CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN *Escherichia coli* ISOLATED FROM RIVER BASIN OF KATHMANDU VALLEY

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF MICROBIOLOGY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

BY

BINDU GHIMIRE

MAY 2024

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Institute of Science and Technology

DEAN'S OFFICE

Kirtipur, Kathmandu, Nepal

Institute of Science & Technology Dean's Office Kirtipur 2045

ference No.:

EXAMINERS

The Title of Ph.D. Thesis: " Characterization of Antimicrobial Resistance in *Escherichia coli* Isolated from River Basin of Kathmandu Valley "

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September 11, 2024

(Dr. Surendra Kumar Gautam)
Asst. Dean

DECLARATION

Thesis entitled **"Characterization of Antimicrobial Resistance in *Escherichia coli* Isolated from River Basin of Kathmandu Valley"** which is being submitted to the Central Department of Microbiology, Institute of Science and Technology (IoST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Prakash Ghimire of Central Department of Microbiology, Tribhuvan University and co-supervised by Assoc. Prof. Dr. Komal Raj Rijal of Central Department of Microbiology, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

Bindu Ghimire

RECOMMENDATION

This is to recommend that **Ms. Bindu Ghimire** has carried out research entitled **"Characterization of Antimicrobial Resistance in *Escherichia coli* Isolated from River Basin of Kathmandu Valley"** for the award of Doctor of Philosophy (Ph.D.) in **Microbiology** under our supervision. To our knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

.....
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May 2024

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE AND TECHNOLOGY
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Date: 2024/05/31

LETTER OF APPROVAL

On the recommendation of Prof. Dr Prakash Ghimire (Supervisor) and Assoc. Prof. Dr. Komal Raj Rijal (Co-Supervisor), this Ph.D. thesis submitted by Bindu Ghimire, entitled **"Characterization of Antimicrobial Resistance in *Escherichia coli* Isolated from River Basin of Kathmandu Valley"** is forwarded by Central Department Research Committee (CDRC) to the Dean, IoST, T. U. .

.....

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Bindu Ghimire

May 2024

शोध सार

जीवाणुले गर्ने सङ्क्रमणको उपचारमा प्रयोग गरिने प्रतिजैविकहरु प्रति जीवाणुले देखाउने प्रतिजैविक प्रतिरोधको अवस्था विश्वव्यापी चुनौतीको रुपमा देखा परेको छ । नेपाल भित्र वातावरण, पशुपंक्षी र सङ्क्रमित मानिसहरुको नमुनाबाट भेटिएका जीवाणुहरुले प्रतिजैविक प्रतिरोध गर्ने पुष्टि गरेका छन् । यो अध्ययन जनवरी, २०२० देखि सेप्टेम्बर, २०२३ सम्म काठमाडौं उपत्यका भित्र वागमती जलधार क्षेत्रमा गरिएको थियो । वागमती नदी अन्तर्गत उपल्लो नदी खण्ड (७), मध्य नदीखण्ड (३७), र तल्लो नदीखण्ड (१६), सहायक नदीहरु (६), तथा ढुंगेधाराहरु (१४), र इनार (८) बाट पानीका तेहोरो नमुनाहरु सङ्कलन गरिएका थिए । राष्ट्रिय जन स्वास्थ्य प्रयोगशालाबाट मूत्रनलीमा शंकास्पद सङ्क्रमण देखिएका विरामीहरुको मूत्रको नमुनाहरु (१,२२०) र १३ वटा कुखुराको फार्मबाट ३९० कुखुराको सुलीको नमुना पनि सङ्कलन गरिएको थियो । पानीका नमुनाहरुबाट सर्वाधिक संभावित संख्या गणना विधिको प्रयोग गरी तथा उपयुक्त जीवाणु वृद्धि माध्यमको प्रयोग गरेर मानिसको मूत्रको नमुना र कुखुराको सुलीबाट जीवाणुहरु अलगाव गरिएका थिए । जीवाणुहरुलाई उचित जैवरासायनिक परीक्षण गरी प्रजातिको पहिचान पश्चात् *E. coli* हरुको प्रतिजैविक संवेदनशीलता परीक्षण गरिएको थियो, जसमा १७ वटा प्रतिजैविकहरुलाई ११ वटा श्रेणी र ५ वटा परिक्षण प्रतिवेदन समुहमा विभाजित गरिएको थियो (CLSI, 2020) । विस्तारित स्पेक्ट्रम बिटा ल्याक्टामेज उत्पन्न गर्ने क्षमता भएका *E. coli* को लक्षण प्रारुप पुष्टिका लागि संयोजन डिस्क र जीव प्रारुप पुष्टिका लागि वहुसंयुक्त पोलिमरेज शृंखला प्रतिक्रिया विधिको प्रयोग गरिएको थियो । माथि उल्लेखित सबै नमुनाहरुको विश्लेषण कार्य त्रिभुवन विश्वविद्यालय अन्तर्गतको सुक्ष्मजीवविज्ञान केन्द्रीय विभागको प्रयोगशालामा गरिएको थियो ।

यसरी सङ्कलित नमुनाहरुको विश्लेषण गरी इन्टेरोब्याक्टेरिएसी परिवार भित्र १२ र मोरग्जेलेसी परिवार भित्र १ प्रकारका गरी १,२९५ जीवाणुहरु पहिचान गरीयो । पानीबाट २८८, कुखुराको सुलीबाट २६६ तथा मानवमूत्रबाट ६३ गरी कुल ६१७ *E. coli* पहिचान भए । यस अध्ययनमा *E. coli* बाहेक *Acinetobacter* spp., *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella* Typhi, *Salmonella* Paratyphi, *Shigella dysenteriae* जीवाणुहरु पनि भेटिए । कुल १,२९५ जीवाणुहरु मध्ये करिब ६ % (८०/१,२९५) ESBL *E. coli* को रुपमा पहिचान भए । विशेषतः वागमती नदीको उपल्लो नदी खण्ड, ढुंगेधारा र इनारबाट सङ्कलन गरिएका नमुनाहरुमा ESBL *E. coli* भेटिएनन् । सय मिलिलिटर पानीको नमुनामा; उपल्लो नदी खण्डमा सबैभन्दा कम (३१) र सहायक नदीहरुका संगमस्थलहरुमा (७०,०००) सबै भन्दा बढी कोलिफर्म्स पाइयो । पानीका नमुनाहरुमा ७९७ वटा जीवाणुहरु फेला परेका थिए, जसमध्ये करीब ३६ % (२८८/७९७) *E. coli* र करिब ५ % (४०/७९७) ESBL *E. coli* थिए । *E. coli* हरु मध्ये करिब १४ % (४०/२८८) ESBL *E. coli* भेटिए । पानीका नमुनाको विश्लेषण हेर्दा करिब ९७ % (८५/८८) नमुना संग्रहस्थलहरुमा *E. coli* भेटिए भने करिब ३० % (२६/८८) नमुना संग्रहस्थलहरुमा ESBL *E. coli* पाइए । प्राप्त *E. coli* र ESBL *E. coli* हरुको प्रतिजैविक प्रतिरोध परीक्षण गर्दा परीक्षणमा प्रयोग गरिएका १७ वटा प्रतिजैविक औषधिहरु मध्ये ९५ % भन्दा बढी non ESBL *E. coli* हरुले एम्पिसिलिन, इराइथ्रोमाइसिन, पाइपेरासिलिन, एमोक्सिलिन क्ल्याभुलिनिक एसिडए र टेट्रासाइक्लिनका विरुद्ध प्रतिरोध देखाए भने करिब १०० % (४०/४०) ESBL *E. coli* हरुले एम्पिसिलिन, एमोक्सिलिन क्ल्याभुलिनिक एसिड, सेफ्टाजिडाइम,

