Antimicrobial Resistance in *Escherichia coli* and other Coliform Bacteria Isolated from Bagmati River

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ABSTRACT

Objectives: To explore the presence of fecal indicator bacteria and assess antibiotic resistance status in Bagmati river water.

Methods: In a cross sectional study during a year 2020; a total of 180 water samples were collected from the Bagmati River's upstream, midstream, and downstream sources. Maintaining reverse cold chain, the samples were transferred to the laboratory of central department of Microbiology, Tribhuvan University. Organisms were isolated on Eosin Methylene Blue (EMB) and Nutrient Agar. The organisms were further identified based on the biochemical properties and antibiotic susceptibility testing was performed following CLSI (2020) guidelines.

Results: Of the 546 isolates, 209 (or 38%) were *Escherichia coli*. Other coliforms isolated were *Enterobacter* spp (2%), *Citrobacte*r spp (37%), and *Klebsiella* spp (23%). Upstream source revealed least percentage 3% (7/209) of *E. coli*. All the recovered *Citrobacter* spp. were resistant and *E. coli* showed >99% resistance towards Tetracycline, Ampicillin and Amoxiclav antibiotics. *Klebsiella* spp. was 100% resistant towards Ampicillin and Amoxiclav antibiotics. The coliforms exhibited least resistance (10%) towards Chloramphenicol. Based on antibiotic resistance percentage pattern, *E. coli* showed 27% similarity to *Citrobacter* spp.

Conclusion: Coliforms showed maximum resistance towards first line antibiotics prescribed in human infection. Immediate water safety plans should be instituted to improve the water quality.

Key words: Bagmati river, *E. coli*, Antibiotic resistant

INTRODUCTION

The necessity of water for life is essential. Water exists in numerous forms, nevertheless a mere one per cent of water sources are accessible to humans, whereas only around three percent of water sources are pure (Dinka 2018). One of the primary sources of drinking water in the Hindu Kush region, the river flows through a region of considerable importance. These regions' water supplies are beneficial to Nepal as well (Scott et al. 2019). Historic and religious significance is likely to be recognized in the Bagmati river, which travels through Nepal's capital city, Kathmandu (Platman 2023).

The water of the Bagmati River is vital to the people who live along its basin for a variety of uses. River water became contaminated as a result of increased urbanization and human settlement (Tamrakar & Parajuli 2019). Both the biotic and abiotic communities are harmed by the contaminated water (Singh et al. 2021). All forms of waste products, both liquid and solid, are found in the river. While there is open discharge of community sewers into the river system, main locations for rubbish disposal are along the riverbank (Mishra et al. 2017).

A significant source of microorganisms resistant to several antibiotics is the contaminated water (Kaiser et al. 2022). An organism develops antibiotic resistance when it can no longer be affected by the type and dosage

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of antibiotic employed against it. Antibiotic resistance poses a severe threat to human survival (Cesur & Demiröz 2013). The contaminated river water puts the microorganisms in a pressured environment, which helps them evolve resistance to different antibiotic forms (Taneja & Sharma 2019).

The majority of organisms found in the contaminated water sources are coliforms (Niyoyitungiye et al. 2020). An indicator bacterium for fecal contamination is *Escherichia coli* (Holcomb & Stewart 2020). The Bagmati river flows along inhabited areas in the Kathmandu valley. The residents in the area are connected to this water system, either directly or through an intermediary. The aim of this study was to characterize the antibiotic-resistant coliforms from the Bagmati River's headwaters, midstream, and downstream zones.

MATERIALS AND METHODS

Study area

The study was conducted along the Bagmati river flowing from its origin Baghdwar to Chobar, from where its leaves the valley. The segment of Bagmati river is shown in Figure 1.

