J Nepal Health Res Counc 2023 Oct.-Dec.; 21(61):672-79

Extended Spectrum Beta Lactamase *Escherichia coli* in Bagmati River, Kathmandu Valley

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ABSTRACT

Background: Antimicrobial resistance organisms in the peripheral communities of an environment can be predicted by the presence of extended-spectrum beta-lactamase *Escherichia coli* in that environment. The close connectivity between humans and water sources can facilitate the entry of antimicrobial resistant organisms into the human ecosystem. The aim of this study was to assess beta lactamase producing *Escherichia coli* from Bagmati river within Kathmandu valley.

Methods: In the year 2020, a cross-sectional study was conducted on water samples collected from 66 locations along the Bagmati River. Coliforms were isolated by five tubes dilution method and identified by cultural and biochemical tests. Further *Escherichia coli* was isolated in eosin methylene blue agar at 44.5 ^{0}C. Antibiotic susceptibility test was performed by Kirby Bauer disk diffusion methods. Beta lactamase gene types were detected by using conventional multiplex polymerase chain reaction.

Results: A total of 615 bacterial isolates were identified among which 39 % (n=241) were *Escherichia coli*. Extended spectrum beta lactamase producing *Escherichia coli* was confirmed in 16.6 % (40/241) of total *Escherichia coli* isolates. Among 66 sites this isolate was detected in 26 (40 %) sampling sites excluding upstream regions. All the *Escherichia coli* isolates were multidrug resistance showing higher percentage (>99 %) of resistant for penicillin, tetracycline and erythromycin antibiotics. There were significant differences in resistance rate for cefotaxime and ceftazidime by extended spectrum beta lactamase producing and non-producing *Escherichia coli* ($p<0.05$).

Conclusions: Presence of multidrug resistance extended spectrum beta lactamase producing *Escherichia coli* in river streams suggests the chances of circulating within river system and hence transmitting in human community.

Key words: Bagmati river; drug resistance; *Escherichia coli*; human.

INTRODUCTION

The Hindu Kush Himalayan region is considered as major source of drinking water for millions of people in south Asia. However, rapid urbanization has affected quality of water in many countries within this region.[1] The water is polluted with increased human settlement.[2] As a result of direct fecal deposition in the river sources, there is additive increment proportionality in the resistance group of organisms, resulting in variety of gene resistance variants.[3] The polluted water sources in the south Asian countries are recorded as contributors of the extended spectrum beta lactamase producing *Escherichia coli* (ESBL EC).[4] The drug resistance organisms in aquatic environment have propensity to spread infection between humans and animals. In the Kathmandu Valley, capital city of Nepal, the Bagmati River flows amidst populated settlements and is heavily polluted. Therefore, this study was conducted to determine the presence of *E. coli*, ESBL EC, its antibiotic resistance pattern and ESBL EC gene in the Bagmati river water of the Kathmandu valley.

METHODS

For this research, ethical acceptance was obtained from the National Health Research Council (NHRC) (Ref. No.: 1572), and permission for the collection of water samples was granted by the Department of National Parks and Wildlife Conservation, Government of Nepal (Ref. No.: 1018). A cross-sectional study was conducted along the Bagmati River in Kathmandu over a one-year period from January to December 2020. For this study, the segment of the Bagmati River was divided into upstream, midstream, and downstream sections. The section from Sundarijaal to Baghdwar, which encompasses the water

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source area of the Bagmati River, was designated as the upstream section. The stretch from Sundarijal to the confluence with the Manohara tributary was identified as the midstream of the Bagmati River. From this location to the Chobhar area, the section of Bagmati river is regarded as downstream (Figure 3). Additionally, sampling locations were chosen at the confluence points where tributaries join the Bagmati River. As per the World Health Organization's proposal for global integrated surveillance of ESBL EC, the sample size can be calculated using the following formula: sample size = 1 city × sampling sites × 3 rounds per year.[5] For this study, the sample size (n) was determined as follows: 1 (Kathmandu city) × 66 (sampling sites) × 3 (rounds per year) = 198.