सेफोट्याक्जिमी र पाइपेरासिलिनका विरुद्ध प्रतिरोध देखाए । तर इमिपेनेमका विरुद्ध भने करिब १८ % (७/४०) ESBL *E. coli* हरुले मात्र प्रतिरोध देखाए । त्यस्तै मानवमूत्रका जम्मा १,२२० वटा नमुनाहरुमध्ये करिब ८ % (९६/१२२०) नमुनाहरुमा उल्लेखनीय रुपमा जीवाणुहरु भेटिए जस मध्ये करीब ६६ % (६३/९६) *E. coli* र करीब १९ % (१८/९६) ESBL *E. coli* थिए । शत प्रतिशत (१८/१८) ESBL *E. coli* हरुले सेफोट्याक्जिमी, सेफ्टाजिडाइम र सिप्रोफ्लोजासिनका विरुद्ध प्रतिरोध देखाए भने इमिपेनेमका विरुद्ध करिब १७ % (३ / १८) प्रतिरोध देखाए । अर्को तर्फ कुखुरा फार्मबाट सङ्कलित कुखुराका सुलीका ३९० वटा नमुनाबाट ४०२ वटा जीवाणुका पहिचान गरियो जसमा करिब ६६ % (२६६/४०२) *E. coli* र करिब ५ % (२२/४०२) ESBL *E. coli* थिए । कुखुराका सुली सङ्कलन गरिएका कुखुरा फार्म मध्ये करिब १५ % (२/१३) कुखुरा फार्ममा र ९४ % (३६८/३९०) नमुनाहरुमा ESBL *E. coli* पाइएन । कुखुराका सुलीबाट प्राप्त ESBL *E. coli* हरुको प्रतिजैविक प्रतिरोध परीक्षण गर्दा परीक्षणमा प्रयोग गरिएका १७ वटा प्रतिजैविक औषधिहरु मध्ये ESBL *E. coli* हरुले १०० % (२२/२२) एमोक्सिलिन क्ल्याभुलिनिक एसिड, सेफ्टाजिडाइम र सेफिपिमिका विरुद्ध प्रतिरोध देखाए तर इमिपेनेमका विरुद्ध भने करिब २३ % (५/२२) मात्र प्रतिरोध देखाए । बहुसंयुक्त पोलिमरेज शृंखला प्रतिक्रियाबाट प्राप्त नतिजा अनुसार कुल ८० वटा ESBL *E. coli* मध्ये करिब ९० % (७२/८०) मा *bla CTX M-1*, करिब ४० % (३२/८०) मा *bla TEM* र १५ % (१२/८०) मा *bla SHV* आनुवनंशिक तत्त्व देखियो । नदीको पानीको एउटा नमुनामा *bla CTX M-9* आनुवनंशिक तत्त्व भेटियो तर मानवमूत्रमा *bla SHV* आनुवनंशिक तत्त्व भेटिएन । नमुना सङ्कलन गरिएका स्रोतहरु (पानीका स्रोत, मानवमूत्र र कुखुराको सुली) का आधारमा ESBL *E. coli* बिचको सहसम्बन्ध गुणांक विश्लेषण गर्दा मानवमूत्र र पानी (r=0.3), पानी र कुखुराको सुली (r=0.3), र मानवमूत्र र कुखुराको सुली (r = 0.13) मा कमदेखि मध्यमस्तरसम्मको सहसम्बन्ध भेटियो । ESBL *E. coli* को प्रतिजैविक प्रतिरोध प्रतिशतको आधारमा तयार गरिएको समानता मानचित्रमा पानी र मानवमूत्रमा नमुनमा भेटिएका ESBL *E. coli* मा पानी र कुखुरोको सुलिको नमुनमा भेटिएका ESBL *E. coli* भन्दा कम दुरी भेटियो । यस अध्ययनको नतिजाले मानिस, पशुपंक्षी र वातावरणका विभिन्न स्रोतहरुमा व्याप्त जीवाणु बिचको सम्भावित अन्तरसम्बन्धलाई उजागर गरेको छ ।

त्यसर्थ यस अध्ययनले प्रतिजैविक प्रतिरोधी जीवाणु जुनसुकै क्षेत्रबाट मानिसमा सर्न सक्ने सम्भावनालाई औंल्याएको छ । प्रतिजैविक प्रतिरोधी जीवाणुको सङ्क्रमणबाट जोगिनका लागि सर्वप्रथम आमजनमानसमा प्रतिजैविक प्रतिरोध सम्बन्धी जानकारी पुर्‍याउन र नतीजा उन्मुख अध्ययन अनुसन्धानलाई प्राथमिकता दिन आवश्यक छ । यसका साथै उचित सर्भिलेन्सको व्यवस्था हुन जरुरी देखिन्छ । यसका लागि एक स्वास्थ्यका प्रमुख घटक अन्तर्गत पर्ने स्वास्थ्य वातावरण, स्वास्थ्य मानिस र स्वास्थ्य पशुपंक्षीको अवधारणालाई मूर्त रुप दिन जरुरी देखिन्छ ।

संकेत शब्दहरु: *E. coli*, *ESBL E. coli*, *ESBL आनुवनंशिक तत्त्व* (*bla CTX -M 1*, *bla CTX- M 9*, *bla TEM*, *bla SHV*), *सहसम्बन्ध*

ABSTRACT

Antimicrobial resistance, a global burden, leads to treatment failures and increased healthcare challenges. Antimicrobial resistance in bacterial isolates from water, poultry feces and human pathological specimens has been published in some scattered small studies in Nepal, without any specific correlation. To understand the commonality between the isolates, their antimicrobial resistance pattern and possible correlation between the isolates from human, animal, and environment, the current study has been conducted during Jan 2020 till Sept 2023. The study aimed to characterize *Escherichia coli* and ESBL *E. coli* within the Katmandu valley river basin (Bagmati river water, stone spouts, shallow well), poultry feces, and human urine samples and determine their correlation. A total of 264 water samples from 88 sampling sites: upstream (7), midstream (37), downstream (16) and tributaries (6) of Bagmati river, stone spouts (14) and shallow wells (8); 390 poultry feces from 13 poultry farms spread within 5 clusters all along the Bagmati river basin were analyzed following standard methodology, at Central Department of Microbiology Laboratory; while 1,220 human urine samples from patients visiting National Public Health Laboratory, were analyzed at NPHL following CLSI guideline.

Out of the 1,295 bacterial isolates detected from the samples, 48 % (617/1,295) were *E. coli*; of which 288 were from water, 63 were from human urine and 266 were from poultry feces. Analysis revealed that 6 % (80/1295) were ESBL *E. coli*. A total of 797 bacteria were isolated from water samples: *E. coli* (36%), *K. oxytoca* (16%), *C. freundii* (21%), *C. koseri* (14%), *K. pneumoniae* (9%), and *E. aerogenes* (4%). A total of 402 bacteria were isolated from Poultry feces: *E. coli* (66%), *C. freundii* (18%), *C. koseri* (8%), *P. mirabilis* (3%), *P. vulgaris* (1%), *S. Typhi* (1%), *S. Paratyphi* (0.7%), *S. dysenteriae* (2%), and *Acinetobacter* spp. (0.3%). A total of 96 bacteria were isolated from urine samples: including *E. coli* (66%), *C. freundii* (17%), *C. koseri* (5%), *K. pneumoniae* (4%), *E. aerogenes* (3%), *K. oxytoca* (2%), *P. mirabilis* (2%), and *P. vulgaris* (1%).ESBL *E. coli* was not detected in upstream, stone spout, and shallow well water samples. The MPN count revealed increasing trend from upstream to tributaries: upstream (31), stone spout (69), shallow well (160), midstream (2,400), downstream (17,000), and tributary (70,000), indicating human activity effects across the human inhabitation. In river water 36 % (288/797) of the isolates were *E. coli* and 5 % (40/797) were ESBL *E. coli*. *E. coli* was not detected from about 3 % (3/88) and ESBL *E. coli*

was not detected from 70 % (62/88) of water sampling sites. Among non ESBL *E. coli*, 99 % (246/248) were resistant to erythromycin; 98 % (244/248) to amoxicillin clavulanic acid and about 98 % (242/248) to ampicillin and 96 % to piperacillin and tetracycline. All ESBL *E. coli* (40) were resistant to ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime and piperacillin antibiotics, whereas only about 18 % (7/40) ESBL *E. coli* were resistant to imipenem. From urine samples, significant growth of Enterobacteriaceae was detected in 8 % (96/1,220) of the samples, with 96 bacterial isolates detected; among which 66 % (63/96) were *E. coli* and 19 % (18/96) were ESBL *E. coli*. All ESBL *E. coli* (18) isolates were resistant to cefotaxime, ceftazidime and ciprofloxacin whereas about 17 % (3/18) ESBL *E. coli* were resistant to imipenem. Among the poultry feces isolates, 66 % (266/402) were *E. coli* and 5 % (22/402) were ESBL *E. coli*. ESBL *E. coli* was not detected in fecal samples from 15 % (2/13) of the poultry farms. All ESBL *E. coli* (22) were resistant to amoxicillin/clavulanic acid, cefepime, ceftazidime and piperacillin tazobactam antibiotics whereas about 23 % (5/22) of ESBL *E. coli* were resistant to imipenem. The ESBL gene *bla CTX M-1* was detected in 90 % (72/80), *bla TEM* was detected in 40 % (32/80) and *bla SHV* was detected in 15 % (12/80) of the ESBL *E. coli* isolates. A *bla CTX M-9* gene was detected only in ESBL *E. coli* from one river water sample, while *bla SHV* gene was not detected in *E. coli* from human urine samples. The correlation coefficient showed low to moderate correlation between ESBL *E. coli* in urine and water (r = 0.3), water and feces (r = 0.17) and urine and feces (r = 0.13). The similarity map based upon antibiotic resistance pattern of ESBL *E. coli* revealed less distance in ESBL *E. coli* isolates from water and human urine in comparison to water and poultry fecal isolates. The study unveils low but significant correlation in the antibiotic resistance profiles of ESBL *E. coli* isolates from river water, human urine, and poultry feces, indicating the possible cross-linkage between the bacteria from all 3 sources: human, animal, and environment. The findings of the study clearly indicated the need for strengthening surveillance, monitoring, and containment programs, enhancing sanitation, hygiene, and infection prevention & control practices, and promoting rational use of antibiotics, for public health protection. Addressing the key components of One Health (a healthy environment, healthy humans, and healthy animals) is crucial to reducing the burden of antimicrobial-resistant organisms.