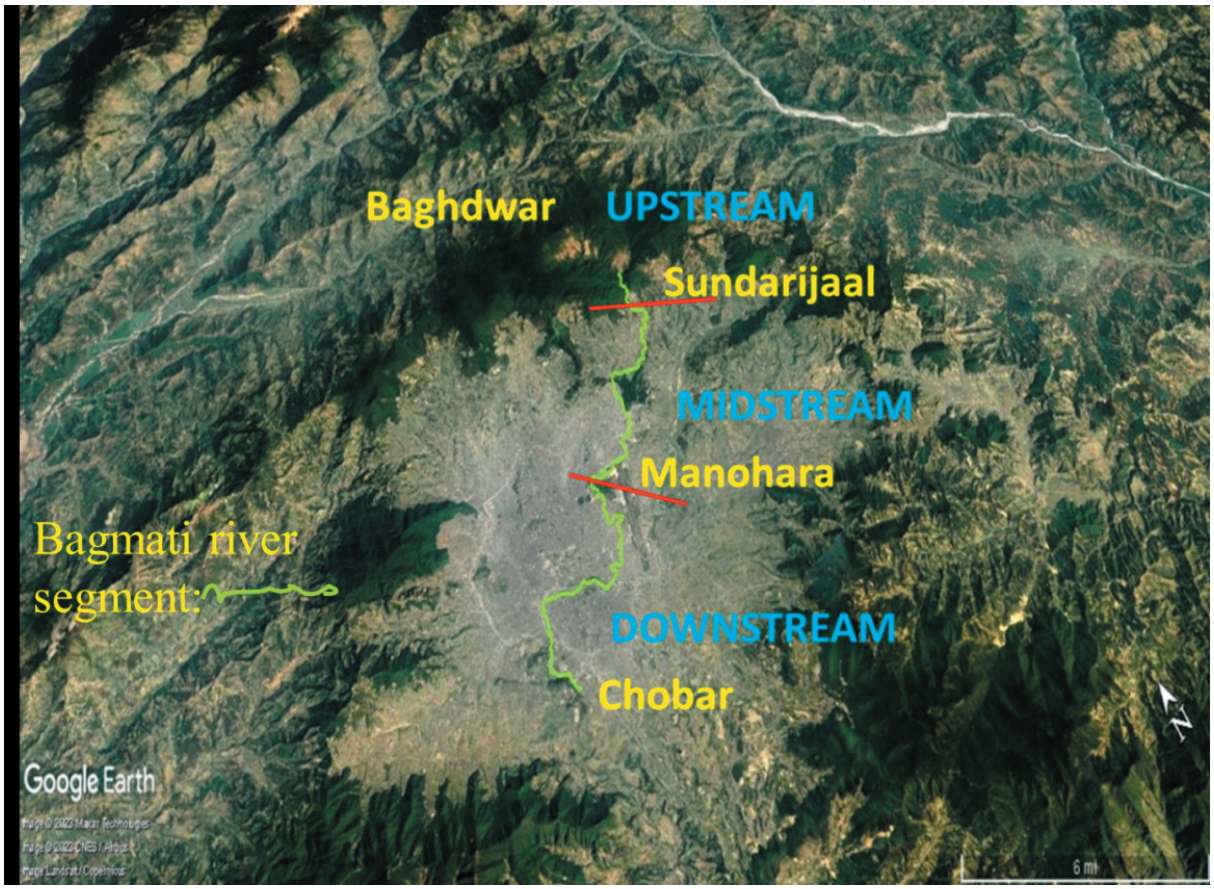

Figure 1: Map area of the Bagmati river basin showing upstream, midstream and downstream river segments.

Ethical consideration

The study received ethical approval from Nepal Health Research Council with ERB protocol number 936/2019. The permission for the water sample collection from the upstream of the Bagmati river was obtained from the Department of National Park and Wildlife Conservation (DNPWC) Ministry of Environment and Forest Conservation, Nepal with reference no: 1018/2020.

Sample collection

The grab sample collection technique was applied to collect the water from the subsurface area of the Bagmati River. The sample was collected from the convenient region of the Bagmati River (Murphy et al 2017). The Bagmati River flowing along Shivpuri National Park was regarded as the upstream area. From the Sundarijal outlet to the point where major tributary Manohara mixes with the Bagmati river was

considered as midstream and the area ascending from below the junction of Manohara and Bagmati upto Chobar is considered the downstream region.

Sample size

Triplicate samples were collected from the different segments of the Bagmati river. The sample size was calculated as: 1 city X sampling sites X 3 round a year (WHO 2023). Sample size=1 X 60 X 3=180.

