatograms of the sequences followed by identification by BLAST search. Multiple sequence alignment was performed using the ClustalW Multiple Alignment algorithm in BioEdit 7.2.6 software and submitted to the NCBI for accession numbers. Phylogenetic relationship of 16S rRNA gene sequences were analyzed using the partial sequence of these amplicons and reference sequences with MEGA 11. Neighbor-joining method was employed to build the phylogenetic tree using the bootstrap replicates (1000).

Results

Identification of *E. coli* from the water samples

Temperature of the water samples were in the range from 28.9℃ to 30.5℃, whereas pH values were observed from 6.3 to 7.8. The total viable bacterial count of the samples varied from 5.0×10^5 cfu/ml to >TNTC, with total Enterobacteriaceae ranging from >TFTC to 8.1×10^5 cfu/ml and presumptive ESBL-producing isolates ranging from >TFTC to 5.1×10^5 cfu/ml. According to the phenotypic and genotypic methods, 51 isolates were screened as *E. coli* from the seven surface water samples.

Antibiotic resistance pattern of the isolates

100% of the study *E. coli* isolates were observed as multidrug-resistant, with the highest resistance against ampicillin (100%), AZI (100%), and NA (100%), followed by CIP (98%), CFM (96.1%), CXM (92%), LEV (90%), CTX (88%), AK and AMC (74%), GN and CAZ (72%), CRO (66%), C (43.14%), and the lowest resistance observed against MEM (1.96%) (Fig. 1).

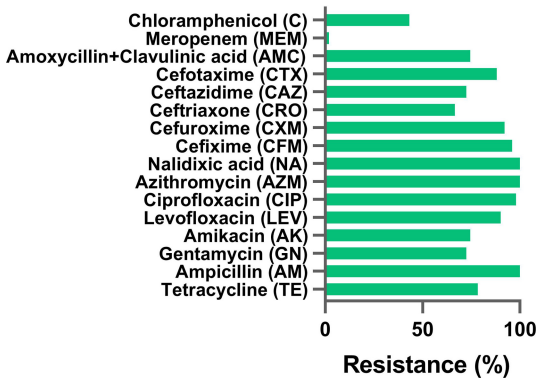

Fig. 1. Antibiotic resistance percentage of the *E. coli* isolates (n = 51) from seven surface water samples against eight different group of antibiotics. Highest percentage of resistance was found against ampicillin, azithromycin, and nalidixic acid (100%) and the lowest resistance against meropenem (1.96%).

Characterization of β-lactamase genes and virulence genes

Out of the total 51 *E. coli* isolates, 69% (n = 35) were confirmed positive for β-lactamase activity by Double Disk Diffusion Synergy Test (Fig. 2). The presence of four different classes of β-lactamase genes was detected as follows: bla_{TEM} gene in 70.6% (n = 36) of the isolates, $bla_{CTX\text{-}M\text{-}13}$ in 60.8% (n = 32), $bla_{CTX\text{-}M\text{-}15}$ in 54.9% (n = 28), and bla_{SHV} in 1.96% (n = 1) (Fig. 3). Using multiplex PCR, the presence of virulence genes *Lt* (ETEC), *eagg* (EAEC), and *ipaH* (EIEC) was detected in 7.8%, 5.9%, and 1.9% of the *E. coli* isolates, respectively

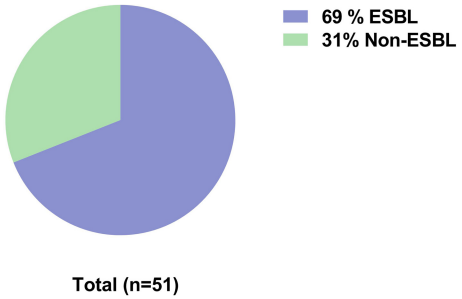

Fig. 2. Phenotypically 69% of the *E. coli* (n = 51) isolates were ESBL producing as detected by DDST method.

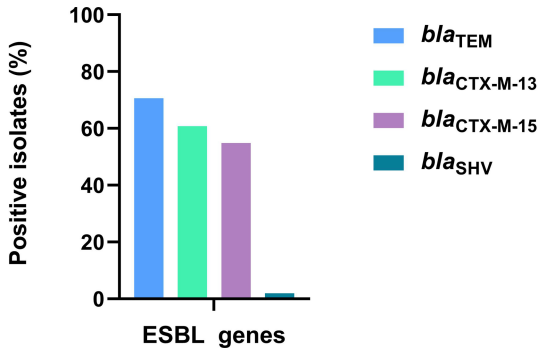

Fig. 3. Prevalence of the ESBL genes among the surface water *E. coli* isolates, prevalence was calculated as percentage for total number of the isolates (n = 51). bla_{TEM} was the most prevalent one with highest percentage, whereas lowest percentage was observed for bla_{SHV}.