Keywords: *ESBL E. coli, ESBL gene* (*bla CTX M-1*, *bla CTX M-9*, *bla TEM*, *bla SHV*), *correlation*

LIST OF ACRONYMS AND ABBREVIATIONS

AMC	: Antimicrobial Consumption
AMR	: Antimicrobial Resistance
AMU	: Antimicrobial Use
APEC	: Avian Pathogenic *Escherichia coli*
AST	: Antimicrobial Susceptibility Testing
BEL	: Bordeaux Extended Spectrum Beta Lactamase
bla	: Beta Lactamase
CES	: Chronobacter Extended-Spectrum
CIA	: Critically Important
CLSI	: Clinical and Laboratory Standard Institute
CTX-M	: Cefotaxime Munich
DAEC	: Diffusely Adherent *Escherichia coli*
DNA	: Deoxyribonucleic Acid
DNPWC	: Department of National Parks and Wildlife Reserve
EHEC	: Enterohemorrhagic *Escherichia coli*
EIEC	: Enteroinvasive *Escherichia coli*
EPEC	: Enteropathogenic *Escherichia coli*
ESBL	: Extended Spectrum Beta Lactamase
ETEC	: Enterotoxigenic *Escherichia coli*
FAO	: Food and Agriculture Organization
GES	: Guiana Extended Spectrum (GES)
GLASS	: Global Antimicrobial Resistance and Use Surveillance System
GoN	: Government of Nepal
glp T	: Glycerol 3 Phosphate Transporter
HPCIA	: Highest Priority Critically Important Antibiotics
IRT	: Inhibitor Resistant TEM
ISEcp1	: Insertion Sequence of *Escherichia coli* Promoter
LMICs	: Low- and Middle-Income Countries
MDR	: Multiple Drug Resistance
MNEC	: Meningitis Associated *Escherichia coli*
uhp	: UDP-N-Acetylglucosamine Enol Pyruvyl Transferase

NPHL : National Public Health Laboratory
NRL : National Reference Laboratory
NCC : National Coordinating Center
OTC : Over the Counter
OXA : Oxacillin
OXY : Oxacillinase
PCR : Polymerase Chain Reaction
PPE : Personal Protective Equipment
PER : Pseudomonas Extended Resistance
Qnr : Quinolone Resistance Protein
rRNA : Ribosomal Ribonucleic Acid
SFO : Serratia Fonticola
SHV : Sulphydryl
ST : Sequence Type
STEC : Serotoxigenic *Escherichia coli*
TEM : Temonaria
TLA : Temoneira Like Activity
Uhp : Uptake Hexose Phosphate
UPEC : Uropathogenic *Escherichia coli*
UPEC : Uropathogenic *Escherichia coli*
UTI : Urinary Tract Infection
VEB : Vietnamese Extended Spectrum Beta Lactamase
VIM : Verone Integron Encoded
WHO : World Health Organization
XDR : Extensive Drug Resistance

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CHAPTER 1

1. INTRODUCTION

1.1 Introduction

Antimicrobial resistance (AMR) occurs when pathogens that are normally sensitive to specific antibiotics develop the ability to resist their effects. This resistance is a natural phenomenon that occurs as all organisms evolve to survive in hostile environments (Seukep *et al*., 2023). The concept of the "survival of the fittest" applies to the development of resistance in organisms that are exposed to harsh conditions over an extended period. This is because these organisms possess the ability to reproduce and pass on their resistance traits to future generations (Gregory, 2009). When an organism is exposed to adverse conditions repeatedly but for brief periods, it quickly develops adaptive responses. This rapid adaptation enables the organism to survive in environments with antibiotics, complicating the selection of effective treatments due to the development of AMR (Mancuso *et al*., 2021). Frequent exposure to sub-optimal doses of antibiotics results in the development of resistance to those specific drugs (Bassetti *et al*., 2022). Additionally, factors such as the use of subpar antibiotics, their easy access in pharmacies, the absence of essential antimicrobial susceptibility testing prior to prescription, administering antibiotics in insufficient doses or for inadequate durations to treat microbial diseases, their use as feed additives, and poor socioeconomic conditions significantly contribute to the acceleration of antibiotic resistance in organisms (Knobler *et al*., 2003).

AMR extends beyond pathogens and infectious diseases. AMR microbes can be found in various components of the environment, both living (biotic) and non-living (abiotic). The resistance microbes are also detected from pristine environments (Nappier *et al*., 2020). Globalization, rising urbanization, extensive pollution, and limited interactions among human populations have all contributed to the emergence of resistance in organisms (Zhu *et al*., 2022). In this challenging environment, resistance genes actively transfer between and within communities of humans and microbes (Liguori *et al*., 2022). The One Health approach to tackling AMR emphasizes the necessity of conducting integrated research involving the environment, humans, and animals to establish effective strategies for controlling the AMR population (McEwen & Collignon, 2018). Water, a chemical compound, possesses the ability to dissolve various substances and is also considered the origin of biological life (Brack, 1993).

Since the dawn of civilization, humans have tended to settle near sources of water, meeting their diverse needs for water in various aspects of life (Wang & Gao, 2020). The expansion of human settlements is leading to increased pollution of water sources and their surrounding areas in low- and middle-income countries (LMICs) (Fuller *et al*., 2022). Basin areas suffer from severe pollution, with direct sewer discharge into water bodies and their transformation into open dumping grounds for all types of waste. Significant mixing of chemicals and antibiotics into these waters is also observed (Wang *et al*., 2020). Such polluted environments create ideal niches for antibiotic-resistant organisms to thrive. Polluted water sources create a pressured environment that enables microorganisms to develop resistance and transfer resistance genes among similar or different entities (Kraemer *et al*., 2019). The biological community, which relies directly on water sources for survival, is at risk as AMR bacteria from these water bodies can readily move into human and animal populations. The rapid emergence of antimicrobial-resistant organisms in these communities is fueled by the excessive use of various antibiotics for treatment and prophylaxis. This situation fosters an environment that facilitates the exchange of resistant bacteria among water, animals, and humans (Graham *et al*., 2019). Although antimicrobial resistance has been acknowledged since 1959, it is only recently that the surveillance of resistance development and its transmission to human communities through various pathways has been actively explored (Ventola, 2015; Murrary *et al*., 2022). Since environmental components are interconnected, there is a high likelihood of genetic transfer within the same species of bacteria. Controlling AMR generation is achievable only by studying all environmental factors within a unified framework (Baquero *et al*., 2019).

The concept of integrated health initially emerged from a unified approach to the health of humans and animals, incorporating both biotic and abiotic elements of the environment (Gyles, 2016). Since 2015, the World Health Organization (WHO) has prioritized the “One Health approach”, as a fundamental strategy for tackling AMR organisms. In 2017, WHO segregated critical pathogens according to antimicrobial drug categories, and by 2022, had completed this classification. Furthermore, in 2021, WHO introduced the tricycle protocol for AMR surveillance across environmental, animal, and clinical settings, using extended spectrum beta-lactamase (ESBL) *Escherichia coli*, resistant to third-generation cephalosporin, as an indicator organism (WHO, 2022). *E. coli*, a Gram-negative organism, possesses dual characteristics as both commensal and pathogenic, and is ubiquitous in distribution. First reported by

Escherich in 1885, *E. coli* has since demonstrated its vigilant presence on every continent (Etymologia, 2015). The various subtypes of this bacterium have the capability to exhibit a wide range of physiological functions, leading to a multitude of infections in different body parts and developing resistance to antimicrobials (Braz *et al*., 2020). *E. coli* can resist a wide array of antibiotics. Globally, *E. coli* strains that exhibit multidrug resistance, extensive drug resistance, and pan-drug resistance pose significant challenges to management with antibiotic therapy (Ozma *et al*., 2022). The recent alarming spread of the ESBL gene in the environment has the potential to spread across the globe (Husna *et al.*, 2023). The whole genome sequence of *E. coli* K-12 was published in 1997 (Blattner *et al*., 1997). Up until June 28, 2023, the National Center for Biotechnology Information (NCBI) database has recorded a total of 215,289 genome types of *E. coli*. Among these, the ESBL gene type *bla CTX M-15*, found in Sequence Type 131, is considered a global pandemic threat. *bla CTX M-15*, a subtype of the *bla CTX M-1* gene, shows distinct resistance to cefotaxime. In addition, the *bla SHV* and *bla TEM* gene types have been frequently reported across diverse ecosystems (Castanheira *et al*., 2021).

1.2 Rationale

Escherichia coli, commonly known as *E. coli*, is widely dispersed across varied environments, including the guts of humans and animals, water and food. Consequently, it plays a crucial role as a marker organism in understanding the transmission pathways of antibiotic resistance (Larrson *et al*., 2023). The detection of extended spectrum beta lactamase (ESBL) genes in *E. coli* originating from various pedigrees offers valuable understanding into how resistance have spread in the ecosystems and the possible paths of transmission between humans, animals, and their surroundings (Castanheira *et al*., 2021). ESBL enzymes have the capacity to deactivate a broad spectrum of beta-lactam antibiotics, such as penicillin, cephalosporins, and monobactams. This ability greatly reduces the usefulness of these vital antibiotics, making infections caused by ESBL producing bacteria remarkably challenging to manage (Husna *et al*., 2023). Considering the extensive utilization of beta lactam antibiotics in human healthcare and agricultural practices, the rise and dissemination of ESBL resistance presents a significant threat to both public health. Mapping out the prevalence and distribution of ESBL *E. coli* across these interconnected environments, could shed light on the pathways for transmission of antibiotic resistance in Nepal. This

information is vital for designing targeted policy, guideline, protocols and practice related interventions to mitigate the spread of resistance and preserve the antibiotics for humankind.

1.3 Objectives

1.3.1 General objective

To characterize extended spectrum beta-lactamase producing *Escherichia coli* from the river basin of Kathmandu valley and correlate its occurrence among water, human urine, and poultry fecal samples.