Sample transportation

All the samples from midstream and downstream segments were collected in 300ml sterile BOD bottle and transported to Laboratory of Central Department of Microbiology in an ice box within two hours of sample collection (Saxena et al. 2011). All the samples were collected within 8:00am-10:00am.

Sample processing

The organisms were isolated by the completed test of the Most Probable Number Count method. The serial dilution of the sample was performed in lactose broth and Brilliant Green Lactose Bile Broth. From the BGLB broth, one loopfull of sample was transferred onto Eosin Methylene Blue Agar media. The isolates with different colony characteristics were streaked onto a nutrient agar plate (FDA 2023). The Gram's stain was performed. Organisms were identified from the nutrient agar plate through enzymatic testing (Catalase, Oxidase) and the panel of biochemical tests (Indole, Methyl Red, Voges Proskauer, Citrate, Oxidative/Fermentative, Triple Sugar Iron, and Urease) (Chauhan et al. 2017).

Antibiotic susceptibility testing

All the isolates were further tested for the antibiotic resistance pattern using Mueller-Hinton agar. A panel of 10 different antibiotics, comprising 17 different types, was used for AST testing. The antibiotics types used are Ampicillin (AMP, 10 µg), Amoxicillin clavulanic (AMC,50/10 µg), Pipericillin (PI, 100 µg), Pipericilin tazobactam (PIT,100/10 µg), Cefipime (CPM,30 µg), Cefixime (CFM,5 µg), Ceotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Imipenem (IPM, 10 µg), Amikacin (AK,30 µg), Tetracycline(TE, 3 µg), Ciprofloxacin (CIP, 5 µg), Nalidixic acid (NA,30 µg), Chloramphenicol (C,30 µg), Erythromycin (E,15 µg), Nitrofurantoin (NIT,300 µg), Co-Trimoxazolae (COT,25µg,1.25/23.75 µg). For quality control, the ATCC 25922 culture was used. The inoculation of the organism was prepared in normal saline and compared with 0.5 Mac Farland Standard. The inoculum was swabbed onto the MHA plate and let dry for 5 minutes before placing the antibiotic disc. For the 90-mm plate, five different antibiotics were inoculated. The plates were incubated for 18 hours, and the inhibition zone was measured with the scale. The inhibition zones were compared with the standard values. The antibiotics were tested following clinical laboratory standard guidelines (CLSI 2020).

Data analysis

All the results obtained from lab were entered onto Excel worksheet. The table and column diagram was prepared by using Microsoft Excel, 2010. The Chi square association test was calculated by using Statistical Package for Social Science (SPSS) version 21.0. The similarity map was constructed using R package 4.3.0.

RESULTS

Colony characteristics in Eosin Methylene Blue (EMB) agar media

From the 180 river water sample, through serial dilution in Brilliant Green Lactose Bile Broth (BGLB) and plating on Eosin Methylene Blue (EMB) agar media, the four colony types were recovered. Only the *E. coli* isolate possessed green metallic sheen. The colony types were distinguished on the basis of their morphological features (Table 1).

Table 1: Diversified colony morphology in Eosin Methylene Blue (EMB) agar

S.N.	Size (mm)	Colour	Metallic Sheen	Consistency	Margin	Configuration	Elevation
1.	2	Voilet	-	Mucoid	Smooth	Round	Flat
2.	4	Brown	-	Mucoid	Smooth	Round	Convex
3.	3	Green, Black centered	+	Mucoid	Smooth	Round	Flat
4.	4	Pink	-	Mucoid	Smooth	Round	Convex

Total coliforms identified from EMB agar media

A total of 546 isolates were recovered from EMB media, which were identified further by biochemical testing after plating on nutrient agar media. *Escherichia coli* and *Citrobacter* spp. were the highest isolates, accounting for 38% and 37%, respectively (Figure 2).