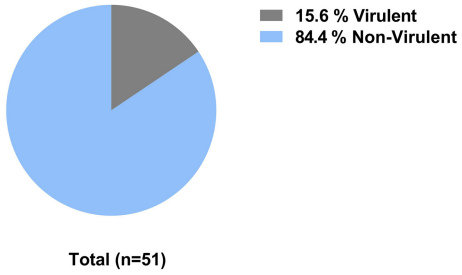

Fig. 4. 15.6% of the isolates (n = 51) were virulent strains with the presence of *E. coli* specific virulence genetic determinants.

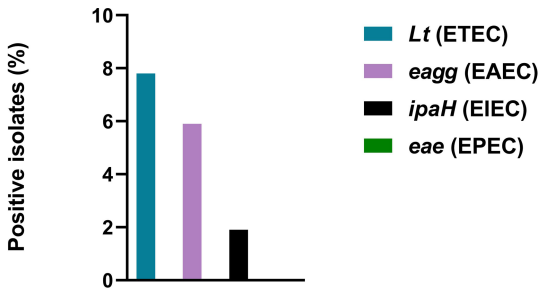

Fig. 5. Frequency distribution of virulence genes (*eae, ipaH, eagg* and *Lt*) among the *E. coli* isolates. *Lt* was present among highest pe1rcentage of the isolates, whereas none of the isolates was positive for *eae* gene.

(Fig. 4). Herein, 15.6% of the total isolates were found to contain virulence gene determinants, whereas none of the isolates were positive for *eae* (EPEC) gene (Fig. 5).

Phylogenetic analysis

The results of 16S rRNA analysis indicate that the majority of the samples are closely related to a strain of *E. coli,* specifically strain E4742. Interestingly, strain A18 shows a high similarity percentage of 99.71% with *Achromobacter insuavis*, belonging to the *Betaproteobacteria* class, *Burkholderiales* order, and *Alcaligenaceae* family. This result indicates a distinct taxonomic classification compared to the *Escherichia* strains and suggests a different genetic background and potentially different pathogenic properties. (Fig. 6). Strain L29 is corresponds to *Franconibacter helveticus*, a member of the *Proteobacteria* phylum, *Gammaproteobacteria* class, *Enterobacterales* order, and *Enterobacteriaceae* family. However, the similarity percentage is slightly lower at 96.46%. This discrepancy might indicate some genetic divergence or unique characteristics present in this particular strain compared to the *Escherichia* strains. The completeness values of the samples range from

Table 2. Antibiotic resistance phenotypes, presence of ESBL genes, and virulence pattern of *E. coli* isolated from surface water samples.

Isolates ID	Antibiotic resistance Phenotypes[a]	MAR index	ESBL resistance gene	Virulence gene
L1	AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L2	GN, AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L3	AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.7	*blaCTX-M-13, blaCTX-M-15*	-
L4	AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.7	$bla_{CTX\text{-}M\text{-}13}$, *blaCTX-M-15*	-
L5	AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L6	AMP, TE, LEV, NA, AZI, CFM, CIP, CRO, CAZ, CTX, AMC/CLV	0.7	*blaCTX-M-13, blaCTX-M-15*	-
L7	AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13*	-
L8	AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.7	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L9	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	$bla_{CTX\text{-}M\text{-}13}$, *blaCTX-M-15*	-
L10	AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13*	-
L11	AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, AMC/CLV	0.6	*blaTEM*	-
L12	AMP, TE, LEV, NA, AZI, CIP, C, CTX, AMC/CLV	0.6	*blaTEM, blaCTX-M-13,*	-
L13	GN, AMP, TE, LEV, NA, AZI, CFM, CIP, C, CTX	0.6	*blaTEM, blaCTX-M-13*	-
L14	GN, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, AMC/CLV	0.7	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L15	AMP, TE, NA, AZI, CFM, CXM, CIP, C, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM*	-
L16	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CIP, C, CAZ, CTX, AMC/CLV	0.8	*blaTEM*	*eagg, Lt, ipaH*
L17	GN, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CRO, CAZ, CTX	0.8	-	*eagg*

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Table 2. Continued.