1.3.2 Specific objectives

i. To isolate, identify and characterize the bacterial isolates from water bodies within river basin of Kathmandu valley
ii. To isolate, identify and characterize the bacterial isolates from urine samples collected at National Public Health Laboratory
iii. To isolate, identify and characterize the bacterial isolates from chicken feces from poultry farms in Kathmandu valley
iv. To evaluate the antibiotic resistance pattern in the *E. coli* isolated from water, poultry and clinical isolates
v. To detect targeted genes responsible for conferring ESBL resistance in ESBL *E. coli* isolates
vi. To investigate the possible linkage between *E. coli* from water, clinical and poultry samples within Kathmandu valley

CHAPTER 2

2. LITERATURE REVIEW

2.1 Antimicrobial Resistance

Organisms naturally produce antibiotics, substances that have the ability to inhibit the growth and metabolism of various other microorganisms. The term 'antibiotics' derives from the Latin words 'anti' (against) and 'bioticus' (relating to life), indicating their ability to suppress life functions (Poirel *et al*., 2018). Global resistance to commonly used antibiotics for treating infectious diseases is increasing, suggesting that failures in treating antibiotic resistance could result in 10 million deaths annually by 2050 (Yam *et al*., 2019).

Antibiotic resistance in bacteria has been documented since 1928, following the use of Penicillin to treat wound infections in humans. Furthermore, commensal bacteria can develop resistance to antibiotics when individuals fail to take the full prescribed course of an antibiotic for their condition (Huttner *et al*., 2013). The misuse of medications through underuse, overuse, and self-medication, along with the easy access to drugs without verifying the pathogens and their resistance profiles, has significantly increased the prevalence of antimicrobial-resistant (AMR) organisms (Nadgir & Biswas, 2023).

Antibiotic resistance in organisms falls into three categories: multidrug resistance (MDR), pan drug resistance (PDR), and extensive drug resistance (XDR). An organism qualifies as MDR when it resists three or more categories of drugs. It achieves PDR status when it shows resistance to all treatment drugs, except for last resort antibiotics like polymyxin and tigecycline. Finally, an organism is considered XDR when it has developed resistance to nearly all antibiotics, leaving very few treatment options (Ozma *et al*., 2022).

In 2019, worldwide, around 4.95 million deaths were attributed to *E. coli* resistant to third generation cephalosporins (Murray *et al*., 2022). Southeast Asian countries experience a notably higher burden of antimicrobial-resistant (AMR) bacteria, with India witnessing some of the highest mortality rates associated with AMR (Yam *et al*., 2019). The pooled prevalence of fecal ESBL *E. coli* carrier in South Asian countries range from 10% - 60% (Bezabih *et al*., 2022). A study carried out in Nepal between 2019 and 2020 found that out of 122 cases of death from infections by multi-drug-resistant Gram-negative bacteria, 4.5% were due to *E. coli* infections (Bhattrai *et al*., 2021). The presence of *E. coli* in infections is often linked to fecal contamination.

Antimicrobial resistant (AMR) *E. coli* strains are increasing and have developed effective survival strategies in humans and animals (Arbab *et al*., 2022). Using targeted microbiological methods to quantify bacterial counts, alongside molecular techniques, can assist in identifying AMR-related genes and understanding their evolution in these organisms (Cave *et al*., 2021).

2.1.1 Antimicrobial resistance in *E. coli*

Commensal *E. coli* is initially susceptible to a broad spectrum of antibiotics. However, through continuous evolution, these organisms have become diverse pathogenic entities, developing antibiotic resistance mechanisms to survive in harsh environmental conditions. As a result, these organisms have acquired resistance to nearly all drug categories used in clinical practice (Poirel *et al*., 2018). The general mechanism of drug resistance adopted by *E. coli* is outlined as below:

i. Penicillin: Two main mechanisms deactivate penicillin: beta-lactamase enzyme production and drug efflux pump activation. Chromosomally located or plasmid-based AmpC beta-lactamases hinder ampicillin's action (Gregova & Kmet, 2020).
ii. Cephalosporins: These broad-spectrum antibiotics are critically important. Plasmid or chromosomally encoded Ampicillin C beta-Lactamase (AmpCs) and Extended Spectrum Beta-Lactamase (ESBLs) enzymes mediate resistance to this group of antibiotics (Zamudio *et al.*, 2022).
iii. Fluoroquinolones: Mutations in the enzymes DNA gyrase and topoisomerase represent significant mechanisms of resistance in *E. coli*. Additionally, efflux pumps, the fluoroquinolone-altering enzyme acetyltransferase (aac(6')-Ib-cr), and Qnr proteins (QnrA, QnrB, QnrC, QnrD, and QnrS) are effectively involved in conferring resistance against this antibiotic class (Gregova & Kmet, 2020).
iv. Carbapenem: The carbapenemase enzyme encoded by VIM-1 was initially detected in *E. coli* from pigs. The enzyme types most frequently reported include NDM-1 and NDM-5. Additionally, IMP-4, OXA-48, OXA-181, and β-lactamase KPC (blaKPC-2) have been isolated from both humans and animals (Poirel *et al*., 2018).
v. Aminoglycosides: Changes in either the 16S rRNA or the S5/S12 ribosomal proteins can lead to heightened resistance to aminoglycosides. Methylation occurring at G1405 and A1408 positions within the A site of 16S rRNA renders aminoglycosides inactive, resulting in resistance to medications such as netilmicin, gentamicin, tobramycin, and amikacin (Gauba & Rahman, 2023). Both animals and

humans exhibit the concurrent presence of the Rmt and ArmA genes, showcasing their resistance capabilities. Enzymes such as acetyltransferases, nucleotidyltransferases, and phosphotransferases further support the inactivation of these compounds (Bodendoerfer *et al*., 2020).

vi. Fosfomycin: Fosfomycin disrupts bacterial cells by inhibiting peptidoglycan metabolism. Resistance to fosfomycin develops through mutations in the glpT, uhpA/T, and murA genes. Plasmid-mediated enzymes that inactivate fosfomycin include fosA, fosH, fosX, and fosB (Ríos *et al.*, 2022).

vii. Tetracycline: Tetracycline resistance is facilitated by various efflux genes such as tetB, tetC, tetD, tetE, tetG, tetJ, tetX, and ribosomal protection genes tetM and tetW (Perewari *et al*., 2022).

viii. Phenicols: Resistance to phenicols emerges through enzymatic modifications of the target compound, efflux pump activations, and rRNA target site methylations. Both the chloramphenicol resistance gene (cmlA) and the florfenicol resistance gene (pp-flo) have been detected in humans and poultry (White *et al*., 2000).

ix. Sulfonamides and Trimethoprim: Mutations in the genes that encode dihydropteroate synthase or dihydrofolate reductase led to resistance to sulphonamide and trimethoprim. Additionally, the presence of Sul and Dfr genes actively mediates resistance to these compounds (Skold, 2001).

x. Polymyxin: The polymyxin resistance gene can reside either on a chromosome or a plasmid. Mutations in the PmrA/B genes of the pmrCAB operon in the PhoPQ two-component system led to resistance to colistin. Additionally, the plasmid-borne mcr-1 gene, which encodes for phosphoethanolamine transferase, modifies the lipid A region of *E. coli*'s lipopolysaccharide, thereby causing resistance to the antibiotic (Moffatt *et al*., 2019).

2.2 *Escherichia coli*

E. coli, a facultative anaerobic Gram-negative bacterium, has gained significant recognition in the field of genomics. This bacterium, short and rod-shaped, measures between 0.5 and 2µm in length and duplicates itself every 30 minutes through binary fission (El-Hajj & Newman, 2015). The motility of bacteria is favored by polar flagella and adhesion enhanced by pili and fimbriae (Wahda & Berg, 2022).

In 1885, Austrian pediatrician Dr. Theodor Escherich discovered a bacterium in the stool of newborns, which he initially named Bacterium coli commune, recognizing it

as a common gut commensal (Meric *et al*., 2016,). By 1895, Migula had renamed it *Bacillus coli*, and it was later renamed again by Castellani and Chalmers in 1919 to *Escherichia coli*, a name that has since been universally adopted alongside the equivalent *Escherichia/Shigella coli*. This bacterium is classified within the Kingdom: Bacteria, Division: Pseudomonadota, Class: Gammaproteobacteria, Order: Enterobacterales, and Family: Enterobacteraceae (NIH, 2024). It is divided into six phylogenetic group (A, B1, B2, D, E, F) and five clades (CI-CV) (Cobo-Simón *et al*., 2023). The proposal, based on the genomic hybridization technique, suggests reclassifying the four *Shigella* species; *E. sonnei*, *E. boydii*, *E. flexneri*, and *E. dysenteriae* under the single category of *E. coli* BIO (Cobo-Simon *et al*., 2023).

E. coli tests negative for oxidase, citrate, urease, and Voges-Proskauer reactions but positive for catalase, methyl red, and indole, showcasing its fermentative abilities. It ferments glucose and lactose, forming pink-colored colonies on MacConkey agar and displaying characteristic green metallic sheen colonies on eosin methylene blue agar media (Batt, 2014; Mishra *et al*., 2017).

The strain *E. coli* K-12 is typical commensal type which is not present in environment (Kuhnert *et al*., 2000). The uidA gene, responsible for β-glucuronidase production, and the ferrichrome receptor gene (fhuA) are found in *E. coli*. Additionally, the Type 1 Fimbria gene (fimA) occurs in both commensal and pathogenic strains of *E. coli* (Molina *et al*., 2015; Kuhnert *et al*., 2000). *E. coli* possesses six types of Universal stress protein (Usp) gene viz. UspA, UspC, UspD, UspF, UspG and UspE (Siegele, 2005).