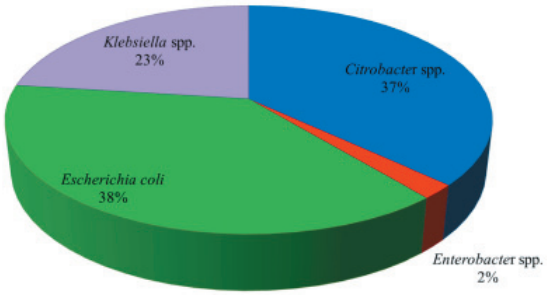

Figure 2: Distribution percentage of coliforms in different water samples

Distribution of coliforms in different water streams

The frequency distribution of isolates differed in different water stream. *E. coli* was least identified from upstream river water source accounting for 3.3 % of the isolates. All the coliforms were highly recovered from midstream water sample as shown in Table 2. In the Upstream water source *Klebsiella* (50%) was the highest isolates. On the other hand, *Citrobacter* spp. and *E. coli* outweighed other coliforms in the midstream and downstream. The percentage distribution of isolates in each river water stream is shown in Figure 3.

Table 2: Distribution of the bacterial isolates within river water streams

Isolates (N=546)	River water streams (n%)			Total
	Upstream	Midstream	Downstream	
Citrobacter spp.	13 (6.4)	125 (61.8)	64 (31.6)	202
Enterobacter spp.	5 (45.5)	3 (27.2)	3(27.2)	11
E. coli	7 (3.3)	140 (67)	62 (29.6)	209
Klebsiella spp.	25 (20.1)	70 (56.4)	29 (23.3)	124

