Final report of small research grant project on

“Phylogrouping and Antibiogram of *Escherichia coli* from river water sample”

Submitted by

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Preface

This final project report has been prepared as a part of the Small Research Grant (Dean Grant 2080/081) funded by Dean office, Institute of Science and Technology, Tribhuvan University in the year 2080/81. This was a 6-month project. In this project, microbiological analysis of water was done and the bacteria isolated was subjected to antimicrobial susceptibility testing. Amplification of specific gene sequence of DNA was done using specific primer and then their phylogroup were identified. This study was conducted to determine the status of thermotolerant *E. coli* of fecal origin in the Bagmati river from entry to exit area and characterize them based on antimicrobial resistance and phylogroup.

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Declaration

This final project report entitled **"Phylogrouping and Antibiogram of *Escherichia coli* from river water sample"** is being submitted to the Dean Office, Institute of Science and Technology (IOST), Tribhuvan University as a requirement of Dean Research grant of the year 2080/2081. This project was carried out by **Ms. Suchitra Thapa** under my supervision. I hereby approve this final report and ensure that this work is original and has not been submitted earlier in part or full in any institution or elsewhere.

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Most importantly this project would not have been possible without the guidance of Dr. Dev Raj Joshi who has been guiding me from the very beginning of this study. Further, I would like to acknowledge Dr. Tista Prasai Joshi for her continuous suggestions for the improvement of the project. Also, it is necessary to mention the persistent support and encouragement of our Campus Chief Dr. Lok Bahadur Baral, Amrit Campus and all the administrative staff of Amrit Campus. Besides, I would like to acknowledge our colleagues and laboratory staff of department of Microbiology, Amrit Campus for their constant and unbiased support in this research study. Additionally, it is utmost necessary to acknowledge the students (Mr. Umesh, Ms. Diya and Ms. Sushmita) involved in this project for their hard work throughout the project. Finally, I would like to acknowledge all the person who directly or indirectly supported for this research work.

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Summary

Water borne diseases like diarrheal infection caused by *E. coli* is a serious threat to public health. However, in Nepal there are no clear data on characterization of these *E. coli* based on their virulence or antimicrobial resistance. And phylogrouping will help to track the microbial source and help us understand the route of transmission. Also, antibiotic resistant profile will help to design a better treatment regime for the patients. Therefore, this study aims to characterize the diarrheagenic *E. coli* that are prevalent in the water sources and gauge their transmission potential to humans. For this *E. coli* was isolated and identified from water sample following the conventional culture techniques and enumerated by standard membrane filtration method. Further antibiotic susceptibility was determined by disc diffusion method towards different classes of antibiotics that are used in clinical settings following the procedure recommended by CLSI. Further, gene detection was done by PCR using specific primers for phylogroup. The result was then analyzed using SPSS and WHONET software. Descriptive analysis as well as inferential analysis was done to fulfill the objective of the study. The result revealed a high occurrence of thermotolerant *E. coli* of phylogroup B1 (58.2%) in Bagmati river which is commensal in nature and originates from either human or animals. Also, isolates conferring resistance to multiple antibiotics (n= 6) were detected along with high priority isolates (n=11). Thus, circulation of such high risk isolates in aquatic settings of Nepal is concerning so a comprehensive study of such isolates should be done at genomic level so that to better understand their transmission potential and intervene their transmission.

Keywords: E. coli, Diarrhea, Pathotype, Phylogroup, Antibiotic resistance

CHAPTER 1: Introduction

1. INTRODUCTION

1.1. Background of the study

Fecal contamination in water sources are very common in developing countries where proper drainage system is poorly managed. Since *E. coli* inhabits the digestive tract of both animals and humans, their presence in water is indicative of fecal contamination (Ishii et al 2008) Several outbreaks related to exposure to contaminated water are well documented (WHO 2017) and *E. coli*, apparently are the primary cause in most diarrheal cases (Kaper et al 2004). However, not all *E. coli* strains are pathogenic to human (Ishii et al 2008). The pathogenic nature is attributed by the prevalence of virulent traits that they have acquired and this acquired trait render them capability to cause diseases in human (Sidhu et al 2013, Kuhnert et al 2000). According to WHO 2017 report it is estimated that nearly 1.7 billion cases and 0.5 million deaths of children under 5 age group are caused by diarrheal disease every year (WHO 2017). Among several pathogens, the enteric strain of diarrheagenic *E. coli* are considered the most important because of their public health relevance (Kaper et al 2004). And they are the major cause of morbidity and mortality among children in developing countries (WHO 2014, Kaper et al 2004) like Nepal. Specifically, traveler diarrhea and acute infant diarrhea is the leading reason for morbidity and mortality among diarrheal cases in Nepal (Shrestha et al 2022). Normally, the diarrheagenic *E. coli* are categorized into pathotypes based on the prevalence of virulence attributes and this attribute is conferred by the presence of specific virulence gene (Sidhu et al 2013, Kaper et al 2004). Many studies have shown that diarrheagenic *E. coli* contains one or more virulence gene (Sidhu et al 2013). The presence of such strains in water and wastewater environment is not only indicative of circulating pathogen in the community but also a potential hazard of possible outbreaks. However, the status of diarrheagenic *E. coli* in environmental matrix is largely elusive in Nepal but very few studies have been done in Nepal.

Antimicrobial resistance is a global threat and their prevalence within diarrheagenic *E. coli* will exaggerates the problem by limiting the therapeutic options. And reportedly, AMR among diarrheal *E. coli*, has been reported to be continuously emerging globally (Martak et al 2020, Murphy et al 2019, Alikhani et al 2012). But, studies focusing on diarrheagenic *E. coli* based on their pathotypes and AMR profile is rare and their phylogroup is unknown. Therefore, given the importance of such studies in epidemiological surveillance, more such research works are necessary which could further help in prevention and control of disease.