2.2.1 *E. coli* in human

In humans, *E. coli* acts as both a benign resident and a pathogen. It helps produce Vitamin K2, enhances metabolic processes, strengthens the immune response, and secretes bacteriocins to ward off competing pathogenic strains (Kaushik *et al*., 2023; Mazurek-Popczyk *et al*., 2020). *E. coli*, a pathogenic bacterium, is divided into ten biovars known for causing both intestinal and extraintestinal diseases The intestinal pathogenic forms of *E. coli* (IPEC) are categorized into several types: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC), and avian pathogenic *E. coli* (APEC). These are known to cause diarrhoea with severe outcome (Leimbach *et al*., 2013).

The extraintestinal pathogenic strains of *E. coli* (ExPEC) include those associated with meningitis (MNEC), septicemia (SEPEC), urinary tract infections (UPEC), and avian diseases (APEC) (Robbens *et al*., 2014). These pathotypes are distinguished by their disease symptoms' progression, evolutionary relationships, and the genes they share within pathogenicity islands (Leimbach *et al*., 2013).

Ongoing DNA recombination, facilitated by transposable genes, plasmids, and phages through mutations or horizontal gene transfer, drives the evolution of virulent *E. coli* strains from commensal types (Puente & Finlay, 2001).

The first pathovar, EPEC, was identified in 1940 as the cause of summer diarrhea in infants. In 1982, EHEC was identified from cases of human bloody diarrhea. The serotype O157:H7 is associated with hemorrhagic colitis and kidney failure in children. ETEC causes diarrhea through toxins similar to those of the cholera bacterium, making it a common cause of traveler's diarrhea (Pakbin *et al*., 2021).

Pathotypes are categorized into two groups based on their utilization of the Type III secretion system (T3SS): those that deploy the T3SS (EHEC, EPEC, EIEC) and those that do not (ETEC, EQEC, STEC, DAEC, AIEC) (Clements *et al*., 2012).

Diarrheagenic *E. coli* causes approximately 5 million deaths annually, including 43,000 children under the age of five. The lack of access to safe drinking water and inadequate sanitation practices, especially in low and middle income countries, significantly contributes to the spread of infections (Gomes *et al*., 2016).

Urinary tract infections (UTIs) lead to increased bacteremia incidents, predominantly caused by *E. coli*. These infections may originate in hospitals or the community (Zhou *et al*., 2023). Although they affect both sexes, females face a higher risk due to the anatomical closeness of the rectum to the vulva (Scholes *et al*., 2000). UPEC affects 4 million people globally, with a 60.14 % increase since 1990. ASMR due to UTI increased from 29.9 to 66.7 in South Asian region within 1990 - 2019; indicating a higher rate of recurrence during this period (Yang *et al*., 2022).

In 1988, ETEC was reported in a Kathmandu hospital from patients with diarrhea and travelers visiting Nepal (Taylor *et al*., 1988). Matsushita *et al.* in 2001 reported fluoroquinolone resistance ETEC. In 2005, *E. coli*, the organism showing the lowest sensitivity to fluoroquinolone antibiotics, was identified in urinary tract infections with a prevalence of 49% in hospitals across the Kathmandu Valley (Jha & Bapat, 2005). UTIs are identified in 23.1% to 37.45% of patients visiting tertiary care hospitals in Nepal (Ganesh *et al*., 2019). A variety of antimicrobial agents, including sulfonamides,

fluoroquinolones, nitrofurantoin, macrolides, β-lactams, and carbapenems, are prescribed for UTI treatments based on the severity of the infection (Zagaglia *et al*., 2022).

2.2.2 *E. coli* in poultry

E. coli is characterized as a commensal organism within the gastrointestinal tract of chickens, playing a role in the synthesis of essential vitamins (Mourand *et al*., 2020). As a pathogen, APEC causes various extraintestinal diseases in chickens, collectively termed colibacillosis (Apostolakos *et al*., 2021). The disease causes substantial economic losses, lowers productivity, and necessitates the widespread use of antibiotics (Kromann & Jensen, 2022). Poor hygiene, overcrowded environments, contaminated feed practices, insufficient veterinary services, and substandard farming practices contribute to worsening the disease's impact (El-Tahawy *et al*., 2022).

Virulence genes associated with adhesins, invasins, iron acquisition systems, protectins, toxins, and various other types-often included in the ets operon, microcin, colicin, and cell cycle inhibitor factors-play a key role in the pathogenesis of APEC (Kravik *et al*., 2023). Strain types ST95 and ST131, identified as foodborne pathogens, originate from animal sources (Kathayat *et al*., 2021).

E. coli primarily resides in the gastrointestinal tract as its main habitat and adapts to the external environment as a secondary residence. The design and management of poultry farms create an ideal environment for APEC to thrive (Stromberg *et al*., 2017). *E. coli* of the commensal type can be obtained from the cloacal swab, cecum, or litter of chickens (Dawadi *et al*., 2021).

Veterinary practices currently use antibiotics such as β-lactams (penicillin and cephalosporins), aminoglycosides, tetracyclines, sulfonamides, and fluoroquinolones for treating colibacillosis and for prevention (Aberkane *et al.*, 2023).

Traditional farming practices, emphasizing conventional breeding and maintenance, sustain the poultry industries in low and middle-income countries (Birhanu *et al.*, 2023). In low and middle income countries, the rapid growth of poultry industries is expected to lead to a 67% increase in antibiotic use by 2030 (Hedman *et al.*, 2020*)*. The estimated prevalence of *E. coli* isolation from poultry farms ranges between 55 % and 91 % in south Asian countries (Dawadi *et al*., 2021).

From the National Disease Investigation Laboratory in Chitwan, 94 % of *E. coli* isolated from liver samples of chickens suspected of colibacillosis were multidrug-resistant. The

highest sensitivity was to amikacin (84 %), and the lowest was to ampicillin (98 %) (Subedi *et al*., 2018). *E. coli* exhibiting 100 % resistance to amoxicillin antibiotics has been reported in chicken meat according to a study conducted in eastern Nepal (Bantawa, *et al*., 2019). In Nepal, poultry farming is widely adopted as a source of income generation. However, the substandard production and distribution practices increase the likelihood of isolating pathogenic bacteria such as *E. coli* from poultry farms, slaughterhouses, and butcher shops (Thapa Shrestha *et al*., 2020). *E. coli* was the most frequently recovered isolate from broiler birds in Nepal. Among these isolates, 37.44 % were multidrug-resistant (MDR), exhibiting the highest rates of resistance to ciprofloxacin, trimethoprim, and sulphamethoxazole (Pal *et al*., 2022). Avian pathogenic *E. coli*, making up 91 %, have been identified in broilers and layers in Nepal. The pathogenicity determining gene fin (H) was associated with all the *E. coli* isolates detected in poultry (Bhattarai *et al*., 2023).

2.2.3 *E. coli* in environment

E. coli can grow, replicate, exchange genetic material, and colonize new hosts across generations, despite facing challenging environmental conditions (Petersen & Hubbart, 2020). *E. coli* acquires micro-elements and thrives in a wide temperature range, surviving in low-oxygen environments and breaking down organics for nutrition, facilitating its global presence (Ishii & Sadowsky, 2008). *E. coli* serves as a marker for fecal pollution in food and water supplies, gaining entry through soil contaminated with feces. The practice of discharging sewage directly into water bodies has escalated the levels of *E. coli* in river waters, which in turn can seep into groundwater (Jain, 2023).

E. coli is the members of total coliforms in Enterobacteriaceae family, commonly used as indicators of water quality including several genera such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia*, and *Hafnia*. The presence of total coliforms in water does not inherently mean the water is harmful, it signals potential fecal contamination (Charles *et al*., 2020). Fecal coliforms are a group of bacteria predominantly found in the intestines of warm-blooded animals, including humans. These bacteria serve as key indicators of fecal contamination in water sources, making them crucial for assessing environmental and public health risks. The most well-known organism within this group is *E. coli*, which is often used as a primary indicator due to its abundance in the intestinal tract (Xu *et al*., 2022). The term "thermotolerant *E. coli*" refers to strains of *Escherichia coli* that can survive and grow at higher temperatures, typically around

44.5°C, which is above the optimum temperature for most *E. coli* strains (which is around 37°C). This characteristic is often used in microbiology to differentiate *E. coli* from other coliform bacteria in water testing, as it indicates the presence of fecal contamination (Hachich *et al*., 2012).

The presence of the glycosyltransferase gene (ycjM), exclusive to intestinal *E. coli*, helps distinguish it from *E. coli* species that have naturalized to environments outside the intestine (Yuan *et al*., 2018).