Isolates ID	Antibiotic resistance Phenotypes[a]	MAR index	ESBL resistance gene	Virulence gene
L18	AK, AMP, TE, NA, AZI, CFM, CIP, CAZ, CTX, AMC/CLV	0.6	*blaTE*	-
L19	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CIP, CTX, AMC/CLV	0.7	*blaCTX-M-13*	*eagg*
L20	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CTX	0.7	*blaTEM*	*Lt*
L21	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CAZ, CTX	0.8	*blaTEM*	-
L22	AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CTX	0.7	*blaTEM, blaCTX-M-13*	-
L23	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, AMC/CLV	0.8	*blaTEM, blaCTX-M-15*	*eagg*
L24	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	*blaTEM, blaCTX-M-15*	*Lt*
L25	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L26	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CRO, CAZ, CTX, AMC/CLV	0.9	*blaTEM, blaCTX-M-13*	-
L27	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L28	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L29	AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX	0.8	*blaTEM, blaCTX-M-15*	-
L30	GN, AK, AMP, LEV, NA, AZI, CFM, CXM, CIP, CAZ, AMC/CLV	0.7	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L32	GN, AK, AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L33	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L34	AK, AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX	0.6	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L35	GN, AK, AMP, LEV, NA, AZI, CFM, CXM, CIP, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-15*	-
L37	GN, AK, AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L38	GN, AK, AMP, LEV, NA, AZI, CFM, CXM, CIP, CRO, CTX, AMC/CLV	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
L39	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, MEM, C, CRO, CAZ, CTX, AMC/CLV	1.0	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
A7	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX	0.8	*blaTEM, blaCTX-M-13, blaCTX-M-15*	-
A8	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	*blaCTX-M-13, blaCTX-M-15*	-
A9	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CTX, AMC/CLV	0.8	$bla_{\text{CTX-M-13}}$, *blaCTX-M-15*	-
A10	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	$bla_{\text{CTX-M-13}}$, *blaCTX-M-15*	-
A11	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CRO, CAZ, CTX, AMC/CLV	0.9	*blaCTX-M-13, blaCTX-M-15*	-
A12	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, CRO, CAZ, CTX, AMC/CLV	0.9	*blaTEM, blaCTX-M-13*	-
A13	GN, AK, AMP, TE, LEV, NA, AZI, CIP, C, CTX,	0.6	*blaTEM*	-
A15	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CIP, C, CRO, CAZ	0.8	*blaSHV, blaCTX-M-13*	-
A16	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CTX	0.8	*blaTEM*	-
A18	GN, AK, AMP, TE, NA, AZI, CFM, CXM, C, CRO, CAZ, CTX, AMC/CLV	0.8	-	-
A19	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CIP, C, CAZ, CTX, AMC/CLV	0.8	-	-
A23	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CRO, CTX, AMC/CLV	0.9	-	-
A24	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CIP, CRO, CAZ, CTX, AMC/CLV	0.8	*blaTEM*	-
A27	GN, AK, AMP, TE, LEV, NA, AZI, CFM, CXM, CIP, C, CRO, B22CTX, AMC/CLV	0.9	-	-

[a] Abbreviations: AM-Ampicillin, Amoxycillin+Clavunic acid-AMC, AZM-Azithromycin, AK-Amikacin, GN-Gentamycin, C-Chloramphenicol, CFM-Cefixime, CXM-Cefuroxime, CRO-Ceftriaxone, CAZ-Ceftazidime, CTX-Cefotaxime, CIP-Ciprofloxacin, LEV-Levofloxacin, Nalidixic acid-NA, MEM-Meropenem, Tetracycline (TE).

92.8% to 96.6%, as shown in Table 3. This table provides a summary of the taxonomic and sequence similarity information for the microbial samples analyzed. However, further investigation and analysis of this data may be required to fully comprehend the biological significance of these findings.

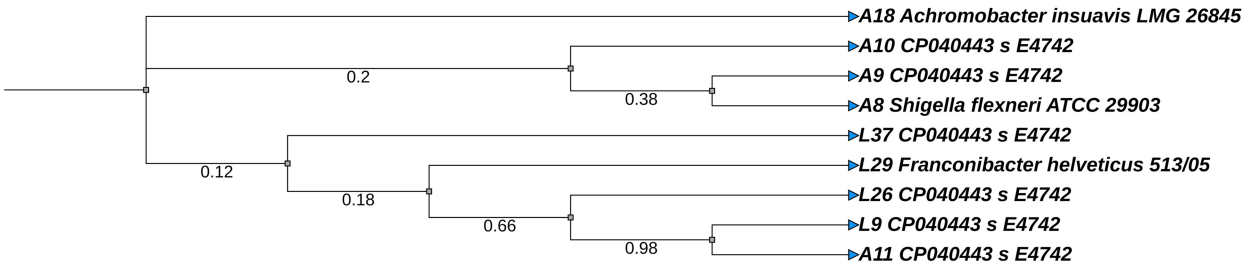

Fig. 6. Phylogenetic relationship of the *E. coli* isolates based on nucleotides sequence of 16S rRNA gene.