For the river water sampling process, river samples were taken starting at the Bagmati river's source in Baghdwar and going all the way to the Chobhar, where the river empties out of the valley. Additionally, confluence points of tributaries in the Bagmati River were selected for sampling. Stratified type of sampling techniques was applied where the samples were collected purposively (homogenous) from the tributaries and conveniently from river source.[6] A total of 66 sites were categorized into 7 upstream, 37 midstream, 16 downstream locations, and 6 major tributaries. The coordinates of the sampling points are provided in Table 1 of supplementary material S1. Subsurface sampling was conducted by collecting grab samples in sterile glass bottles positioned beneath the surface at a depth of 15-20 cm, using a clamped stick. [1,7] All the sampling bottles were labelled. All the samples were transferred to the laboratory within 2 hours of sample collection in an icebox and processed immediately. Due to the distance of the sampling sites and transportation feasibility, some samples were refrigerated at 4°C for 24 hours before processing in the laboratory.[8]

The water samples were processed using the most probable number (MPN) count method. The volume of 10, 1, 0.1, 0.001 and 0.0001 mL of water sample were prepared.[9] For the presumptive identification of an organism, 10 mL double dilution lactose broth (5 tubes) and 5 mL single dilution lactose broth (10 tubes) were used which were incubated at 37 ºC for 48 hours. Then, lactose broth showing positive growth of organism was processed for confirmatory testing. Confirmatory testing was done by using brilliant green lactose bile broth (BGLB). From the positive BGLB broth 0.1 mL of inoculum was plated on eosin methylene blue (EMB) agar and incubated at 37 ºC and 44.5 ºC for 24 hours. The representative colonies were taken for identification. Gram's stain, enzymatic test (catalase and oxidase) and biochemical tests (indole, methyl red, Voges Proskauer, citrate, urease, fermentative test) were used for the identification of coliforms.

Antibiotic susceptibility testing was done for *E. coli* isolates, by using the clinical and laboratory standard institute guidelines and Kirby and Mueller methods on the Muller Hinton agar plates.[10,11] A panel of 17 different antibiotics was tested for the *E. coli* which included into eight different categories of antibiotics. Multi drug resistant (MDR) category was allocated as given by Wolfensberger, 2019.[12] All the *E. coli* isolates showing resistant towards cefotaxime and ceftazidime were processed for ESBL confirmatory testing using combination disc method as indicated in CLSI guidelines using Muller Hinton agar.[10] The dose of an antibiotic is tabulated in Table 2 in supplementary material S2. ATCC 25922 and ATCC 760023 were taken as the ESBL negative and positive control strain. All the instruments in the lab were calibrated regularly.

For the molecular detection of the ESBL EC gene types, DNA was extracted by using Spin Star™ total DNA extraction kit (ADT, Biotech). For this 1mL of ESBL positive *E. coli* culture was prepared on Luria Bertani broth with 24 hours incubation. Cica Geneus™ ESBL genotype detection kit2 (Kanto chemical Tokyo, Japan) was used for multiplex polymerase chain reaction (PCR). The protocol provided in the kit was followed for preparing the PCR reaction mixture and running the PCR cycles. The PCR products were subjected to electrophoresis as instruction provided in the kit and visualized using gel doc system. The band size of the sample was compared with the 100 base pair DNA ladder and positive control. For the negative control, PCR reaction mixture was run without DNA extract.

All the observed results were recorded in daily log book in the laboratory. The data were entered and analyzed in Statistical Package for Social Science version 21.0. The Chi square test was applied to determine the significant association between the *E. coli* and ESBL EC isolates from different river streams. The test was regarded significant at $p < 0.05$, 95% confidence interval. R software version 4.2.1 was used to prepare the Venn diagram and Euler diagram.

RESULTS

Altogether 615 total coliforms were isolated from Bagmati river water samples. A total of 615 coliforms were identified, with 47, 338, 158, and 72 isolates of

coliforms segregated from the upstream, midstream, downstream, and tributaries of the Bagmati river segment respectively.

Among the total coliforms, 374 were other coliforms, 201 were *E. coli*, and 40 were ESBL EC (Figure 1).

