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Research Article

EXTENDED SPECTRUM ß-LACTAMASE PRODUCING *Escherichia coli* FROM BAGMATI RIVER WATER

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ABSTRACT
Aquatic environments can be the sources for the spread of antibiotic-resistant microorganisms and resistance genes. *Escherichia coli* is one of the bacteria taken as an indicator of water contamination with human faecal matter. *CTX-M* producing *E. coli* is the most common type of extended-spectrum ß- lactamase (ESBL) producing *E. coli* worldwide. This study was conducted from October 2019 to December 2020 to determine the proportion of *CTX-M* gene among ESBL *E. coli* isolated from the Bagmati River. Thirty-nine water samples in triplicates were collected from 13 different points of Bagmati River in Kathmandu Valley and analyzed for isolation of *E. coli*. Antimicrobial susceptibility test was performed by modified Kirby- Bauer disc diffusion method. ESBL was confirmed phenotypically by the combination disk method recommended by CLSI guidelines. Alkaline hydrolysis method was used for plasmid DNA extraction and *CTX-M* gene was detected by a polymerase chain reaction and agarose gel electrophoresis. *E. coli* was isolated from 76.9% (n= 30) samples and 80% (n= 24) of *E. coli* isolates were multidrug-resistant (MDR). Out of 24 MDR *E. coli*, 33.3% (n= 10) were ESBL producers. Among 10 ESBL *E. coli*, 70% (n= 7) had *CTX-M* gene. It shows that Bagmati River water is polluted due to anthropogenic activities. The resistant bacteria may circulate from water to the community posing a potential threat of infection. Effective treatment of river water is recommended to prevent the spread of antibiotic-resistant microorganisms.

Keywords: Bagmati River water, *CTX-M* gene, *E. coli*, extended spectrum ß-lactamase

INTRODUCTION
The urban areas of developing countries directly dispose excess wastewater into surface water, which impairs the quality of water (Ismail & Abed, 2013). Faecal contamination of river water has a direct impact on catchment communities (Woldemichael *et al.*, 2016). Water has been identified as a significant reservoir of antibiotic resistant bacteria (Rizzo *et al.*, 2013). The excessive use of antibiotics in human, animal health, and food sectors may accelerate the development of antibiotic resistant bacteria (ARB). Antibiotic resistant genes (ARGs) in bacteria from water is becoming an increasing concern. Many ARGs that encode resistance to a variety of antibiotics in bacteria which are present not only in hospital and animal production wastewater, but also in wastewater treatment plants, sewage, surface water, ground water, and even in drinking water (Kelly *et al.,* 2023).

Escherichia coli is a normal flora of the gastrointestinal tract in both humans and animals and most often used as an indicator bacterium of fecal contamination of water. *E. coli* is a very common cause of nosocomial and community-acquired infections in humans. Extended-spectrum ß-lactamases (ESBLs) are an important cause of ß-lactam antibiotics resistance in gram-negative bacteria (Bradford, 2001) and are spreading globally (Thenmozhi *et al.*, 2014). Among nine distinct structural and evolutionary families of ESBL (Bajpai *et al.*, 2017), the main types of ESBL variants include *TEM, SHV, CTX-M*, and *OXA*. The *CTX-M* enzymes have been found disseminated among a wide range of clinical bacteria within and across the species worldwide (Ruppe *et al.*, 2009). *CTX-M* ß- lactamase in commonly isolated bacteria *E. coli* is a global public health problem. The studies conducted in Nepal revealed high proportion of ESBL producers in wastewater and river water (Khanal *et al.*, 2024; Ghimire *et al.*, 2024; Tandukar *et al.*, 2018) and *blaCTX-M* gene as more frequent in *E. coli* (Khanal *et al.*, 2024).