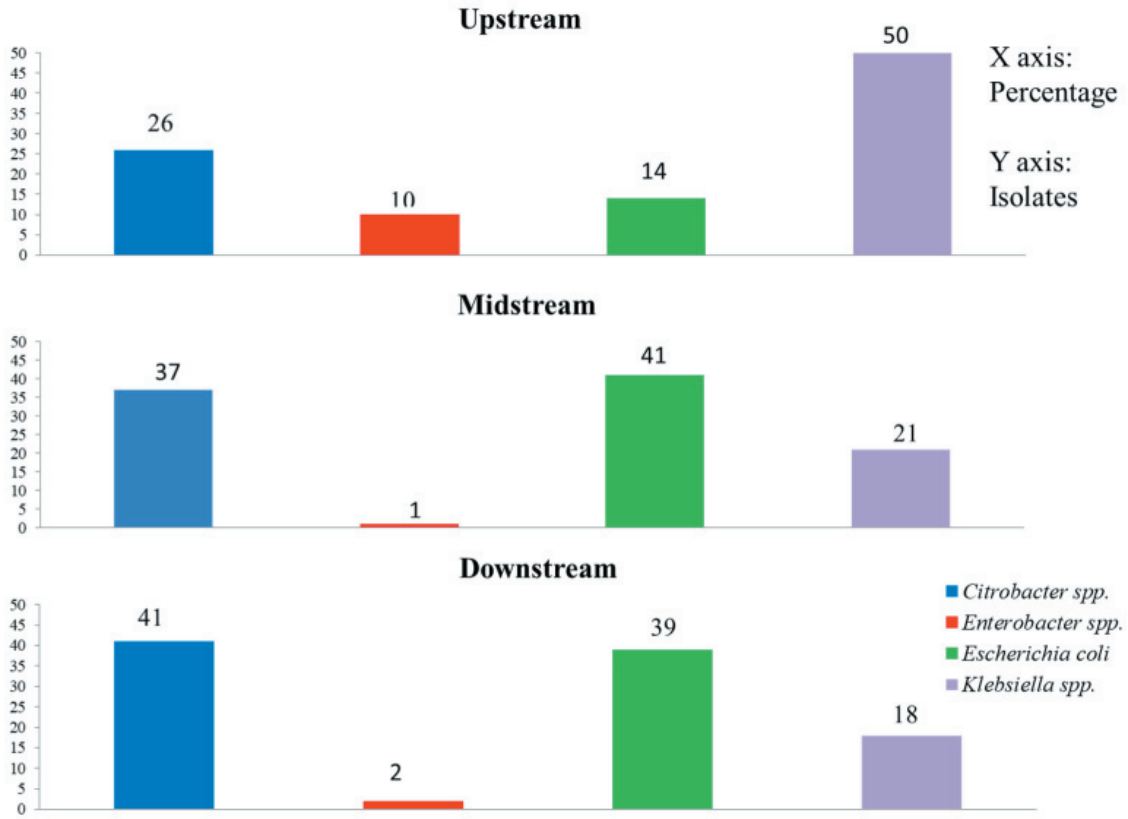

Figure 3: Distribution pattern of bacterial isolates in water streams

Antibiotic resistant pattern of coliforms

All the isolates were Multi Drug Resistant as tested against 17 different antibiotics within 13 categories. More than 95% of coliforms were resistance towards Amoxiclav (AMC), Ampicillin (AMP), Erythromycin (E) and Tetracycline (TE) antibiotic. The least resistant group of an antibiotic was Imipenem (13.7 %) and Chloramphenicol (10%). There was significant difference in antibiotic resistant pattern exhibited by coliforms for different antibiotics except Amoxiclav, Cefepime and Choramphenicol antibiotics (Table 3).

Table 3. Association of antibiotic resistance percentages in an isolates

Antibiotics	*Citrobacter* spp. (N%)		*E. coli* (N%)		*Enterobacter* spp. (N%)		*Klebsiella* spp. (N%)		Total (N%)		p value
NIT	43	21.3	68	32.5	6	54.5	59	47.6	176	32.2	0.0000
TE	202	100	206	98.6	10	90.9	115	92.7	533	97.6	0.0000
E	201	99.5	207	98	9	81.8	123	99.2	540	98.9	0.0320
AK	63	31.2	78	37.3	6	54.5	28	22.6	175	32	0.0150
CIP	42	20.8	48	23	7	63.6	21	16.9	118	21.6	0.0120
PI	202	100	207	99.9	8	72.7	121	97.6	538	98.5	0.0030
AMP	202	100	207	99	10	90.9	124	100	543	99.5	0.0480
C	26	12.9	33	15.8	4	36.4	13	10	76	13.9	0.1360
AMX/C	202	100	208	99.5	10	90.9	124	100	549	99.6	0.0700
PTZ	121	59.9	122	58.4	4	36.4	94	95.8	341	62.5	0.0020
COT	58	28.7	60	28.7	5	45.5	15	12.1	138	25.3	0.0000
NA	74	36.6	71	34	5	45.5	27	21.8	177	32.4	0.0230
IPM	14	6.9	22	10.5	6	54.5	17	13.7	59	10.8	0.0010
CFM	154	76.2	160	76.6	4	36.4	93	75	411	75.3	0.0520
CPM	125	61.9	153	73.2	7	63.6	106	85.5	391	71.6	0.0000
CAZ	72	35.6	97	46.4	5	45.5	40	32.3	215	39.4	0.0460
CTX	60	29.7	96	45.9	5	45.5	44	35.5	205	37.5	0.0070

Antibiotic resistance pattern of bacterial isolates in varied water streams

A significant association was observed for Ceftazidime, Cefipime, Cefixime, Tetracycline and Pipericillin Tazobactam antibiotics among the coliforms isolated from the upstream water sources. Also the coliforms exhibited variable pattern of resistance for Cefotaxime, Ceftazidime, Imipenem and Cotrimoxazole antiibotic isolated from downstream river water sources. Whereas, the resistance percentage differed for Ceftazidime, Cefepime, Imipenem, Amoxicillin and Tetracycline antibiotics for the coliforms of midstream river segments (Table 4).