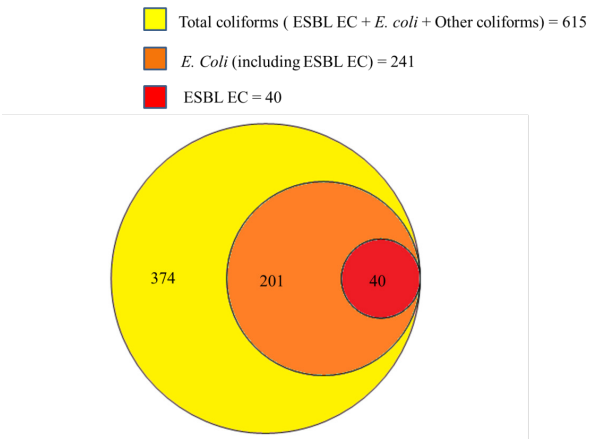

Figure 1. Prevalence of ESBL EC and E. coli within isolated total coliforms.

Other coliforms represented 85 %, 58.6 %, 60.7 % and 56 % in upstream, midstream, downstream and tributaries respectively. *E. coli* represented nearly 15 %, 37 %, 30.3 % and 29 % in upstream, midstream, downstream and tributaries respectively. While ESBL EC was not detected in upstream water sample however, ESBL EC represented 4.4 %, 9 % and 15 % of total coliforms in midstream, downstream and tributaries (Figure 2).

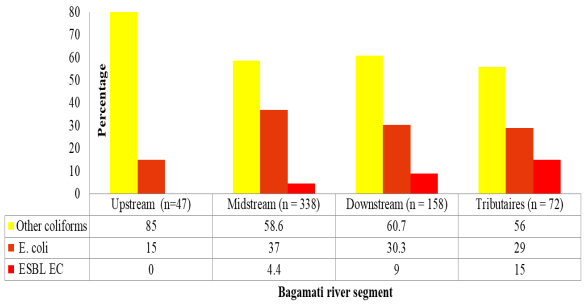

Figure 2. Prevalence of isolates in different segments of Bagmati river.

All the samples collected from 66 water sampling sites showed the presence of *E. coli* together with other coliforms except from the 2 (3 %) sites located in the upstream. ESBL EC was isolated from the lower ends of the midstream to downstream. All the tributaries showed presence of ESBL EC. ESBL EC was detected from 26 (39.4 %) of water sampling sites (Figure 3).

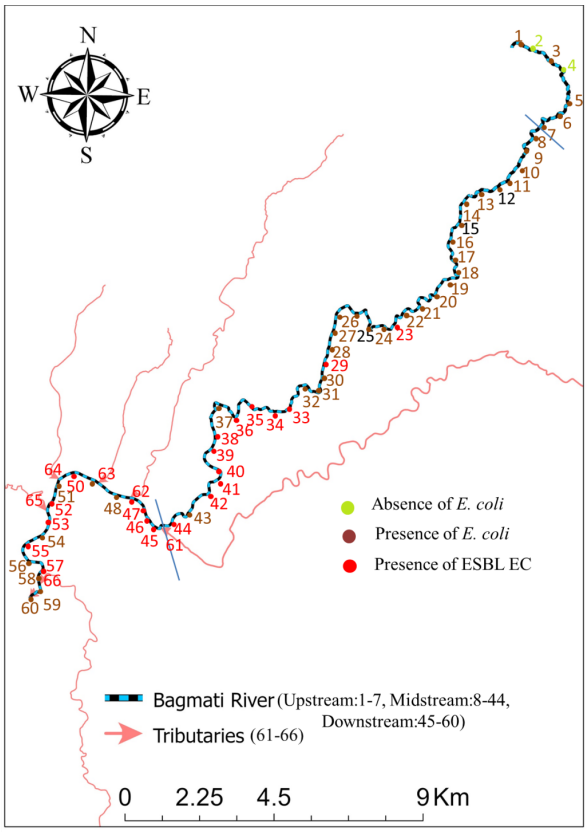

Figure 3. Map showing the sites with the presence of E. coli and ESBL EC.

All the *E. coli* isolates (N = 241) were multidrug resistance. More than 95 % of the isolates were highly resistant towards penicillin group of an antibiotic, erythromycin and tetracycline. The isolate was least resistant towards imipenem (11.2 %) and chloramphenicol (15.4 %) antibiotics. The resistance against cephalosporins ranged from 47 % - 78 %. There were no significant differences in the antibiotic resistance percentages of the *E. coli* isolated from different water sources ($p<0.05$) (Table 1).