1.2. Statement of the problem

In South Asian region, the mortality rate due to diarrheal disease among children under 5-year age is estimated to be 35% (Walker et al 2012). In a case study involving children, *E. coli* was present in 96% of the diarrheal cases (Kotloff et al 2013). Evidently *E. coli* is the leading cause of bacterial diarrheal cases among adults and children in developing countries including Nepal. Also, AMR *E. coli* are in rise (Murphy et al 2019) making the treatment of diarrheal cases difficult. Several studies focusing on different pathogenic types of *E. coli* have depicted to cause human illness and outbreaks from different water source (Martak et al 2020, Park et al 2018). Despite the significant disease burden linked to contaminated water exposure, the prevalence of *E. coli* pathotypes, phylogroup and AMR profile in aquatic environment is still not well characterized. Considering the high mortality rate and high risk of co-existence of virulence and AMR, the data on such epidemiological characters is crucial to plan and implement proper intervention of disease. But in Nepal, the identification of different pathotype, phylogroup and antibiotic resistance of diarrheagenic *E. coli* is still unknown. This insufficient information may limit our understanding to identify the source of contamination and determine the pathogenic strains that prevail in those sources.

1.3. Objective of the study

General objectives

- The overarching goal of study was to understand transmission potential of antibiotic resistant pathogenic *E. coli* from environment to community via surface water.

Specific objectives:

- To isolate and identify fecal *E. coli* from water sample
- To determine the antibiotic susceptibility profile of the isolate
- To determine the phylogroup of the isolate

1.4. Rationale of the study

Water are the possible source of contamination of human diarrheal diseases and often considered as outbreak source. Also, *E. coli* are regarded as the indicator of fecal contamination. Therefore, to understand the epidemiological aspect of the pathogenic *E. coli* that prevail in the environmental matrices it is essential to determine their pathotype, phylogroup and AMR profile. This study will detect the various virulent gene that are present and help to identify the potentially pathogenic isolates as well as their diversity in the specific niches. Further, this study will also detect the possible source of contamination and their antibiotic susceptibility towards the common treatment regime. All of this information could basically help in microbial source tracking, virulence mechanism determination, vaccine development planning and ultimately addressing the wider aspect of disease prevention and control.

1.5. Research questions /hypothesis

- Whether particular pathotype of *E. coli* is circulating in water environment of Kathmandu?
- Whether phylogroup and antibiotic resistant profile of water circulating *E. coli* are associated?
- Whether pathogenic *E. coli* from environmental niche are multi-drug resistant?

1.6. Limitation of the study

This study will focus only on environmental niches but not clinical samples. The bacterial pathogens between environmental and clinical set up will not be compared. The present study is based on culture and PCR analysis of target genes using particular primer sets. However, sequencing techniques could detect mutational modifications of the isolates.

2. LITERATURE REVIEW

Water are the source of many infection and reportedly water-borne diseases are the major cause of deaths worldwide (Agingu et al 2020). Specifically, diarrheal disease alone is estimated to cause 1.8 million deaths every year in low-income countries (WHO 2014). The majority of diarrheal etiology studies indicates that *E. coli* is the most common bacteria responsible for causing the disease which are called

Diarrheagenic *E. coli* (DEC) (WHO 2014). This diarrheagenic bacteria are highly pathogenic due to their virulent determinants (Sidhu et al 2013, Kuhnert et al 2000, Kaper et al 2004). Based on these virulent determinants, they are categorized into different groups known as pathotypes. The 6 common pathotypes are enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC/STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al 2004). The prevalence of these pathotypes vary by geographic regions and source of infection. Globally, the most common pathotype is ETEC followed by EPEC and EHEC (Sidhu et al 2013, Kuhnert et al 2000,). ETEC causes diarrhea in young children from developing countries and in adults from industrialized countries traveling to these regions (Kaper et al 2004). While EPEC are responsible for causing diarrhea in children below 2 years of age from developing countries. Further, EHEC/STEC infections represent typical diseases of industrialized countries and severe forms of infection are observed mainly in young children and the elderly. In case of Nepal, diarrhea is the leading cause of child morbidity and mortality (Shrestha et al 2022) and evidently ETEC is the major etiological agent among children and travelers. Several cases of ETEC has been reported especially focusing on international travelers (Murphy et al 2019, Margulieux et al 2018, Pandey et al 2011) and children (Shrestha et al 2022, Park et al 2018, Margulieux et al 2018, Lama et al 2007, Hoge et al 1995). These pathotypes are important to understand the virulence mechanism and disease surveillance caused by diarrheagenic *E. coli* but extensive data on their pathotype are rare.