Table 4. Antibiotic resistance pattern of coliforms in river streams

Stream	Up					Mid					Down				
Antibiotic/ Isolate	*Citrobacter* spp. (n=13)	*Enterobacter* spp. (n=5)	*E. coli* (n=57)	*Klebsiella* spp. (n=25)	p value	*Citrobacter* spp. (n=125)	*Enterobacter* spp. (n=3)	*E. coli* (n=140)	*Klebsiella* spp. (n=70)	p value	*Citrobacter* spp. (n=64)	*Enterobacter* spp. (n=3)	*E. coli* (n=62)	*Klebsiella* spp. (n=29)	p value
	%					%					%				
CTX	7.7	0	28.6	32	**0.116**	35.2	66.7	49.3	37.1	**0.077**	21.9	100	40.3	34.5	0.012
CAZ	0	0	28.6	48	0.001	43.2	66.7	48.6	34.3	**0.206**	28.1	100	43.5	13.8	0.001
CPM	69.2	40	100	100	0.001	60	66.7	75.7	88.6	0	64.1	100	64.5	65.5	**0.455**
CFM	84.6	20	42.9	76	0.024	83.2	33.3	83.6	81.4	**0.29**	60.9	66.7	64.5	58.6	**0.947**
IPM	15.4	20	14.3	20	**0.976**	7.2	66.6	11.4	12.9	**0.057**	4.7	100	8.1	10.3	0.001
NA	23.1	40	14.3	32	**0.697**	38.4	33.3	29.3	11.4	0.001	35.9	66.7	46.8	37.9	**0.485**
COT	24	20	80	24	**0.649**	28.8	33.3	23.6	5.7	0.001	26.6	100	38.7	17.2	0.007
PTZ	92.3	0	71.4	96	**0**	64.8	66.6	62.9	81.4	0.035	43.8	66.7	46.8	44.8	**0.88**
AMC	100	80	100	100	**0.187**	100	100	100	100	**0.144**	100	100	98.4	100	**0.598**
C	23.1	20	28.6	12	**0.713**	12	33.3	12.1	4.3	0	12.5	66.7	22.6	24	**0.106**
AMP	100	80	100	100	**0.187**	100	100	100	100	0	100	100	96.8	100	**0.286**
PI	100	40	100	92	**0.008**	100	100	100	100	0	100	100	96.8	96.6	**0.34**
CIP	30.8	60	28.6	28	**0.597**	21.6	66.7	21.4	7.1	0.007	17.2	66.7	25.8	31	**0.169**
AK	30.8	40	57.1	96	**0.205**	28.8	66.7	33.6	100	0	35.9	66.7	43.5	100	**0.598**
E	92.3	60	85.7	32	**0.646**	100	100	100	14.3	0.009	100	100	98.4	34.5	**0.578**
TE	100	80	100	76	0.047	100	100	100	98.6	**0.368**	100	100	95.2	93.1	**0.122**
NIT	15.4	40	42.9	68	**0.015**	22.4	66.7	36.4	45.7	0.003	20.3	66.7	22.6	34.5	**0.206**

Similarity matrix of the coliforms

The isolates were divided into three clusters based on the antibiotic resistance pattern of the isolates. The similarity map of the coliforms revealed the least distance measure of 26.7 for *Citrobacter* spp. and *E. coli*. Also *E. coli* measured the distance of 53.4 and 92.7 for the *Klebsiella* spp. and *Enterobacter* spp. respectively (Figure 5).

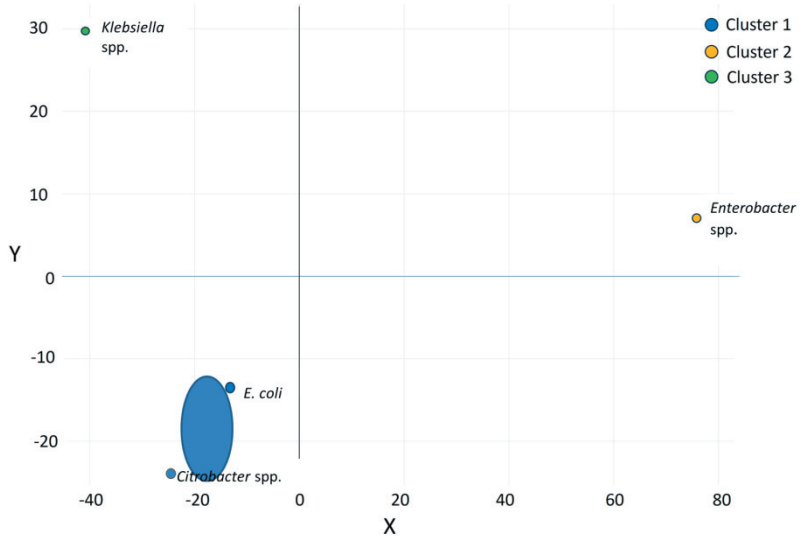

Figure 5: Similarity map of coliforms based on similarity index

DISCUSSION

The primary cause of the declining amount of potable water sources is contamination in river water. There are several domestic, agricultural, and industrial uses for the river's water. However, the direct release of sewage and other wastes (chemical, biological, and physical) has rendered the river water unfit for human consumption (Babuji et al. 2023). River contamination in the Kathmandu Valley has been escalating as a consequence of growing populations and city-centered development. The primary sources for hazards in urbanization processes are river watersheds. Waste disposal presently takes place in the valley beside the revered Bagmati River (Mishra et al. 2017).