The presence of the uidA, gadAB, and fimH genes in both *E. coli* and the *Escherichia* clade highlights the conservation of lineage traits and dynamic adaptation to various niches (Jang *et al*., 2017). *E. coli's* symbiotic relationships with microorganisms, nutrient assimilation, temperature adaptability, biofilm production, and coexistence with local organisms bolster its survival in diverse environments beyond endothermic hosts (Osińska *et al*., 2023). Environmental stress can lead to the emergence of diverse *E. coli* strains from a single organism, exhibiting significant variations in phenotypic traits (Janezic *et al*., 2013). Organisms adapt to harsh environments by mutating, acquiring plasmids, transposons, and mobile genetic elements through horizontal gene transfer. They can transmit highly conserved genetic loci that express heat resistance traits to their offspring and resist toxic substances (Kamal *et al.*, 2021). Rapid urbanization and centralization in numerous Asian countries have significantly contaminated their river ecosystems. The direct discharge of untreated sewage and medical waste into these waters has increased the presence of antibiotic-resistant pathogenic *E. coli*. (Bong *et al*., 2022). Antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) are frequently found in polluted water. *E. coli* was often identified as the main marker in detecting antimicrobial resistance (AMR) through both phenotypic and genotypic approaches (Ibrahim *et al*., 2023). Enteric bacteria associated with humans have been confirmed in the surface water of the Bagmati River, indicating probable fecal contamination in the river water (Tandukar *et al*., 2018). The biological oxygen demand in the Bagmati River of Kathmandu is elevated, and the ratio of dissolved oxygen to chemical oxygen demand has decreased due to waste deposition (Panthi *et al*., 2017). In Nepal, water pollution poses a critical challenge, primary cause of water pollution in these areas is human activity (Karn & Harada, 2001). The study conducted from water sample of Bagmati river showed, of all the isolates, 70 % were *E. coli*, and among these, 80 % were resistant to multiple drugs (Shrestha *et al*., 2023).

2.3 Extended spectrum beta lactamase (ESBL) *E. coli*

Extended-spectrum beta-lactamases (ESBLs) are enzymes that neutralize the effects of penicillin, first and second generation cephalosporins, and aztreonam, thus conferring resistance. However, they do not inherently deactivate cephamycin or carbapenems. Substances like clavulanic acid, however, can inhibit them (Paterson & Bonomo, 2005). *E. coli* produced ESBL enzyme before the introduction of the first antibiotic, indicating a natural mechanism for developing resistance to inhibitory substances (Bradford, 2001). ESBL producing *E. coli* poses a significant threat to human health because they exhibit strong resistance to a wide range of antibiotics, making them difficult to treat. Thus, the WHO has classified them as critically important pathogens (Sunarno *et al*., 2023).

In 1968, Sawai *et al*. began classifying beta-lactamase enzymes into three types based on their serological properties. Richmond and Jack expanded this classification to five types in 1970, using ionization and enzymatic properties. Richmond and Sykes reclassified these five types in 1973 based on the enzymatic recognition of specific substrates. Sykes and Matthew further differentiated these enzymes into five groups (I, II, III, IV, and V) in 1976, based on ionization potential (Bush, 2018).

Ambler proposed the classification of beta-lactamases in 1980, which divides them into four groups: A, B, C, and D. This classification system helps researchers understand the different mechanisms and characteristics of these enzymes to combat antibiotic resistance effectively (Chaudhary & Aggarwal, 2004).

Bush and Jacoby proposed a reclassification of beta-lactamases based on their substrate specificity and inhibitory activity. They categorized resistance to cephalosporins as 1(C) and 1e (C), resistance to penicillin as 2a (A), 2b (A), and 2br (A), resistance to extended-spectrum cephalosporins as 2be (A), 2ber (A), 2de (D) and 2e (A), and resistance to carbapenems as 2df (D), 2f (A), 3a(B1) and 3b(B2) (Bush & Jacoby, 2010).

The ST131 sequence type of ESBL *E. coli* is widely recognized for its detection and expression of various pathogenic phenomena. The most commonly observed gene types associated with ESBL include *CTX M*, *TEM*, and *SHV*, which are found across diverse ecosystems. The gene types that represent ESBL families include *TEM*, *SHV*, *IRT*, *CMT*, *CTX M*, *GES*, *PER*, *VEB*, *BEL*, *TLA*, *SFO* and *OXY* (Castanheira *et al*., 2021).

i. *bla CTX M*: In recent years, the *CTX M* beta lactamase has surpassed the *bla TEM* and *bla SHV* lactamases in prevalence. These enzymes are known for their ability

to rapidly break down cefotaxime and ceftazidime antibiotics. They have gained significant importance in the field of clinical medicine as they have been linked to hospital-acquired infections and environmental spread (Jones *et al*., 2009). The *bla CTX M* gene type is distinct from the *bla TEM* and *bla SHV* gene types. Unlike sulbactam or clavulanate, tazobactam is highly effective in deactivating these enzymes. The *bla CTX M* enzymes were initially discovered and identified in *Kluyvera* spp in 1980 and have since been classified into five different categories (Shaikh *et al*., 2015). *CTX M* gene types are widely distributed and considered pandemic. The most frequently reported types of *E. coli* belong to five families: *CTX M-1*, *CTX M-2*, *CTX M-8*, *CTX M-9*, and *CTX M-25*. Two newer categories, *CTX M-74* and *CTX M-75*, have been identified and are associated with the *bla* gene found in *Klyuvera ascorbata* and *Kluyvera georgia* (Bialvaei *et al*., 2016). *Klyuvera ascorbata*, which is commonly found in the roots of plants, has been identified as having intrinsic resistance to third generation cephalosporins encoded in its chromosome. The insertion sequence ISEcp1 is known to play a crucial role in facilitating the transfer of resistance genes to *E. coli*, which is often associated with human infections (Bevan *et al*., 2017).

ii. *bla TEM*: The name of the enzyme, *TEM-3*, was derived from the patient named Temoneira in 1989. According to Castanheira *et al*. (2021), there are over 243 known gene types in this enzyme family. While *TEM-1* and *TEM-2* are known to deactivate penicillin and cephalosporins, *TEM-3* and its derivatives are specifically effective against cefotaxime and ceftazidime antibiotics. The arrangement of amino acids such as Glutamate, Lysine, Serine, Histidine, Glycine, and Arginine play a significant role in determining the different types of TEM enzymes (Bradford, 2001). The variant types *bla TEM-48*, *bla TEM-49*, *bla TEM-68*, and *bla TEM-25* are all known to have resulted from mutations in the *bla TEM-1A* gene (Baraniak *et al*., 2005).

iii. *bla SHV*: The beta-lactamase enzyme known as *SHV* is derived from the *SHV-1* gene type found in *Klebsiella pneumoniae. E. coli* is known to acquire the *SHV-1* gene through horizontal transfer mechanisms, which is highly efficient (Jones *et al*., 2009). The *SHV-1* gene, also known as Sulfhydryl reagent variable, was first identified in *E. coli* in 1970 and is commonly found on plasmids. These enzymes are classified into 2b, 2br, and 2be classes, with the latter two classes specifically encoding for ESBL genes (Liakopoulos *et al*., 2016).

In 2006, *E. coli* strains producing extended-spectrum beta-lactamase (ESBL) were reported at Tribhuvan University Teaching Hospital in Kathmandu. The prevalence of ESBL-producing *E. coli* accounted for 11.6 % (Pokharel *et al*., 2006). In 2020, a 100% resistance to amoxicillin and third-generation cephalosporins was observed in MDR ESBL producing *E. coli* strains in hospitals (Thapa Shrestha *et al*., 2020). The presence of *bla CTX M*, bla *TEM*, and bla *SHV* plasmids has been reported in clinical settings in the years 2021 and 2023 (Sah *et al*., 2021; Chaudhary *et al*., 2023).

ESBL-producing *E. coli* were reported in poultry and human from the Kaski district of Nepal, with *bla CTX M-15* being the most prevalent gene detected. None of the isolates were found to be carbapenemase producers. Among the isolated *E. coli* strains, two belonged to the same clade, C (Subramanya *et al*., 2021). The detection rates of colistin resistance in *E. coli* from poultry and clinical sources were 31.6 % and 21.4 %, respectively. The gene responsible for colistin resistance, *OXA-48*, was identified in both clinical and poultry samples (Muktan *et al*., 2020). The study conducted along the Manohara river in the Kathmandu valley, involving samples from humans, animals, and the environment, identified *E. coli* with the bla *SHV* gene in all settings. This finding suggests the possibility of transmission of similar organisms across these interconnected ecosystems (Young *et al*., 2022).

2.4 AMR, ESBL *E. coli* and ESBL *E. coli* gene detection

Detection of antimicrobial resistance (AMR), extended spectrum beta lactamase (ESBL) producing *E. coli*, and ESBL associated genes is crucial for understanding the prevalence, transmission dynamics, and mechanisms of resistance, which is essential for effective surveillance, prevention, and control of antibiotic resistance (Ribeiro *et al*., 2024).

2.4.1 Detection of antimicrobial resistance (AMR)

Detection of AMR involves laboratory testing of bacterial isolates to determine their susceptibility or resistance to specific antibiotics. These tests identify bacterial isolates that are resistant to one or more antibiotics, providing insights into the prevalence, patterns, and trends of resistance in different settings and populations (Yamin *et al*., 2023). Methods commonly used for AMR detection include culture-based methods, such as disk diffusion, agar dilution, and broth microdilution, and molecular methods, such as polymerase chain reaction (PCR) and whole-genome sequencing (Salam *et al*., 2023).