Due to the large diversity of pathogenic and commensal microorganisms and continuous discharge of antibiotic resistant bacteria and antibiotic resistance genes into the environments, aquatic environments have been identified as hotspots of antibiotic resistance. Hospital and municipal wastewater, effluents from pharmaceutical companies (Khanal *et al.*, 2024; KC *et al.*, 2024), poultry, animal and aquaculture facilities (Young *et al.*, 2022) are the sources for antibiotic resistance. Wastewater treatment plants do not completely remove antibiotics and resistant microorganisms. Municipal wastewater treatment does not exist in Nepal to address the dumping of wastewater in Bagmati River. Municipal wastewater treatment plants absorb human fecal waste carrying a variety of ARB, and they have been regarded as key reservoirs for several ARGs that are linked to human infections (Rizzo *et al.*, 2013). Although wastewater treatment reduces upto 99% bacteria (Papajova *et al.*, 2022), remaining bacteria are discharged in surface water bodies. Most treated wastewater is dumped into rivers, which help spread ARB and ARGs

further (Amos *et al*., 2018). The presence of ARB and ARGs cause the serious complications in humans as well as animals and increase the morbidity and mortality. In Nepal, research shows that there is high prevalence of ESBL producing *E. coli* in clinical samples and a couple of research have been performed in river water samples. It needs to generate more evidence to recommend developing effective strategies and guidelines for wastewater treatment in our context before discharging it into river. Monitoring of ARGs for β-lactam, quinolone, and vancomycin may help to identify antibiotic resistance determinants in water (Amarasiri *et al*., 2022). Therefore, this research was conducted to determine the proportion of *CTX-M* gene in ESBL producing *E. coli* isolated from the Bagmati River. This helps in estimating the emergence of *CTX-M* linked ESBL producers and their spread in the community and hospitals.

MATERIALS AND METHODS

Research Design

This was a field and laboratory based cross-sectional study. Water samples were collected from Bagmati River and were processed at the laboratory of Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu. Water samples from Bagmati River were collected from the five sites where anthropogenic activities are relatively low and eight sites where anthropogenic activities are high.

Study Sites and Duration

The study sites were 13 different sampling points of the Bagmati River starting from Chobhar to Sundarijal. The sampling point was taken 2 km apart as the physicochemical composition of water may vary at this distance. The study was conducted from October 2019 to December 2020.

Sample Collection and Measurement of Physical Parameters

A total of 39 river water samples were collected from 13 sampling points of Bagmati River. Triplicate samples were collected in acid washed sterile plastic bottles from each sampling point as grab samples. The points with turbulence were avoided during sample collection. Water samples were collected facing the mouth of the collection bottle towards the current of water. The bottle had left few spaces at the top for aeration. Collected water samples were labeled clearly and transported in an insulated cold box. pH was measured using pH meter (APHA/AWWA/WEF, 2017). Temperature was measured with a thermometer and turbidity with turbidimeter (APHA/AWWA/WEF 2017).

Detection of Total Coliforms by Membrane Filtration (MF) Method

Membrane filtration technique was used for detection of bacteria. 100ml of water sample was used for filtration and membrane filter was placed on the Eosin-Methylene blue agar plate. It was incubated for 24 hours at 37°C for total coliforms and 44°C for fecal coliforms. After incubation, pink colonies with dark center having greenish metallic sheen at 37°C indicated total coliforms and growth at 44.5°C with colony characteristics specific to coliforms confirmed fecal coliforms. For the completed test, the colonies with metallic sheen at different points at the media were transferred to lactose broth and incubated at 37°C for 24 hours and observed for gas production (APHA/AWWA/WEF 2017).

Identification of Isolates

Identification of bacterial isolates was done following Bergey's Manual from the cultural characteristics and biochemical tests including catalase, oxidase, sulfide indole motility medium (SIM), methyl red, Voges Proskauer, citrate utilization, triple sugar iron (TSI) and urease test (HiMedia Laboratories Private Ltd, India).