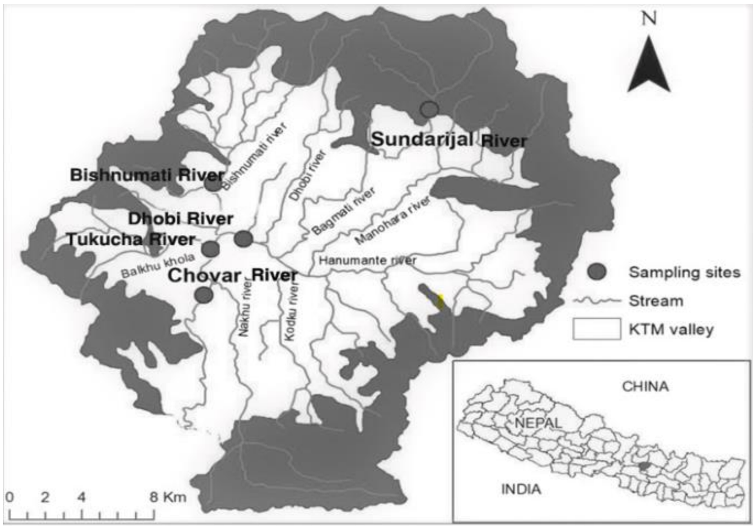
E. coli can also be categorized based on phylogenetic cluster which is known as phylogrouping. This classification constitutes 6 major phylogroups namely, A, B1, B2, C, D, E, F, and G (Clermont et al 2013, 2019). In general, phylogroups A and B1 strains are ubiquitous and associated to humans or animals. Studies have shown that strains belonging to phylogroup A are predominant in humans while B1 strains are predominant in animals (Martak et al 2020). Further, strains belonging to phylogroups B2, D, and F are commonly found in human ExPEC infections (Clermont et al 2013) while STEC strains are found in phylogenetic groups A and B1(Ishii et al 2008). This phylogroups is useful in microbial source tracing but unfortunately there is no data related to phylogroups of any clinical or environmental *E. coli* from Nepal.

Antimicrobial resistance is a global threat to clinical medicine and a burden to developing countries where the impact is higher (Alikhani et al 2012). The continuous evolution and dissemination of AMR bacteria within the clinical and community setting is becoming a serious problem (Alikhani et al 2012). Even, Diarrheagenic *E. coli* has been reported to display antimicrobial resistance (Murphy et al 2019, Margulieux et al 2018, Alikhani et al 2012). In Nepal, few studies on diarrheal *E. coli* have indicated emergence of ciprofloxacin resistance and azithromycin resistance (Murphy et al 2019) among ETEC. Additionally, presence of ESBL-positive ETEC isolates as high as 30% (Margulieux et al 2018) was reported, which is alarming. However, due to the resource poor setting of many laboratories in Nepal, comprehensive data on pathotyping, phylogrouping or AMR profiling of diarrheal cases are usually unavailable. Therefore, studies that primarily focuses on the different virulence traits and distribution of pathogens in environmental matrices will be helpful to intervene in the diarrheal disease prevention, and control. Further, antibiotic resistant profiling can help in disease treatment.

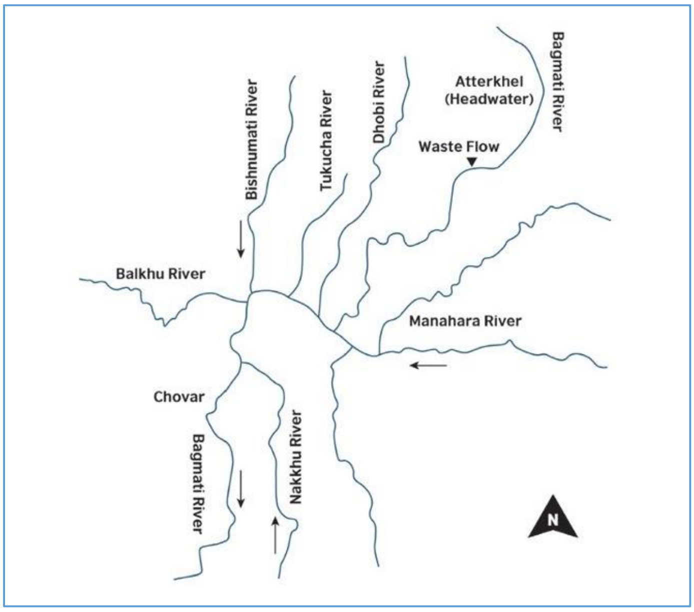
CHAPTER 2: Study design, Methods, and Data analysis

2.1. STUDY DESIGN:

- **Study design:** This was a cross-sectional study which was done in microbiology laboratory to collect data. Primary data was generated from laboratory analysis and secondary data was extracted from different publications/reports/articles for literature review and comparative study.
- **Sample type and size:** The sampling area under study was Kathmandu district and the sample was water of Bagmati river. This river enters the valley from Sundarijal and exits from Chobar so the sampling was done upstream from Chobar area. A total of 97 water sample in two different time period (March and December 2023) was sampled. During the first phase a total of 49 water samples from 5 point sites (particularly the sites of tributaries joining points) while a total of 48 samples from 24 point sites in second phase were collected from Bagmati river and tributaries. The first sampling sites are shown below:



While the second phase sample was collected from 16 different sites from the main Bagmati river and 8 sites from the tributary that leads into Bagmati river. The second sampling sites are shown below:



2.2. METHODOLOGY:

- **Sampling collection and transport:** The water sample (1-liter) from different sources of the study site was collected in a sterile screw capped bottle and transported to the laboratory for further processing by maintaining cold chain within 2 hours of collection
- **Physiochemical analysis of sample**: After collection of sample, the physiochemical analysis for determining temperature and pH was carryout on the site while turbidity of the sample was determined in the microbiology laboratory.
- **Enumeration, Isolation and identification:** Each sample was diluted to 100 times and filtered using standard membrane filtration procedure [Walter 1961] as recommended by APHA and incubated at 44^0C for 24 hrs. 1-2 colonies from EMB plates which are distinct for *E. coli* was selected and sub cultured to obtain pure culture. Preliminary identification was done by staining techniques and confirmatory identification was done on the basis of their biochemical characteristics according to Bergey's manual of systematic bacteriology [Forbes 2007].
- **Antibiotic susceptibility testing:** Antibiotic susceptibility test of *E. coli* was performed by Kirby-Bauer disk diffusion method as recommended by Clinical Laboratory Standards (CLSI 2018). A range of antibiotic belonging to different classes was used. The classes included were Aminoglycosides (Amikacin 30µg, Gentamycin 30µg), Cephalosporin (Cefotaxime 30µg; Ceftazidime 30µg; Cefixime