The Clinical and Laboratory Standards Institute (CLSI) guidelines categorize antibiotics into several groups to optimize antimicrobial susceptibility testing and reporting. Group A (Primary Agents) includes the primary antibiotics recommended for routine testing and reporting for all bacterial isolates of a specific organism group. These agents are typically the most effective and safest options for treating common infections, and routine testing ensures timely results, facilitating swift clinical decisions. Group B (Secondary Agents) consists of antibiotics tested selectively and reported in specific situations, serving as backup options when Group A agents are ineffective due to resistance or patient-specific factors like allergies. They are essential for treating certain pathogens or infections where primary agents are unsuitable. Group C (Supplemental Agents) are tested and reported for specific organisms or infections, often requiring additional criteria to justify their use. These agents provide targeted therapy in particular clinical scenarios and help manage cases with known resistance patterns or complex infections. Group U (Urinary Tract Infection Agents) are tested and reported only for urinary tract infections, tailoring antibiotic choice specifically for treating UTIs and optimizing testing resources for these infections. Finally, Group O (Optional Agents) includes antibiotics that may be tested and reported based on institutional needs, formulary restrictions, or local antibiogram data, allowing adaptation to the specific needs and policies of different healthcare settings and addressing local resistance patterns to tailor therapy accordingly (CLSI, 2020). As of May 2024, the WHO has updated its guidelines on antibiotic categorization to manage their use effectively and mitigate the growing threat of antimicrobial resistance. This classification system helps prioritize antibiotics based on their importance in human medicine and their impact on resistance, with additional considerations for their use in both human and animal health (WHO, 2024).

Critically important antimicrobials (CIA) are vital for treating serious bacterial infections in humans and often serve as last-resort options with few or no alternatives. They are critical for treating severe infections and include antibiotics such as third and higher generation cephalosporins, macrolides, quinolones, and glycopeptides. Highly important antimicrobials (HIA) are important for treating significant bacterial infections and play a key role in medical treatment, though they may have some alternatives available. Examples include aminoglycosides, second-generation cephalosporins, and penicillin. Highest priority critically important antimicrobials (HPCIA) are a subset of CIAs that are of the highest priority due to their critical role in

human medicine and the high risk of resistance development. These antibiotics, such as carbapenems and polymyxins (including colistin), are essential for treating severe infections with very limited alternatives. Important antimicrobials (IA) are used to treat a range of bacterial infections but are considered less critical than CIAs and HIAs. They have more alternatives available and are generally used for less severe infections, including antibiotics like tetracyclines, first-generation cephalosporins, and older penicillin. Medically important antimicrobials (MIA) are of medium importance, used for treating infections where other options may be available. Their use is less frequent and often for less severe conditions, with examples including sulfonamides and trimethoprim. The categorization also considers whether these antibiotics are used exclusively in humans or in both human and animal health. Human use only antibiotics are reserved solely for human medicine, helping manage resistance more effectively and preserving their efficacy. In contrast, antibiotics used in both human and animal health require coordinated stewardship efforts to mitigate resistance that could arise from veterinary use and impact human health (WHO, 2024).

2.4.2 Detection of ESBL producing *E. coli*

Detection of ESBL producing *E. coli* involves laboratory testing of *E. coli* isolates to confirm the production of ESBL enzymes as a screening test with cephalosporins. Phenotypic methods, such as double-disk synergy test and combination disk tests, are commonly used for ESBL detection, along with molecular methods, such as PCR, for confirming the presence of ESBL genes (Rawat & Nair, 2010). ESBL-associated genes, such as *CTX-M, TEM, and SHV* are among the most prevalent beta-lactamase genes found in *E. coli*. Detection of ESBL-associated genes involves molecular methods, such as PCR, multiplex PCR, and DNA sequencing, to identify and characterize specific genes or gene variants in bacterial isolates (Verschuuren *et al*., 2021).

Detection of AMR, ESBL producing *E. coli*, and associated genes is crucial for understanding and combating antibiotic resistance. Through testing and analysis, resistant bacteria and their transmission can be tracked, informing surveillance and interventions to preserve antibiotic effectiveness and public health (Ahsan *et al*., 2022).

2.5 Antimicrobial surveillance

Antimicrobial surveillance involves the systematic collection, analysis, and interpretation of data on the resistance patterns of microorganisms to antimicrobial

agents. This process is crucial for understanding the spread of antimicrobial resistance (AMR), informing treatment guidelines, and shaping public health interventions.

2.5.1 Global antimicrobial surveillance

The WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS), launched in 2015, standardizes AMR surveillance globally, providing a unified platform for data sharing. It harmonizes data collection and reporting, focusing on human health while integrating data from animal health, food, and the environment. Countries submit data on pathogens, resistance patterns, and antimicrobial use, which GLASS consolidates into a global repository accessible to researchers and policymakers. GLASS offers technical support and training, publishes annual reports, and influences public health policies. Despite challenges like limited resources and data quality, GLASS aims to expand, incorporate new technologies, and strengthen global collaboration to combat AMR (WHO, 2022).

2.5.2 Tricycle protocol

The model targets monitoring one indicator, the presence and prevalence of ESBL-producing *E. coli*, across human, animal, and environmental sectors using the "Tricycle protocol" developed by WHO (2021).

i. Focused monitoring on ESBL-producing *E. coli*: The model targets monitoring one indicator, the presence and prevalence of ESBL-producing *E. coli*, across human, animal, and environmental sectors using the "Tricycle protocol" developed by WHO (2021).
ii. Data collection and analysis: The Tricycle protocol facilitates standardized data collection, analysis, and reporting on ESBL-producing *E. coli* by employing three components or "wheels" that capture different aspects of its transmission and spread: prescribing indicators wheel, supply indicators wheel, and stewardship indicators wheel (Appling *et al*., 2023).
iii. Standardized methodology: The tricycle protocol provides standardized methods, tools, and indicators for data collection, enabling consistent, comparable, and reliable assessment of ESBL-producing *E. coli* across different sectors and countries (Ruppe *et al*., 2023).
iv. Data collection from human, animal and environmental sources: The initiative collects data on ESBL-producing *E. coli* from clinical samples in humans, animals,

and environmental samples from water, soil, and food to identify sources, transmission pathways, and hotspots (Pormohammad *et al*., 2019).

v. Regular reporting and analysis: The collected data is regularly analyzed, interpreted, and reported to generate insights, trends, and recommendations for informed decision-making, policy development, and interventions to combat ESBL-producing *E. coli* effectively (McDonald *et al*., 2021).

2.5.3 Key components of laboratory-based AMR surveillance

A national antimicrobial resistance (AMR) surveillance system typically comprises three fundamental elements: a central coordinating hub, at minimum one national reference laboratory, and one or multiple surveillance locations. Information collected at these surveillance sites is channeled through the national coordinating center before being transmitted to GLASS (WHO, 2021). Elements of antimicrobial resistance (AMR) surveillance are as below:

i. Sample collection: Samples, such as blood, urine, sputum, and other clinical specimens, are collected from patients showing signs of bacterial infections. These samples are then cultured to isolate the bacteria causing the infection (Malania, 2021).

ii. Isolation and identification: In the laboratory, bacterial isolates are cultured and identified to determine the species and strain of the bacteria. This step is crucial as different bacteria have varying resistance patterns (WHO, 2015) .

iii. Antimicrobial susceptibility testing (AST): Isolated bacteria undergo testing against a range of antibiotics to ascertain their susceptibility or resistance. This process aids in discerning the effectiveness of antibiotics against the bacteria and identifying those to which the bacteria are resistant, serving the purposes of antimicrobial resistance (AMR) surveillance (FAO, 2019).

iv. Data analysis and reporting: The collected data from AST results are analyzed to identify trends, patterns, and emerging resistance profiles. Regular reports are generated to provide insights into the current state of antimicrobial resistance in Nepal (Maharjan *et al*., 2023).

v. Feedback and intervention: The surveillance findings are shared with healthcare providers, policymakers, and other stakeholders to guide antibiotic prescribing practices, develop guidelines, and implement interventions to combat antimicrobial resistance (Aboushady *et al*., 2023).

2.5.4 One Health approach

The One Health approach for AMR surveillance is vital for addressing the complex and interconnected nature of antimicrobial resistance. By integrating efforts across human, animal, and environmental health sectors, this approach provides a comprehensive understanding of AMR and facilitates the development of effective strategies to combat it.

i. Intersectoral collaboration: The initiative fosters collaboration and coordination among human health, animal health, and environmental sectors to address ESBL-producing *E. coli* comprehensively and holistically (Olufadewa *et al*., 2021).
ii. Integration of Data and Insights: By integrating data and insights from different sectors, the initiative aims to provide a more holistic understanding of ESBL producing *E. coli* transmission dynamics, sources, and risks across human, animal, and environmental domains (Djordjevic *et al*., 2024).