Antibiotic Susceptibility Test (AST)

The antibiotic susceptibility test of the isolates was performed using modified Kirby-Bauer disc diffusion method as recommended by CLSI (2018). The antibiotic disc used for AST were cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (30 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), ampicillin (10 µg), amikacin (30 µg), piperacillin (100 µg) and piperacillin/tazobactam (100/10 µg) (HiMedia Laboratories Private Ltd, India), representing different classes of antibiotics. Antibiotic discs were placed onto the medium then incubated at 37°C for 18 hours. After incubation, diameter of zone of inhibition was measured and interpreted as sensitive, intermediate, and resistant as per CLSI (2018).

Detection of Extended Spectrum beta-lactamase (ESBL) Producer

A standard of 0.5 McFarland turbidity was maintained for the inoculum and Mueller Hinton Agar plates were inoculated with test bacteria. Ceftazidime (30µg) and cefotaxime (30 µg) disks (HiMedia Laboratories Private Ltd, India) were placed alone for screening of ESBL. For confirmation, combination disk with clavulanic acid (ceftazidime plus clavulanic acid, 30/10 µg), (cefotaxime plus clavulanic acid, 30/10 µg) disks were applied onto a plate and then incubated for 24 hours at 37°C. Isolates were confirmed as an ESBL producer if it showed an increase of 5 mm or more in the zone of inhibition of the combination disks compared to that of the ceftazidime/cefotaxime disk alone (CLSI, 2018). *E. coli* isolates in pure culture were preserved in 20% glycerol containing tryptic soya broth and kept at -70°C until further processing for molecular detection.

Extraction of Plasmid and PCR Amplification of *CTX-M* Gene

All phenotypically confirmed ESBL producer *E. coli* was analyzed for detection of bla*CTX-M* gene. The plasmid DNA extraction was done from *E. coli* by using alkaline hydrolysis method (Sambrook and Russel, 2001). The extracted plasmid was stored at -20°C. *blaCTX-M* gene (544bp) in extracted plasmids were amplified by PCR using the *CTX-M* specific primers (F: 5'-TTTGCGATGTGCAGTACCAGTAA-3'; R: 5'-CGATATCGTTGGTGGTGCCATA-3') (Edelstein *et*

al., 2003). The PCR mixture of total volume 25µl was prepared which consist of 3 µl of DNA template, 0.5µl of 10Mm each of forward and reverse primer and 21µl of master mix. Thermal and cycling conditions for the *blaCTX*-M gene: Initial denaturation at 94°C for 1 minutes, denaturation at 95°C for 5 minutes of 35 cycles, annealing at 55°C for 1 sec of 35 cycles, extension at 72°C for 1 minutes of 35 cycles, and final extension at 72°C for 10 minutes. After PCR amplification, 15µl of each reaction was separated by electrophoresis in 1.5% agarose gel for 60 minutes at 100V in 0.5x TAE buffer. Positive and negative controls for *CTX-M* gene were used in each PCR. DNA was stained with ethidium bromide (5 µg/ml). Finally, the gel photo was taken in UV transilluminator (Fig. 1). The band size of the PCR products was compared with 100 bp DNA marker and positive control.

Statistical Analysis

All the data were entered and analyzed using Statistical Package for Social Science (SPSS) version 25 software. Mean and standard deviation were calculated for physical parameters and frequency and percentages for microbiological data.

RESULTS

Physical Quality of Water

The temperature of Bagmati water during sampling ranged from 12° to 26° C. The temperature 12° to 16° C was observed during the winter season while 25° to 26° C in post–monsoon. The pH of the Bagmati River water at different sampling sites were between 6.5 to 8.0, mostly around neutral pH in most of the sites. Turbidity of the river water ranged from 3.5 to 557 NTU at different sampling sites and only one upstream site had turbidity less than 10 NTU (Table 1).

Microbiological Analysis of Water Samples

E. coli was detected in 30 (76.9%) water samples. Growth of *E. coli* was seen low in the samples collected from upstream sites while high growth of coliforms was found in the samples collected from downstream sites.