30µg), Penicillin (Amoxicillin 10µg; Piperacillin 30µg), Penicillin/beta lactamase inhibitor combination(Piperacillin-tazobactam 110µg; Amoxicillin-Clavunate 30µg), Phenicol (Chloramphenicol 30µg), Tetracycline (Tigecycline 30µg), (Fluoro) quinolone (Ciprofloxacin 5µg; Levofloxacin 10µg), carbapenem (Imipenem 10µg; Meropenem 10µg), Nitrofurans (Nitrofurantoin 300µg) Lipopeptide (Polymixin B 300µg; Colistin 10µg) and Sulfonamides (Sulfamethoxazole-trimethoprim 25µg)

- **DNA extraction:** The isolates from overnight culture was centrifuged and DNA was obtained by boiling bacterial suspension in sterile water for 10 min. The pellet was used as sample for PCR.
- **Phylogrouping:** The distribution of phylogenetic groups amongst *E. coli* isolates was determined by detection of specific phylogroup gene via PCR method as per Clermont classification (Iranpour et al 2015). The specific genes are namely *chuA, yjaA, TspE4.C2, arpA* and *trpA*. PCR amplifications was carried out on a Bio-Rad thermal cycler and the PCR products was analyzed by electrophoresis in a 2% agarose gel and then visualized using transilluminator.
- **Pathotyping:** The pathogenic category of *E. coli* was determined by a multiplex PCR for the detection of specific virulence gene in *E. coli* (Vidal et al 2005). The virulence genes are namely Shiga toxin-producing (stx_1, stx_2, and *eae*), entero-pathogenic (*eae* and *bfp*), entero-toxigenic (*stII* and *lt*), entero-invasive (*virF* and *ipaH*), entero-aggregative (*aafII*), and diffuse adherent (*daaE*). PCR amplifications was carried out on a Biorad thermal cycler and the PCR products was analyzed by electrophoresis in a 2% agarose gel and then visualized using transilluminator. Primers used in the multiplex PCR for amplification of diarrheagenic *E. coli* genes are given in the table below:

Gene	Primer sequence (5′-3′)	Size of product (bp)	Reference
stx_1	CAG TTA ATG TGG TGG CGA AGG	348	Cebula et al (1995)
	CAC CAG ACA ATG TAA CCG CTG		
stx_2	ATC CTA TTC CCG GGA GTT TAC G	584	Cebula et al (1995)
	GCG TCA TCG TAT ACA CAG GAG C		
eae	TCA ATG CAG TTC CGT TAT CAG TT	482	Vidal et al. (2004)
	GTA AAG TCC GTT ACC CCA ACC TG		

bfp	GGA AGT CAA ATT CAT GGG GGT AT	300	Vidal et al. (2004)
	GGA ATC AGA CGC AGA CTG GTA GT		
lt	GCA CAC GGA GCT CCT CAG TC	218	Vidal et al. (2004)
	TCC TTC ATC CTT TCA ATG GCT TT		
stII	AAA GGA GAG CTT CGT CAC ATT TT	129	Vidal et al. (2004)
	AAT GTC CGT CTT GCG TTA GGA C		
virF	AGC TCA GGC AAT GAA ACT TTG AC	618	Vidal et al. (2005)
	TGG GCT TGA TAT TCC GAT AAG TC		
ipaH	CTC GGC ACG TTT TAA TAG TCT GG	933	Vidal et al. (2005)
	GTG GAG AGC TGA AGT TTC TCT GC		
daaE	GAA CGT TGG TTA ATG TGG GGT AA	542	Vidal et al. (2005)
	TAT TCA CCG GTC GGT TAT CAG T		
aafII	CAC AGG CAA CTG AAA TAA GTC TGG	378	Vidal et al. (2005)
	ATT CCC ATG ATG TCA AGC ACT TC		

Note: *This multiplex primer did not give a conclusive result therefore the pathotyping was not successful as planned. However, a second set of primer is being planned for further analysis.*

- **Quality control and assurance:** ATCC 25922 culture of *E. coli* was used to ensure the quality of the procedure used.

2.3.TOOLS AND DATA ANALYSIS

The primary data was collected from the experimental work and the secondary data was collected from literatures published in the form of book, reports, research article, new paper, proceeding etc. For data analysis, statistical software was used namely SPSS-21 and WHONET-2023. Basically, the qualitative data was presented in charts and tables while the quantitative data was tested for inferential analysis. Descriptive statistics, Frequency tables, chi-square test and probability significance was used for data analysis and interpretation.

CHAPTER 3: FINDINGS

3.1. RESULT AND DISCUSSION

3.1.1. Result

All the 49 samples showed the presence of fecal coliform (100%) during the microbiological culture analysis. *E. coli* was isolated from all the culture positive plates and identified.

A) Multiple antibiotic resistance in *E. coli*

A total of 141 thermotolerant *E. coli* were isolated from the water sample. The antibiotic susceptible test result of thermotolerant *E. coli* showed a diverse resistance pattern (Table 1).