Table 1. Antibiotic resistant pattern of *E. coli* in different water streams.

Water stream	Upstream	Midstream	Downstream	Tributaries	Total	p value
	n = 7	n = 140	n = 62	n = 32	N=241	
Antibiotic	n (%)	n (%)	n (%)	n (%)	N (%)	
Nitrofurantoin	2(28.6)	51(36.4)	20(32.3)	16(50)	89(36.9)	0.287
Tetracycline	5(71.4)	140(100)	59(95.2)	32(100)	236(97.9)	0.382
Erythromycin	6(85.7)	140(100)	61(98.4)	32(100)	239(99.2)	0.356
Amikacin	4(57.1)	47(33.6)	27(43.5)	7(21.9)	85(35.3)	0.177
Ciprofloxacin	2(28.6)	30(21.4)	16(25.8)	3(9.4)	51(21.2)	0.282
Pipericillin	7(100)	140(100)	60(96.8)	32(100)	239(99.2)	0.344
Ampicillin	6(85.7)	140(100)	60(96.8)	32(100)	239(99.2)	0.344
Chloramphenicol	2(28.6)	17(12.1)	14(22.6)	4(12.5)	37(15.4)	0.684
Amoxicillin Clavulanic acid	7(100)	140(100)	61(98.4)	32(100)	240(99.6)	0.504
Pipericllin Tazobactam	5(71.4)	88(62.9)	29(46.8)	26(81.3)	148(61.4)	0.611
Cotrimoxazole	3(42.9)	33(23.6)	24(38.7)	7(21.9)	67(27.8)	0.716
Nalidixic acid	1(14.3)	41(29.3)	29(46.8)	8(25)	79(32.8)	0.367
Imipenem	1(14.3)	16(11.4)	5(8.1)	5(15.6)	27(11.2)	0.857
Cefixime	3(42.9)	117(83.6)	40(64.5)	27(84.4)	187(77.6)	0.786
Cefepime	3(42.9)	106(75.7)	40(64.5)	28(87.5)	177(73.4)	0.282
Ceftazidime	2(28.6)	68(48.6)	27(43.5)	16(50)	113(46.9)	0.838
Cefotaxime	2(28.6)	69(49.3)	25(40.3)	17(53.1)	113(46.9)	0.838

The *E. coli* and ESBL EC isolates from different water streams showed varied percentages of resistance for 17 different types of antibiotics so tested. But there were no significant differences in the resistance percentage of antibiotics in each water stream. The resistant rate differed significantly for cefotaxime and ceftazidime antibiotics only (Table 2).

Table 2. Association between the pattern of antibiotic resistance in *E. coli* and ESBL EC from various water sources.

Antibiotics	Midstream (N=140)		p value	Downstream (N=62)		p value	Tributaries (N=32)		p value
	E. coli	ESBL EC		*E. coli*	ESBL EC		*E. coli*	ESBL EC	
	n (%)			n (%)			n (%)		
Nitrofurantoin	42(33.6)	9(60)	0.052	16(33.3)	4(28.6)	0.739	11(52.4)	5(45.5)	0.714
Tetracycline	125(100)	15(100)	-	46(95.8)	13(92.9)	0.651	21(100)	11(100)	-
Erythromycin	125(100)	15(100)	-	47(97.9)	14(100)	0.589	21(100)	11(100)	-
Amikacin	42(33.6)	5(33.3)	0.984	23(47.9)	4(28.6)	0.235	5(23.8)	2(18.2)	0.707
Ciprofloxacin	27(21.6)	3(20)	0.887	13(27.1)	3(21.4)	0.673	0(0)	3(27.3)	0.533
Pipericillin	125(100)	15(100)	-	46(95.8)	14(100)	0.441	21(100)	11(100)	-
Ampicillin	125(100)	15(100)	-	46(95.8)	14(100)	0.441	21(100)	11(100)	-
Chloramphenicol	14(11.2)	3(20)	0.326	13(27.1)	1(7.1)	0.119	4(19)	0(0)	0.228
Amoxicillin Clavulanic acid	125(100)	15(100)	-	47(97.9)	14(100)	0.589	21(100)	11(100)	-
Pipericillin Tazobactam	81(64.8)	7(46.7)	0.257	22(45.8)	7(50)	1	15(71.4)	11(100)	0.228
Cotrimoxazole	31(24.8)	2(13.3)	0.325	19(39.6)	5(35.7)	1	4(19)	3(27.3)	0.349
Nalidixic acid	36(28.8)	5(33.3)	0.716	22(45.8)	7(50)	1	3(14.3)	5(45.5)	0.491
Imipenem	13(10.4)	3(20)	0.271	3(6.3)	2(14.3)	0.335	5(23.8)	0(0)	0.193
CefIxime	103(82.4)	14(93.3)	0.282	30(62.5)	10(71.4)	0.542	17(81)	10(90.9)	0.9
Cefepime	92(73.6)	14(93.3)	0.093	30(62.5)	10(71.4)	0.752	19(90.5)	9(81.8)	0.489
Ceftazidime	53(42.4)	15(100)	0.0001	13(27.1)	14(100)	0.0001	5(23.8)	11(100)	>0.001
Cefotaxime	54(43.2)	15(100	0.0001	11(22.9)	14(100)	0.0001	6(28.6)	11(100)	>0.001