Table 1. Physical parameters of water samples from Bagmati River

Sites	Temperature (°C)	pH	Turbidity (NTU)
Sundarijal	12.0	7.6±0.01	3.5±0.20
Ghatte	12.0	7.5±0.00	70.5±4.27
Gokarna	13.0	7.3±0.17	127±4.00
Jorpati	12.0	7.7±0.26	557±0.00
Guheswori	11.0	7.3±0.20	177±18.73
Tilganga	12.0	7.1±0.00	353±13.00
Baneshwor	13.0	7.7±0.10	312±13.75
Sankhamul	12.0	7.8±0.20	235±5.57
Thapathali	13.0	7.6±0.00	396±3.00
Teku	16.0	7.5±0.17	318±5.57
Balkhu	26.0	6.7±0.26	404±13.53
Sundarighat	26.0	6.6±0.10	399±15.72
Chobhar	25.5	7.5±0.17	396±20.42

Antibiotic Susceptibility Pattern of *E. coli* Isolates from Bagmati River Water

A total of 30 isolates of *E. coli* from Bagmati water samples were tested for their antibiotic susceptibility. High number of *E. coli* were resistant towards ampicillin (76.6%), sulphamethoxazole (70%) and third generation cephalosporins; cefixime (60%), ceftazidine (46.6%), cefotaxime (53.3%) and ceftriaxone (46.6%). While, less proportion of *E. coli* isolates were resistant to Amikacin (10%), gentamicin (13.3%) and chloramphenicol (23.3%). Out of 30 isolates of *E. coli* from Bagmati River water, 24 (80%) were MDR (Table 2).

Table 2. Antibiotic susceptibility pattern of *E. coli* (n=30)

Antibiotics	Sensitive (%)	Resistant (%)
Ampicillin	7 (23.4)	23 (76.6)
Amikacin	27 (90.0)	3 (10.0)
Amoxicillin clavulanate	15 (50.0)	15 (50.0)
Ceftazidime	14 (46.7)	16 (53.3)
Cefotaxime	14 (46.7)	16 (53.3)
Ceftriaxone	16 (53.4)	14 (46.6)
Cefixime	12 (40.0)	18 (60.0)
Ciprofloxacin	17 (56.7)	13 (43.3)
Chloramphenicol	23 (76.7)	7 (23.3)
Imipenem	16 (53.4)	14 (46.6)
Gentamicin	26 (86.7)	4 (13.3)
Sulphamethoxazole	9 (30.0)	21 (70.0)
Tetracycline	12 (40.0)	18 (60.0)

ESBL Production Profile and *CTX-M* Gene among *E. coli* from River Water

Among 30 *E. coli* isolates, 16 (53.3%) were screened positive for ESBL production. Out of 16 ESBL suspected isolates, 62.5% (n=10) were confirmed as ESBL producer. Among ESBL producer *E. coli*, *CTX-M* gene was detected in 70% (n=7) isolates. ESBL producing *E. coli* were absent in the samples collected from upstream sites (Sundarijal, Ghatte and Gokarna) and also *CTX-M* genes were absent among them (Table 3). There was not any observable linkage of physical parameters of water with isolation of ESBL *E. coli*.

Table 3. Site wise presence of ESBL producing *E. coli* and *CTX-M* gene in *E. coli* isolates

Sites	No. of *E. coli* isolates	No. of ESBL producer *E. coli*	No. of isolates with *CTX-M* gene
Sundarijal	0	0	0
Ghatte	0	0	0
Gokarna	1	0	0
Jorpati	2	1	0
Guheswori	3	1	1
Tilganga	3	1	1
Baneshwor	3	1	0
Sankhamul	3	1	1
Thapathali	3	1	1
Teku	3	1	0
Balkhu	3	1	1
Sundarighat	3	1	1
Chobhar	3	1	1
Total	**30**	**10 (33.3%)**	**7 (70%)**