Table 1: Antibiogram of the isolates towards 10 different antibiotics (N= 141)

Antibiotics name	Class of antibiotic	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Imipenem	Carbapenem	130 (92.20)	11 (7.8)	-
Meropenem	Carbapenem	140 (99.29)	1 (0.71)	-
Amikacin	Aminoglycoside	133 (94.32)	4 (2.84)	4 (2.84)
Nitrofurantoin	Nitrofurans	131(92.92)	5(3.54)	5(3.54)
Gentamicin	Aminoglycoside	135 (95.74)	-	6 (4.26)
Azithromycin	Macrolide	135 (95.74)	-	6 (4.26)
Chloramphenicol	Phenicols	130 (92.20)	5 (3.54)	6 (4.26)
Ciprofloxacin	Fluoroquinolone	107 (75.89)	18 (12.76)	16(11.35)
Cefotaxime	Cephalosporin	90 (63.83)	27 (19.15)	24 (17.02)
Ceftazidime	Cephalosporin	76 (53.90)	31 (21.99)	34 (24.11)

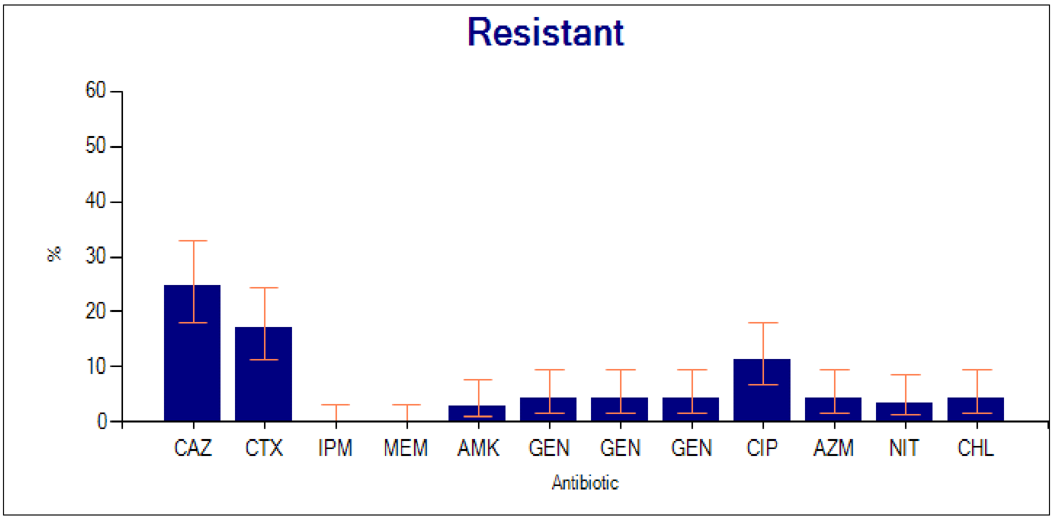


Figure 1: Distribution of resistant isolates within each antibiotic (N = 141)

As per the resistant profile higher resistance was observed towards Cephalosporins class of antibiotic followed by Fluoroquinolones, Phenicols, Aminoglycosides, Macrolide, Nitrofurans and Carbapenem in descending order (Figure 1).

Based on multiple resistance to antibiotics, the distribution of resistance within isolates were grouped into resistant to one antibiotic, to two antibiotics and three or more antibiotics (Figure 2).

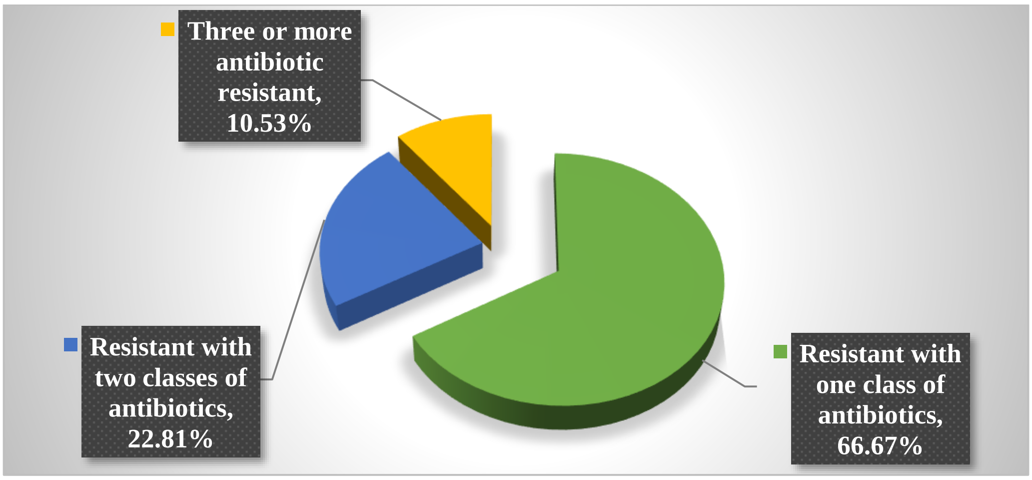


Figure 2: Percentage distribution of resistant isolates (N = 57)

B) Distribution of antibiotic resistant *E. coli* across river sampling sites

As per the sample location, the resistant isolates were abundant in the Bagmati joining point with Tukucha khola. The resistant isolates were observed to rise from entry point to exit point (Downstream) (Table 2).

Table 2: Percentage wise distribution of resistant isolates (N = 57) based on downstream sampling point

Sampling site	**Resistance isolates count (%)**
Sundarijal	0 (0.00%)
Bagmati joining point with Dhobhi khola	14 (42.42%)
Bagmati joining point with Tukucha khola	17 (47.22%)
Bagmati joining point with Bishnumati khola	12 (36.36%)
Bagmati joining point with Chobar khola	14 (38.89%)

C) High priority isolates of antibiotic resistant *E. coli*

Based on WHONET analysis, 11 isolates were identified as high priority isolates as these showed intermediate susceptibility towards Imipenem (Table 3). However, 40 isolates were medium priority due to their possibility of being ESBL producers.