This study found the presence of coliforms from the upstream to the downstream sources. The coliforms were highly present in downstream and midstream river water sources as compared to upstream river water sources. The Bagmati River pollution is severe as it passes by human settlements (Giri et al. 2022). The Shivpuri National Park and Wildlife Conservation Region, which is located upstream of the Bagmati River, provides limited access for the valley's populace (GoN 2023). An extensive network of river sources is affected by the growing quantity of contaminating microorganisms that accompany pollution (Islam et al. 2015).

E. coli is outnumbered in downstream and midstream water sources as compared to upstream water sources. The presence of *E. coli* in the river water sources indicates fecal contamination of the water sources. The presence of other coliforms such as *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. is suggestive of soil contamination in the river water (Patel et al. 2014). As the Bagmati River flows along the Shivpuri National Parks, there are probable chances of the coliforms in the surface water. The presence of coliforms further suggests the presence of infectious organisms in the river water sources. The high recovery of *E. coli* from the midstream and downstream river water sources is due to the direct discharge of the sewer into the river system (Amirat et al. 2012).

The coliforms isolated from the river water sources showed a varied resistance pattern to the different antibiotics tested. Here, the intermediate and resistant types were categorized into a single resistant group. However, the resistance percentages were similar for Amoxiclav, Cefepime, and Chloramphenicol antibiotics. More than 95% of the coliforms were resistant to tetracycline, erythromycin, pipericillin, ampicillin, and amoxiclav antibiotics. The coliforms exhibited high resistance (>99%) to ampicillin antibiotics. The

least resistance was shown for chloramphenicol (14%) and imipenem (11%) antibiotics. All the isolates were multi-drug resistant, showing resistance to three or more drug categories, as were tested in the laboratory (Wolfensberger et al. 2019).

Similar to our study, high percentages of resistance were shown for Cefotaxime, Ciprofloxacin, Erythromycin, Cotrimoxazole, and Tetracycline antibiotics and least towards Carbapenem by coliforms was shown in research conducted by Ho et al. in 2021. The development of bacterial resistance to antibiotics in the river water ecosystem is largely influenced by a number of factors, including improper residential settlement near riverbanks, insufficient waste water management, irrational antibiotic consumption and direct disposal into the river, and fewer waste management guidelines (Mishra et al. 2018). The pattern of antibiotic resistance displayed by *E. coli* and other coliforms in river water from upstream, midstream, and downstream was found to differ significantly. The adaptive response of the organism to geophysical differences, land use pattern, sediment load of tributaries, use of water in the upstream region with less flow towards downstream, and pollution introduced by increased population towards downstream locations can all contribute to the differences in the AMR paradigm towards different antibiotics (Yoon et al. 2015).

The study showed *E. coli* was closely related to *Citrobacter* spp. *Citrobacter* spp. bears genetic adjacency to *E. coli* (Qin et al. 2021). The antibiotic-resistant coliforms serve as a cenote for the antibiotic resistance gene. These genes can easily be acquired by the infectious entity, which creates a threat to the human community (Hartinger et al. 2021). The residents who initially reside within the Kathmandu Valley are in close proximity to the contaminated water of the Bagmati River. In the event that prompt monitoring and pollution mitigation strategies are not implemented, the AMR bacteria can readily spread to the areas nearby.

CONCLUSIONS

The waterways of the Bagmati River are inundated with coliform bacteria. From all of the Bagmati River's water sources, the fecal indicator bacteria, *E. coli*, was found. *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp. species were found in addition to *E. coli*. These coliforms exhibited a high level of resistance to the antibiotic group Penicillin. The delineation of antibiotic resistance pattern shares similarities between *E. coli* and *Citrobacter* spp. AMR organisms pose a concern to the human community because they facilitate the easy spread of AMR bacteria through contaminated water sources.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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