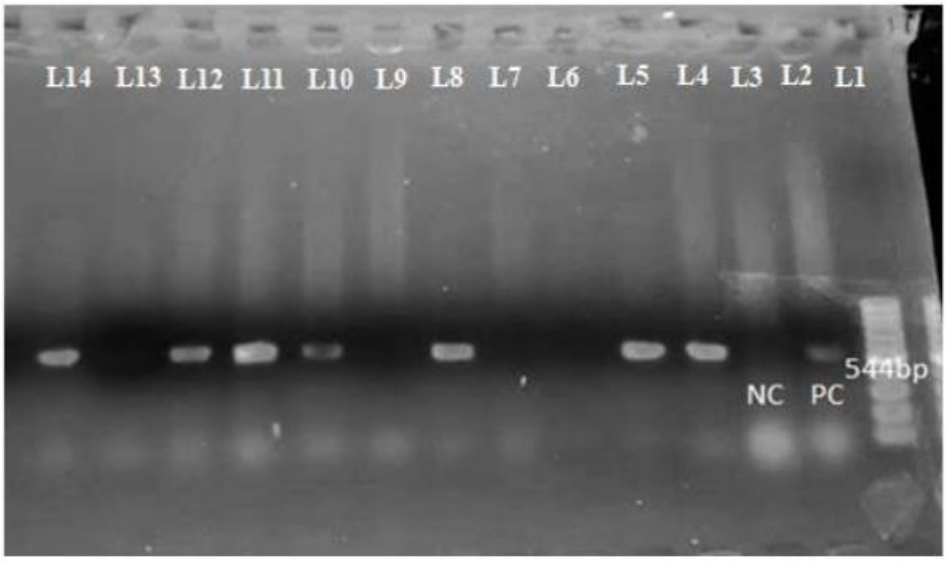

Figure 1. Gel photograph of 544 bp *CTX-M* gene from *E. coli* isolates from Bagmati River water; Lane L1: DNA marker (100-1000 bp), L2: positive control, L3: negative control, L4, L5, L8, L10, L11, L12 and L14: positive bands of *CTX-M* gene

DISCUSSION

Monitoring of river water quality help to understand pollution sources and to design effective mitigation measures. The wastewater treatment plant at Guheshwari is not functional and there is also discharge of wastewater directly from the communities in Bagmati River. Therefore, generating data on ARBs and ARGs could help to understand the extent of Bagmati River pollution and thus enforcing effective treatment of wastewater treatment before discharging into river.

In this study, we found a noticeable difference in river water temperature, the lowest 12°C in winter and the highest 26°C in summer. High temperature enhances microbial growth and activity. Higher temperature in water lowers the dissolved oxygen by enhancing chemical reactions and increasing biological oxygen demand (Chapra *et al.*, 2021). Due to increased amount of water during the monsoon season as compared to others, the Bagmati River water quality is said to be somewhat better (Mishra *et al.*, 2017). High water temperature can also worsen corrosion issues as well as taste, odour, and color as they promote the growth of organisms (Manga *et al.*, 2021). The pH of the Bagmati River was within the given pH standard similar to previous reports (Mahat *et al.*, 2020; Baniya *et al.*, 2019). The pH was relatively high in Shankhamul site which might be due to the high discharge of sewage into the river and presence of high ammonia in water (Soni *et al.*, 2022). The pH for the standard of industrial effluent

ranges from 5.5 to 9.0 (DOI, 2003), and the drinking water standard is 6.5 to 8.5 (National Drinking Water Quality Standard (NDWQS), 2005). River water pH is a significant physico-chemical parameter that has significant impact on all biochemical processes (Tadesse *et al.*, 2018).

The turbidity value of Bagmati River water samples were out of range as recommended by NDWQS 2005 (> 5 NTU). The minimum turbidity 3.5 NTU was observed in the case of the water sample from Sundarijal site and maximum 557 NTU was from Jorpati site. The high turbidity towards the downstream sites must be due to high sediment loading in the downstream. High turbidity provides food and shelter enhancing the growth of microorganisms (Cinque *et al.*, 2004). The turbidity-causing particles shield the bacteria, decreasing the effectiveness of water disinfection (Lechevallier *et al.*, 1981).