Table 3: Resistant profile of high priority isolates (N = 11)

Resistant Profile	Priority	No of isolates
$CTX^RCAZ^RIPM^R$	High priority	3
IPM^R	High priority	3
$AK^RCTX^RCAZ^RIPM^R$	High priority	1
$AK^RCTX^RIPM^RMEM^R$	High priority	1
CAZ^RIPM^R	High priority	1
$CTX^RCAZ^RIPM^RCIP^R$	High priority	1
CTX^RIPM^R	High priority	1

(D) Phylogenetic groups (phylogroup) of antibiotic resistant *E. coli*

Besides antibiogram, these isolates were screened for different genes that are used for phylogrouping and based on the presence /combination of those gene different phylogroup were identified within these isolates (Figure 3).

Quadruplex genotype				
arpA (400 bp)	*chuA* (288 bp)	*yjaA* (211 bp)	TspE4.C2 (152 bp)	Phylo-group
+	−	−	−	A
+	−	−	+	B1
−	+	−	−	F
−	+	+	−	B2
−	+	+	+	B2
−	+	−	+	B2
+	−	+	−	A or C
+	+	−	−	D or E
+	+	−	+	D or E
+	+	+	−	E or clade I
−	−	+	−	Clade I or II
−	(476)[c]	−	−	Clade III, IV or V
−	−	−	+	Unknown
−	−	+	+	Unknown
+	−	+	+	Unknown
+	+	+	+	Unknown
−	−	−	−	Unknown

Figure 3: The identification of different combination of gene for Phylogrouping E.coli.

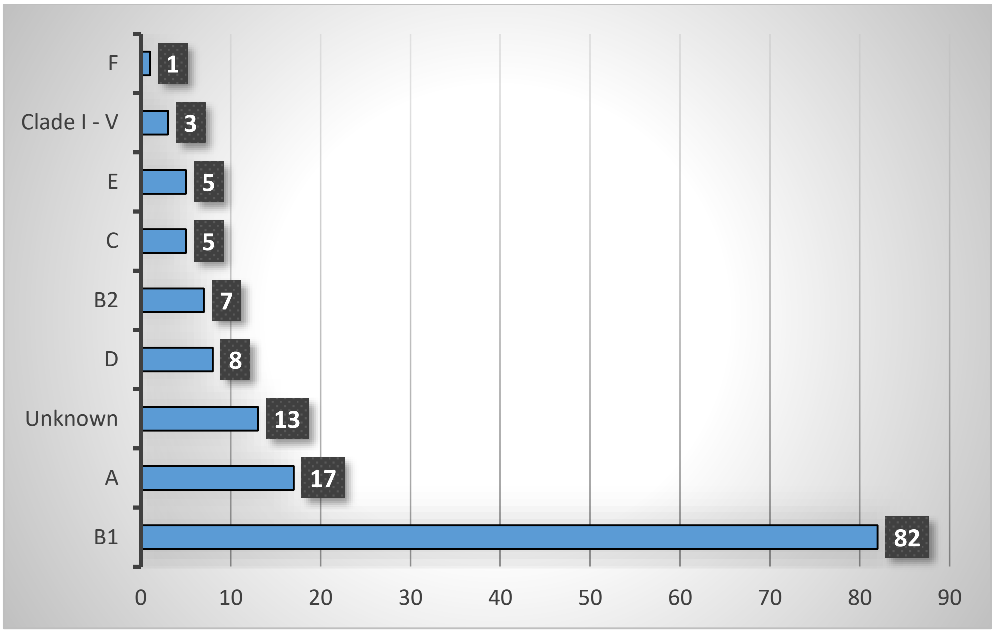


Figure 4: The distribution of E. coli into different phylogroup (N=141)

The predominant phylogroup was B1 (58.2%) followed by A group of *E. coli* (Figure 4). Though the distribution of phylogroup within *E. coli* isolates (N=141) showed no significant correlation statistically ($p > 0.05$). Likewise, no significant correlation ($p > 0.05$) was observed in the distribution of phylogroup within resistant isolates (n=57).

(E) Pathotypes of antibiotic resistant *E. coli*

As for pathotyping, the amplification using the primers (*stx*$_1$, *stx*$_2$, *eae, bfp, stII, lt, virF, ipaH, aafII* and *daaE*) was not successful and the PCR product did not show any bands on the gel. Despite several attempts to conduct pathotyping, the process was not successful. Therefore, a new set of primer has been ordered to start a new and complete the pathotyping. The work will be carried on as per the plan and recommendation from earlier literature.

3.1.2. Discussion

Diarrheal infection is very common in Nepal specially among children. So in this study, we attempted to determine the status of thermotolerant *E. coli* of fecal origin in the Bagmati river sample and characterize them based on antimicrobial resistance, phylogroup and pathotype. In this study, the result revealed that majority of the samples collected from different sites of Bagmati river showed the presence of thermotolerant *E. coli*. This finding specifically indicate that the fecal coliforms are prevalent in Bagmati river which is understandable. Since many sewage systems are drained directly into the river at different human habitat area and the fecal load in water is ought to be high. Besides, several studies on Bagmati water has also similar findings.

Also, the antibiotic resistant isolates were also found higher in majority of isolates. This high occurrence may be due to the exposure of fecal contamination to water via sewage system. Similar higher frequency has also been reported in Nepal. Based on Phylogroup, the most common was B1 group. This group is mostly source tracked to fecal origin so the drainage of sewage system may be their contributor.