Table 3. Taxonomic classification and similarity analysis of isolated strains.

Name	Top-hit taxon	Top-hit strain	Similarity (%)	Top-hit taxonomy	Completeness (%)
A8	*Shigella flexneri*	ATCC 29903	97.94	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	96.6
A9	CP040443_s	E4742	98.27	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	94.9
A10	CP040443_s	E4742	99.71	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	92.8
A11	CP040443_s	E4742	99.12	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	93.2
A18	*Achromobacter insuavis*	LMG 26845	99.71	Bacteria;Proteobacteria;Betaproteobacteria; Burkholderiales;Alcaligenaceae;Achromobacter	94.2
L9	CP040443_s	E4742	98.78	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	95.2
L26	CP040443_s	E4742	99.50	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	96.0
L29	*Franconibacter helveticus*	513/05	96.46	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Franconibacter	95.6
L37	CP040443_s	E4742	98.70	Bacteria;Proteobacteria;Gammaproteobacteria; Enterobacterales;Enterobacteriaceae;Escherichia	94.9

Discussion

Antibiotic resistance have emerged as a serious threat for global public health, as it hinders the efficacy of treatment which results in increased mortality, morbidity, and associated healthcare costs. Aquatic environments connected to industrial wastewater and municipality sewage system become highly polluted with antibiotics and resistant bacteria containing mobile genetic determinants [23, 24]. Wastewaters discharged into environment without any prior or proper treatment promote the dissemination of antibiotic resistance and their genetic determinants in the inhabitant bacterial community [25, 26]. Drug manufacturing facilities has been recognized as a major hotspot for spreading antibiotic resistance as per comparative review analysis on possible source of the resistance from environmental health perspective [27]. *E. coli* is generally a harmless bacteria however, when they are ESBL producing and drug resistant could be a serious threat to public health [28]. Hence, this study focused to investigate the antibiotic resistance profile and molecular characterization of *E. coli* isolates from the surrounding surface water near pharmaceutical industries, on the basis of ESBL resistance and virulence genes.

Here, the temperature of the samples were in the

range of 26.4℃ to 30℃, while the pH values were differed from 6.3 to 7.8 (slightly alkaline). According to the Department of Environment (DoE) standard in Bangladesh, the parameters were within the permissible limit for domestic, irrigation utilization and others [29, 30]. The presence of bacterial community and their distribution could be significantly influenced by the temperature of the water habitat [31]. Moreover, bacterial susceptibility to antibiotic stress is dependent on the abiotic factors like temperature and pH, with having difference among the pathogenic and non-pathogenic strains [32]. In a previous study pathogenic strains of *E. coli* were reported with more frequently develop antibiotic resistance than non-pathogenic strains. Particularly, when they got environmental conditions such as a temperature of 30℃ and pH of 6.5, which supposed to be the optimum values for the acquisition of resistance through selective pressure [33]. Therefore, these physicochemical parameters might promote the high abundance of antibiotic resistance bacterial isolates in the water samples.

Total bacterial count in the water samples were 5×10^5 cfu/ml to TNTC, while the gram-negative bacterial count ranged from TFTC to 8.1×10^5 cfu/ml. This observation is consistent with previous studies on surface water samples, indicating a high population density of bacteria in environmental water samples affected by human and industrial activities [34]. This concur the findings of a study conducted in Borneo that observed higher viable bacterial counts with values from 1.6×10^4 to 3.0×10^4 cfu/ml in groundwater influenced by human or industrial activities, as compared to the counts in water samples distant from such activities [35]. The presumptive count of ESBL isolates ranged from TFTC to 5.1×10^5 cfu/ml, suggesting a high abundance of ESBL-producing bacteria in the water samples. The findings are somewhat similar to other studies, which observed predominance of ESBL bacteria in the aquatic environment and wastewater discharge from various sources [35, 36].