The significant association was observed for the antibiotic resistant pattern of *E. coli* and ESBL EC for 15 different antibiotics so tested except cefotaxime and ceftazidime at $p<0.05$, 95% CI.

From a total of 40 ESBL EC isolated, *CTX-M1* gene type was expressed by 38 (95%) isolates, whereas; *TEM* and *SHV* gene type was expressed in 30% and 25% of isolates respectively. ESBL genes were present either singly or conjunctly with each other. *CTX-M1* was present solely in 21 isolates and the combination with *TEM* was seen in 9 isolates. *CTX-M1*, *TEM* and *SHV* genes were present in 3 isolates whereas *CTX-M1* and *SHV* gene were present in 5 isolates. *SHV* gene was solely present in 2 isolates. *TEM* gene was not expressed solely by the ESBL EC isolates (Figure 4).

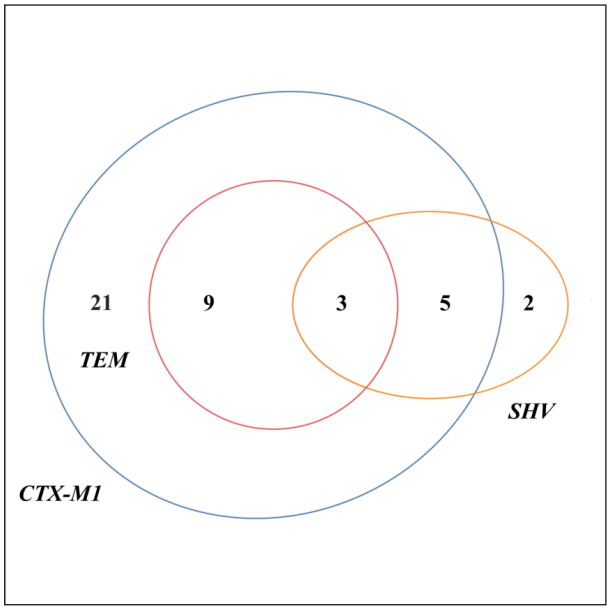

Figure 4. Euler diagram showing the combination of ESBL gene in ESBL EC.

DISCUSSION

The result of the study conducted along the major streams of the Bagmati River and its tributaries showed the presence of *E. coli*, including *E. coli* ESBLs. Fecal contamination of the river water sample is indicated by the presence of fecal indicator bacteria, *E. coli*, in the water either through human or animal source.[13] Whereas, presence of ESBL EC represents antibiotic resistant organism in water sources.[14] The main cause of the presence of fecal indicator bacteria is the direct discharge of raw sewage, open defecation and solid waste dumping along the Bagmati river banks.[2]

Although in lesser numbers, *E. coli* has been detected from the Bagmati river upstream. The upstream portion of the watercourse has a low level of anthropogenic contamination, which accounts for the difference in the microbial load. The Bagmati River's upstream section is contained in the Shivpuri National Park. However, trekking and hiking are permitted in the area. In these locations, open defecation is widespread. Fecal contamination from humans or animals could be the cause of the *E. coli* isolation.[15] The existence of coliform signifies pollution in the immediate environment.[16]