Notable frequency of MDR (6) was observed in this study while majority of the isolates were resistant to at least one antibiotic tested. Since resistance towards the major classes of drug can interfere in the treatment of the infection and even treatment failures, so immediate action is utmost necessary to control the spread and dissemination of such isolates. Some of the MDR isolates were reported as possible ESBL by WHONET software which is further concerning. Undoubtedly these isolates are of human or animal origin, but the question arises whether the antibiotic resistance is acquired or intrinsic. A comprehensive experimental and genomic analysis of mobile genetic element is needed for certainty. However, this antibiotic resistance may be the consequences of uncontrolled prescription, over the counter use and lack of proper surveillance program of AMR in both humans and animal. Thus a proper diagnostic tools to identify such isolates, effective treatment regime, controlled prescription may be pivotal in controlling and spreading such isolates.

Further, 11 isolates were reported as high priority due to their resistance towards Imipenem, which is alarming. Many studies from around the world has also reported similar highly resistant from environmental samples in their studies. Therefore, prevalence of such resistant isolates with resistant towards the last resort drug i.e. Carbapenem is threating to the human and animal life. Besides many isolates showing resistance towards number of antibiotics and rendering multidrug resistance is an alarming situation. Additionally, such resistant isolates infecting vulnerable population specially patients with underlying condition might create a troublesome situation. Antibiotic resistance is a global threat and needs to be addressed in every sector. Thus, this kind of research focusing on phylogrouping and pathotyping of AMR *E. coli*, is utmost necessary to track the source of transmission and highlight the situation into the world. So that the possible risk clones can be intervened in their transmission to humans and animals.

CHAPTER 4: Conclusion and Recommendations

4.1. Conclusion

High occurrence of thermotolerant *E. coli* of phylogroup B1 was observed in different areas of Bagmati river. Also, isolates conferring resistance to multiple antibiotics were detected along with high priority isolates. And phylogrouping suggests that they are of animal and human origin. Thus undoubtedly, such high risk isolates are circulating within human and animal population of Kathmandu valley and is evidently prevalent in the aquatic system of Nepal. This is a threatful situation for Nepalese community.

4.2. Recommendations

Water borne disease like diarrhea is very common in Nepal and diarrheagenic isolates with resistance to multiple antibiotics pose a grave threat to public health. Therefore, based on the findings of this study a number of recommendation can be drawn which are listed below:

- The prevalence of thermotolerant *E. coli* should be determined in all the niches (water, air, soil, humans and animal) to highlight the possible transmission and determine the recent situation analysis, trend analysis and so on.
- The development of MDR, XDR and CRE in stressful condition like environment or human gut is more likely to occur due to the selection pressure. So, a proper control and prevention to avoid AMR evolution and spread should be planned.
- To understand the development and spread of AMR in community and hospital setting, it is utmost necessary to conduct genomic researches.
- AMR surveillance and monitoring should be regularly practiced

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Appendix I: Student training and outcome

Orientation for the student: Since laboratory analysis plays a major role in a wet-lab research work, it was utmost important to orient the student about the difficulties and challenges that students may face during the laboratory analysis and the ways to mitigate the problems. The highlight of the orientation was as follows:

– **Sample collection and on-site analysis:** Since the sample was river water and the sites were not easily accessible. On-site visit as well as sample collection was conducted in presence of the students. They were also practiced for on-site temperature, pH and GPS determination. Since Bagmati river is almost similar to waste water, therefore high precaution was taken to avoid contamination.

– **Laboratory biosafety:** Since microbiology labs deals with all kinds of hazardous samples, therefore biosafety rules and regulation should be strictly followed and the student was given elaborative details about biosafety, which was necessary for their safety and others as well.

– **Sample handling and processing:** Sample handling and processing plays a key role in the study result, therefore students were sensitized about the microbial analysis of water sample based on APHA guideline. Even the antibiotic susceptibility testing was explained to them. As they were of master degree and bachelor degree student of microbiology and they may already have adequate knowledge of this things but it was necessary to orient them about the challenges they might face in the practical world during the sample handling and processing.

– **Handling data collection tools and record keeping:** As data generated after the laboratory analysis should be reported correctly otherwise interpretation of data will be jeopardized. Students were provided observation tables to collect the data after the analysis conduction. They were even given an in-depth explanation about the technicalities of the tabulated data during data analysis.

Outcome:

The students have been able to perform laboratory techniques independently. They have become skilled in sample collection, transport and processing. They have developed competencies like time management, data collection, trouble shooting, project work planning and implementation. This project work will certainly help them in their future research work journey.

Appendix II: Financial Statement Details

S. N	Expense heading	Particular	Estimated (Nrs.)	Actual Expense (Nrs.)	Claimed amount (Nrs.)
1	Personal cost	Travel expense during sampling	6,000	10,800	10,800
2	Student support	Thesis support (3 student each 5000)	15,000	15,000	15,000
3	Wet lab cost (Microbiology)	Laboratory chemical and reagent purchase	60,500	61,000	32,770
4	Office cost	Office supplies	5,000	5,000	5,120
		Printing and binding	5,000	5,000	4,850
		Communication expense	1,000	2,000	2,000
5	Administration cost	Contingency cost (Miscellaneous)	4,500	4,000	4,460
		Total	112,000	120,000	75,000
Amount received as first installment					-37,500
Amount received as second installment					-18,750
Amount agreement of Grant					75,000
Due amount					18,750

Appendix III: List of Scientific activity

The scientific activity that the investigators were involved during the project duration is provided below:

[1] **Invited Talk** entitled “Integrons in Nepal” (Antimicrobial resistant bacteria from clinical and environmental isolates)
Ms. Suchitra Thapa (Principle-investigator)
Mustang Ancient DNA symposium, Organized by Amrit Campus, Dean Office-IOST and KCRE-TU-CAS
Symposium date: March 4th 2024.