Herein our study, 100% (n = 51) of the isolates showed multidrug resistance against eight different group of antibiotics (Table 2). An earlier study reported similarly 100% MDR *E. coli* from environmental and human fecal samples [37]. In contrast, a study conducted in Bangladesh on drinking water sources observed 36% MDR isolates [17], and another study reported 49.48% MDR *E. coli* from water sources [38]. Likewise, another investigation reported a high percentage (81.3%) of MDR *E. coli* from small-scale chicken farms and households in Vietnam [39].

E. coli isolates of the present study were perceived with greater percentage of resistance against Ampicillin, Azithromycin, and Nalidixic acid (100%), followed by Ciprofloxacin (98%), Cefuroxime (96%), Cefixime (92%), Levofloxacin (90%), Amikacin and Amoxycillin/Clavulanic acid (74%), Gentamycin and Ceftazidime (72%), Ceftriaxone (66%), Chloramphenicol (43%), while the lowest resistance was observed against Meropenem (1.96%). β-lactam, quinolone, and fluoroquinolone antibiotics were found with higher frequency of resistance which coincide the findings of other studies carried out in Ethiopia and Bangladesh [40, 41]. In Bangladesh Meropenem is less commonly used antibiotic, whereas the frequent use and production of cephalosporin and β-lactam could be the promoting factor for developing high level of resistance against these group of antibiotics. This in accordance with the findings of a study on antibiotic resistant *E. coli* from water sources in Bangladesh [42].

Multiple Antibiotic Resistance (MAR) value was greater than > 0.2 for all of the study *E. coli*, which implies that the sampling source is heavily contaminated with multiple antibiotics. This finding agree with a previous investigation on drug resistant *E. coli* from water sources of Bangladesh [37]. The high MAR index value of the *E. coli* isolates in our study, imply that the wastewater from the pharmaceutical industries without pretreatment could be attributed to the contamination of surrounding surface water with diverse types of antibiotics.

We found 69% (n = 35) of *E. coli* isolates as ESBL-producing in the Double Disk Synergy Test (DDST). Previously, other studies were reported from Bangladesh with high percentages (43%) of ESBL- *E. coli* isolates from environmental and clinical samples [17, 41]. High number of ESBL-producing bacteria in this study samples may be ascribed to the production of cephalosporins in pharmaceutical industries. This finding directly correlates the influence of pharmaceutical waste on the development of antibiotic resistance in bacterial populations. So, from environmental health perspective, it suggests that the antibiotic pollution from the produc-

tion facilities could exert the selective pressure for the surrounding bacterial biota to particular group of antibiotics.

Four different ESBL genes were targeted in this study to be detected from the *E. coli* isolates, where bla_{TEM} was found as the most prevalent one (70.6%), followed by $bla_{CTX\text{-}M\text{-}13}$ (60.8%) and $bla_{CTX\text{-}M\text{-}15}$ (55%). The predominance of the bla_{TEM} and $bla_{CTX\text{-}M}$ genes among the isolates concur with the previous findings in Bangladesh [42, 43]. Also in some other studies, the prevalence of $bla_{CTX\text{-}M}$ genes has been reported in environmental samples [44], while bla_{SHV} and bla_{TEM} genes have been found mostly in clinical isolates [45]. *E. coli* strains harboring $bla_{CTX\text{-}M\text{-}15}$ has already been recognized as an important concern for both hospital and community-acquired infections [46].

E. coli specific virulence genes were also detected in 15.6 % of the study isolates. In a similar study on supply water resources of Bangladesh, 7% of total *E. coli* isolates were perceived as pathogenic types, including EPEC and ETEC [17]. The ETEC pathotype was the most prevalent among our study isolates, while none was positive for EPEC determinant. Another study reported a high predominance (32.6%) of EIEC (*ipaH*) in the environmental water isolates of Sudan [47]. However, in some other studies reported based on surface waters isolates, virulent types were detected with lower percentage, sometimes as lower than 1% [48]. In these studies, the pathotypes EPEC and EHEC were the prevalent ones encoded by the *eaeA* gene [49].

The wastewater treatment system in Bangladesh is inadequate, resulting in the discharge of poorly treated or untreated effluents that contain a significant number of active antimicrobial agents and antibiotic-resistant bacteria (ARB). Our study provides valuable insight into the extent of antibiotic pollution and distribution of ARB in aquatic environmental sources surrounding pharmaceutical industries in Bangladesh. High MAR index values observed in this study indicate the high risk of antibiotic pollution of the sampling sources, underscoring the urgent need for proper legislation and regulation of waste disposal from industrial activities. Finally, continuous and systematic monitoring of the drug-resistant pattern of the commensal bacterial community is necessary for effective policy formulation and implementation.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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