In our study higher load of ESBL EC had been detected from tributaries. The total microbial population density is impacted by the varying volumes of water that tributaries provide to the main stream, together with the differing amounts of organic and inorganic loading that they carry. Unplanned management, non-implementation of policies and unfair political activities are practiced in the country.[17] Since 2013, the Bagmati River Basin Improvement Project has been underway; however, the deterioration of water quality and erosion of river banks have not yet been effectively addressed.[18] Compared to the other watercourses, the tributaries possess higher levels of hardness, dissolved materials and components.[19] Massive biological and chemical components that work together to form a stressed zone in contaminated water encourage the development of bacterial resistance and an organism's ability to produce ESBL.[20]

In our investigation, every *E. coli* isolate found was multidrug resistant (MDR), with over 95 % of the isolates being resistant to the medication erythromycin, tetracycline, and penicillin. It has been recognized that the contaminated water catchment areas constitute a threat to the MDR organism's persistence, primarily to the ESBL Enterobacteriaceae.[21] The use of beta lactam and beta lactam penicillin in combination with other drugs increased from 34 % to 54 % in Nepal between 2003 and 2019.[22] The valley contains the numerous health care facilities including numbers of public and private tertiary care hospitals. Kathmandu's population density is also increasing annually.[23] The antibiotic residue in the water sources had risen due to the direct discharge of hospital and community waste into the river system.[24]

The pollution of river sources with dense population in lower streams of the Bagmati river is the major contributing factor for the abundance of *E. coli* and ESBL EC in these streams. The unaware use of antibiotics, over the counter availability of the drugs and its massive use in food production system are the main contributing factors for development and increase of AMR population in water system.[25] It has been suggested to treat multidrug-resistant ESBL EC that exhibits carbapenem resistance with cefiderocol alone or in combination

with imipenem vabrobactam and meropenem cliastin relebactam.[26]

The majority (95 %) of the genes detected for ESBL EC were of the *CTX-M-1* type. Also present alone and in combination with *CTX-M-1* were *TEM* and *SHV* genes in our study. The ESBL production in *E. coli* is largely contributed by *CTX-M-1*, *TEM* and *SHV* enzymes, where *CTX-M-1* is predominant in human and animal infection.[27] It is well known that *E. coli* can enter an aquatic ecosystem from humans and animals. AMR develops in contaminated water as a result of the direct release of sewage, feces, and waste from hospitals, businesses, and pharmaceuticals. This pressure zone is subsequently employed by organisms to generate AMR gene complexes.[28]

The detection of approximately 10-12 % of imipenem-resistant *E. coli* in the study indicates the spread of carbapenem resistance *E. coli* in Bagmati river. The polluted water source is considered to be the explicit source of acquisition and transfer of resistance genes within the bacterial community.[29] Therefore, it might have consequences to spread of carbapenem antibiotic resistance through water. Growing crops along the riverbanks, using the river's water for irrigation, sacred practices, and human settlements near the river are all evident. *E. coli* adapts and naturalizes in different types of environments. AMR bacteria enter the human population through close contact with polluted water sources.[30] *E. coli* are known to access different internal and external parts of the plant.[31] Direct entry of the organism into the human population via the food and water chain is also possible if consumed raw or handled improperly.[31,32]

CONCLUSIONS

This study revealed that ESBL EC which is known as prioritized pathogen type, is found to be ubiquitously distributed in Bagmati river. Considering the close association between humans and the water of the Bagmati River, there is a possibility that ESBL EC could be prevalent in the communities living alongside the river. It is essential to assess the nearby human settlement to understand ESBL EC prevalence and curb the spread of drug-resistant organisms. For minimizing the transmission of ESBL EC from river water source into human and animal population, one health practice in pollution reduction, waste water management, and frequent molecular detection of *E. coli* gene types is recommended.

ACKNOWLEDGEMENT

We would like to thank University Grants Commission, Nepal for their funding to complete this research work (Ph. D 77/78-S &T 13).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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