[2] **Research manuscript** is in progress to be published in indexed journal. Currently a data analysis is going on for a comprehensive outcome and the draft is planned to be made ready within three months for publication. This article will definitely play pivotal role in the enhancement in the knowledge regarding the subject matter.

[3] **Abstract preparation** for presentation in international conference. The phylogroup characterization has revealed an interesting outcome therefore based on that an abstract for result dissemination has been prepared for submission to an international conference for poster presentation.

Appendix IV: Photos related to project

Photo 1: Sample collection of Bagmati corridor (Balkhu point) sampling site using a sterile BOD bottle

Photo 2: On-site Temperature and pH determination of the sample using a portable machine at the sampling site

Photo 3: Streaking of *E. coli* on M-endo agar plate for pure culture and greenish metallic sheen colonies of *E. coli* observed.

Photo 4: Biochemical test result of *E. coli*

(from left to right: Oxidation-Fermentation= positive both tubes, SIM= indole positive, Methyl red= positive, VogeusProskeur= negative, Citrate = negative, TSI= acid/acid with gas, Urease=negative)

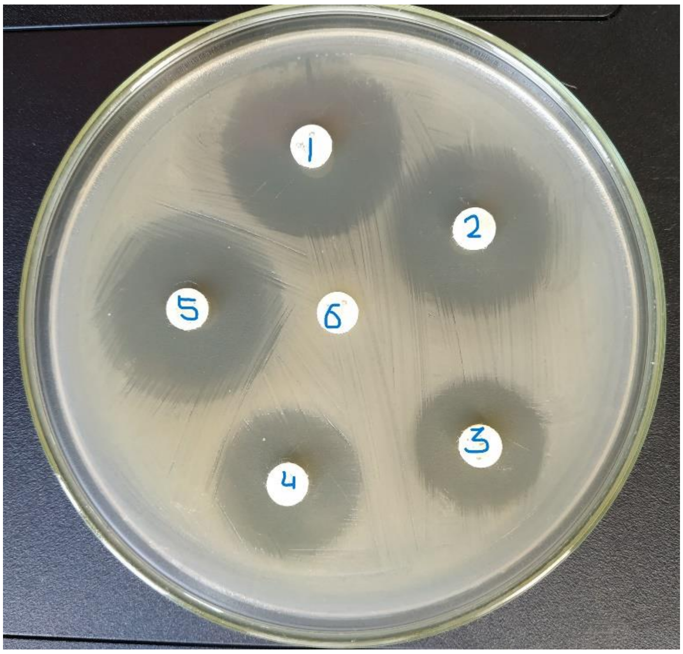

Photo 5: Antibiotic susceptibility plate of *E. coli* isolate

[Clockwise: 1=Meropenem (Susceptible), 2=Amikacin (Susceptible), 3=Cefotaxime (Intermediate), 4=Ceftazidime (Intermediate), 5=Gentamicin (Susceptible) and 6=Ciprofloxacin (Resistant) in the middle].

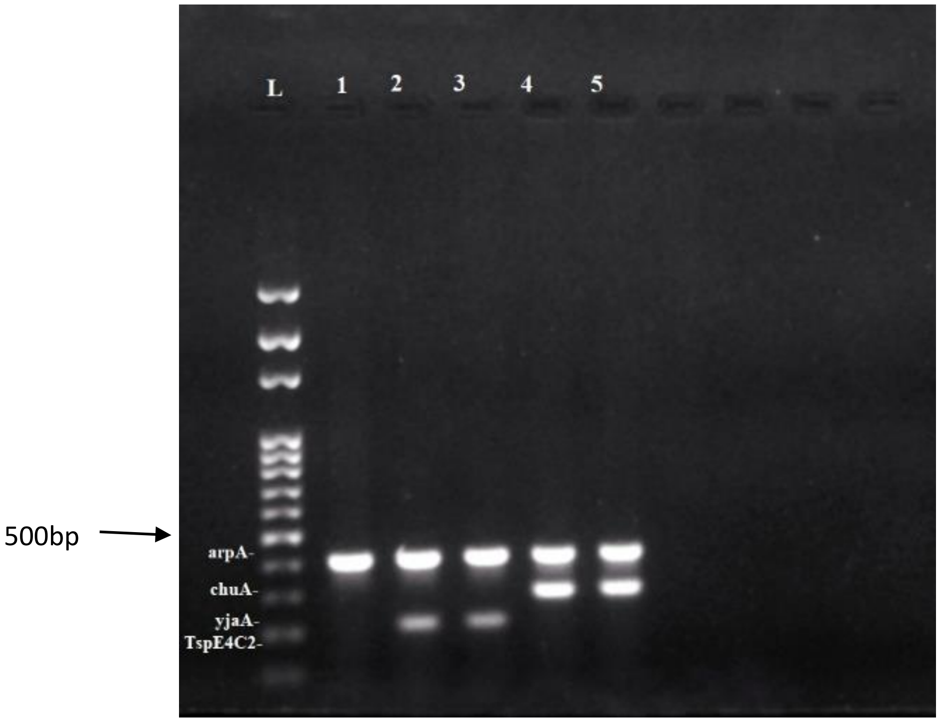

Photo 6: Agarose gel visualization of Phylogroup of *E. coli* for arpA(400bp), chuA(288bp), yjaA(211bp) and TspE4.C2(152bp) genes [From 1= Group A , 2 = Group A or C, 3= Group A or C, 4= Group D or E, 5 = Group D or E)