The microbiological analysis of the Bagmati River water indicated a high level of contamination with a variety of microorganisms similar to other reports (Shrestha *et al.*, 2018; Tandukar *et al.*, 2018; Ghimire *et al.*, 2024; Khanal *et al.*, 2024). The river water contaminates nearby groundwater supplies in addition to polluting the area around it. Chemical study has shown that ground water and surface water are interconnected, allowing for the interchange of pollutants between each water source (Bajracharya & Tamrakar, 2008). There is an increased risk of waterborne infections in the Kathmandu valley due to the same interconnectedness of river water and household ground water. Detection of *E. coli* in Bagmati River water indicates that water quality has declined because of urbanization, inefficient household management, and the unmanaged sewer system (Norman & Michel, 2009).

High numbers of *E. coli* were resistant towards ampicillin, sulphamethoxazole, tetracycline, cefixime and cefotaxime while susceptible to amikacin, gentamicin, chloramphenicol and ciprofloxacin. This type of resistance could be due to production of several beta-lactamase enzymes. In our study, around 90% of *E. coli* isolated from Bagmati River water were sensitive to amikacin. Antibiotics susceptibility pattern of *E. coli* showed that most of the isolates were multidrug resistant (MDR). High proportions of MDR bacteria have been reported from rivers receiving treated effluents and hospital sewage in China (Zhang *et al.*, 2020). Many enteric bacteria from feces that may carry ARGs may enter the water system through a variety of channels. ARB are therefore common in the environment as a result of the use of antibiotics, not only in humans but also in animals.

In this study, one third of *E. coli* were ESBL producers. Other studies reported comparatively very high ESBL rates in *E. coli* from river water from Ghana and Nigeria (Adelowo *et al.,* 2018; Banu *et al.*, 2021), India and Nicaragua (Amaya *et al.*, 2012), Bangladesh (Uddin *et al.*, 2019), but lower rates of ESBL from wastewater in South Africa (Nzima *et al.*, 2020). Nepal has reported a relatively higher percentage of ESBL prevalence in clinical samples (Sharma *et al.*, 2013; Rimal *et al.*, 2017) and Bagmati River water samples (Shrestha *et al.*, 2018; Tandukar *et al.*, 2018; Ghimire *et al.*, 2024) there might be circulation of bacteria between the environment and infections in human.

In this study, ESBL producer *E. coli* had predominantly *CTX-M* genes. *CTX-M* beta –lactamase enzyme is a broad-spectrum beta-lactamase that has been expanding in water such as India (Kaur & Aggarwal 2013; George *et al.*, 2015) and China (Zou *et al.*, 2019). The ability to transfer genetic material, a mutation in plasmid-mediated genes, and mobile resistance genes are all implicated in *CTX-M*. The mobile genetic material that may move resistance genes across DNA molecules either horizontally or vertically is responsible for antibiotic resistant bacteria in diverse environments (Koutsoumanis *et al.,* 2021).

The study has some limitations such as: we collected water samples only one time from different sites of Bagmati River and the season of sample collection varied for different sites. We did not have data on coliform count which limits the understanding of the level of water contamination. We used only *E. coli* for detection of ESBL and *CTX-M* gene. Monitoring of more bacteria in water and various ARGs can provide a broader picture of origin and transmission of antibiotic resistance.

CONCLUSIONS

E. coli isolates from Bagmati River were resistant to most of the commonly used antibiotics and more than half of the resistant isolates were MDR. The presence of *CTX-M* gene in significant number of ESBL producing *E. coli* in water may have ecological and public health implications. Therefore, wastewater from households and industries should be treated before discharging into the river. Monitoring of ESBL *E. coli* in river water and sufficient treatment of wastewater before discharging into river could help minimize the dissemination of *CTX-M* gene in environments, humans and animals.

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AUTHOR CONTRIBUTIONS

MN: designed the study, collected samples, performed the laboratory work, and analyzed data; MRB: designed the study, supervised the study, analyzed data and wrote, reviewed and finalized the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL STATEMENT

The study did not involve human participants and ethical approval was not obtained.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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