2.5.5 Laboratory based AMR surveillance in Nepal

Laboratory based AMR surveillance is a systematic approach to monitor and track the emergence and spread of antimicrobial resistance in bacterial pathogens (Comelli *et al*., 2024). In Nepal, an initiative commenced in 1999 with the establishment of focal center at National public health laboratory (NPHL) in Teku for AMR surveillance. As a part of this initiative, ESBL producing *E. coli* was included in the program in 2009 (Acharya & Wilson, 2019). The One Health hub (OH Hub) in Nepal was established in 2013 as a collaborative platform to address issues related to human, animal, and environmental health through a One Health approach. This approach recognizes the interconnectedness of human health, animal health, and the environment and emphasizes the need for collaborative, interdisciplinary efforts to address complex health challenges (Mckenzie *et al*., 2016).The national antimicrobial resistance containment action plan, Nepal, 2016 is a strategic framework developed by the government of Nepal to address the growing threat of antimicrobial resistance in the country. This action plan outlines the priorities, objectives, strategies, and interventions required to contain and mitigate antimicrobial resistance across human health, animal health, and environmental sectors in Nepal (Ministry of Health, GoN, 2016). Nepal's enrollment in GLASS in 2018, with NPHL as the NCC and NRL, demonstrates its commitment to addressing antimicrobial resistance comprehensively and collaboratively, contributing to global AMR surveillance efforts, and promoting

responsible antibiotic use for current and future generations (WHO, 2023). The Fleming fund country grant for Nepal (2018 - 2020) significantly boosted AMR surveillance capacity, backing government health systems, promoting a One Health approach, and bolstering capacity building and data management. It enhanced AMR data quality, spurred collaboration, and supported evidence-based actions against antimicrobial resistance in Nepal (Acharya and Wilson, 2019). Nepal's National Action Plan for Antimicrobial Resistance, 2021-2026, developed using a One Health approach, represents a comprehensive, strategic, and collaborative framework to address the complex challenge of antimicrobial resistance in Nepal (MoHP, 2024). The Bagmati River Basin Improvement Project is a comprehensive initiative that addresses environmental, social, and economic issues in the Kathmandu Valley. Its successful implementation is crucial for the sustainable development of the region, ensuring a cleaner environment, better management of water resources, and improved quality of life for the residents (MoUD, GoN, 2023). By integrating efforts across human health, animal health, food safety, and the environment, the action plan aims to contain and mitigate AMR, safeguard public health, promote sustainable development, and foster collaboration, coordination, and partnership among stakeholders, sectors, and disciplines to combat AMR effectively for the well-being of current and future generations in Nepal (Chua *et al*., 2021).

2.6 The critical interconnection of AMR: humans, environment, and food Systems

WHO's Integrated Global surveillance on ESBL-producing *E. coli*, launched in 2021, represents a comprehensive and collaborative initiative to monitor and combat the spread of ESBL-producing *E. coli* globally using a One Health approach (WHO, 2021a). By targeting monitoring of ESBL-producing *E. coli* across human, animal, and environmental sectors and employing the Tricycle protocol for standardized data collection and analysis, the initiative aims to enhance understanding, inform decision-making, and support interventions to combat ESBL-producing *E. coli* effectively, mitigate its impact, and safeguard public health across the globe (Gay *et al*., 2023). This initiative employs a One Health approach, targeting monitoring of ESBL-producing *E. coli* across the human, animal, and environmental sectors to understand its transmission dynamics, patterns, and risks comprehensively (Nzietchueng *et al*., 2023).

2.6.1 Bridging the AMR nexus: humans, environment, and food

Detecting and understanding the interlinkage of antimicrobial resistance (AMR) across humans, the environment, and food is crucial for several reasons:

i. Public health impact: AMR poses a significant threat to public health globally. Understanding how resistance spreads among humans, animals, and the environment helps in devising effective strategies to mitigate its impact on human health (Salam *et al*., 2023).

ii. One Health approach: AMR is a classic example of a One Health issue, where the health of humans, animals, and the environment are interconnected. Detecting and understanding the interlinkages of AMR in these three domains allows for a comprehensive approach to tackling the issue (Velazquez-Meza *et al*., 2022).

iii. Transmission dynamics: Identifying how AMR spreads between humans, animals, and the environment is essential for understanding transmission dynamics. This knowledge is crucial for designing interventions to prevent or slow down the spread of resistant bacteria (Lepper *et al*., 2022).

iv. Food safety: AMR can also spread through the food chain. Antibiotics used in food production can lead to the development of resistant bacteria, which can then be transmitted to humans through consumption of contaminated food. Understanding this link is vital for ensuring food safety and reducing the risk of AMR transmission through the food supply (Samtiya *et al*., 2022).

v. Environmental impact: The environment serves as a reservoir for resistant bacteria and antibiotic residues. Pollution from pharmaceutical manufacturing, agriculture, and healthcare facilities can contribute to the spread of AMR genes in the environment. Understanding this connection is important for mitigating environmental contamination and its consequences for human health (Larsson *et al*., 2022).

vi. Antibiotic stewardship: Understanding the connection between AMR in humans, the environment, and food can guide antibiotic stewardship. This insight helps healthcare providers prescribe antibiotics more wisely, and policymakers enact regulations for responsible antibiotic use (Khadse *et al*., 2023).

vii. Global collaboration: AMR is a global problem that requires collaboration across borders. Detecting and understanding the interlinkages of AMR in different settings can facilitate international cooperation in developing strategies to combat this threat effectively (Munkholm & Rubins, 2020).

Detecting and understanding the interlinkage of antimicrobial resistance in humans, the environment, and food is essential for protecting public health, preserving the effectiveness of antibiotics, and ensuring sustainable practices across healthcare, agriculture, and environmental sectors (Graham *et al*., 2019).

2.7 Limitations of AMR surveillance in LMICs

LMICs are seen as significant contributors to the presence of multidrug-resistant (MDR) organisms in the environment. On the other hand, they lack sufficient resources to effectively monitor antimicrobial resistance (AMR) trends. The system urgently needs capacity building across all sectors of development and encounters significant obstacles in adopting a One Health approach for AMR surveillance (Worsley-Tonks *et al*., 2022). Laboratory facilities: In LMICs, certified quality laboratory facilities are significantly lacking. Laboratories that meet the basic requirements for conducting antibiotic susceptibility testing (AST) to monitor AMR bacteria are rarely established, particularly in urban areas. The lack of electricity, self-sufficient staff, equipment, reagents and media, technical personnel, and quality control measures presents major obstacles to achieving AMR surveillance in LMICs. The limited resources available in laboratory settings hinder the effective monitoring of AST in these laboratories (Nkengasong *et al*., 2018).

i. Quality data: In LMICs, the practices of data collection, tabulation, recording, and the management and preservation of data are rarely carried out. Handwritten record sheets in rural areas are often poorly maintained. The collection of data from these sources often depends on personal relationships with the individuals responsible. Additionally, the hierarchical nature of hospital settings can impede the efficient reporting of results within a specific timeframe (Mitchell *et al*., 2020).
ii. Ineffective governance system: LMICs grapple with the challenges posed by a fragile government system. The ineffectual development, dissemination, and implementation of action-oriented plans and policies are common in these regions. There is also a lack of awareness about the emergence and impact of AMR bacteria at the grassroots level, limited cooperation from local communities, restricted scope for broader public participation, an emphasis on urban-centric plans and programs, and a deficiency in moral and ethical values, all of which obstruct the national implementation of the One Health approach in LMICs (Li *et al*., 2023).

iii. Complexity of AMR Dynamics: The complex and multifaceted nature of AMR dynamics, transmission pathways, and contributing factors in LMICs, including human health, animal health, food safety, and environmental factors, require integrated, interdisciplinary, and One Health approaches to address effectively, which may pose additional challenges to AMR surveillance and control efforts (Ikhimiukor *et al*., 2022)

2.8 Data management

i. Data collection

Data typically involves both qualitative and quantitative measurements. In the current study context, the data involves bacterial counts, antibiotic susceptibility profiles, and genetic markers. According to the objectives of the study, the types of data needed, the sources of these data, and the methods for obtaining them should be ascertained. Standardized protocols are essential to ensure the reproducibility and reliability of results. Sample collection methods, storage conditions, and timing play a significant role in preserving the integrity of the data (Saczynsky *et al*., 2014).

ii. Data archiving

Effective data archiving is crucial for the long-term storage and retrieval of microbiological data. Archiving practices must adhere to established guidelines, such as those provided by the World Health Organization (WHO) or the Clinical and Laboratory Standards Institute (CLSI). Digital data, including genomic sequences, should be stored in secure databases that support version control and regular backups. Physical samples, such as bacterial isolates, should be preserved in conditions that maintain their viability, typically in cryogenic storage. Proper archiving ensures that data remains accessible for future research, verification, and meta-analyses (Rani & Buckley, 2012).

iii. Confidentiality and safety

Personal information related to sample sources must be anonymized to protect individual identities. Access to sensitive data should be restricted to authorized personnel only. Protecting the privacy of individuals and the integrity of the research requires strict adherence to established protocols. Data confidentiality is maintained by anonymizing personal identifiers, ensuring that any information that could directly link

samples to specific individuals or animals is removed or coded. This process prevents any potential breach of privacy and safeguards sensitive information. Additionally, access to confidential data must be restricted to authorized personnel only, with stringent access controls in place. Researchers and staff who handle such data are required to be well-versed in data protection policies, ensuring that they follow the best practices for maintaining confidentiality (Donaldson & Lohr *et al*., 1994).

iv. Data curation

Data curation involves the ongoing management of collected data to ensure its quality, usability, and accessibility over time. In the context of microbiological studies, this includes organizing data in a manner that facilitates easy retrieval and analysis. Metadata—information about the data itself, such as the methods used for collection and processing—should be meticulously documented. Curation also involves updating datasets with new findings, correcting errors, and linking related data across different studies. Effective data curation supports reproducibility and enables the integration of data into larger, collaborative research efforts. Data curation involves actions that ensure research data are fit for use, discoverable, and reusable, aligning with the FAIR principles (Findable, Accessible, Interoperable, and Reusable) (Marsolek *et al*., 2023).

v. Data analysis

Data analysis includes statistical analysis to identify trends, patterns, and correlations, as well as comparing findings against known standards or benchmarks. Techniques such as descriptive statistics, inferential statistics, and various data visualization methods are used to process and present data effectively. The goal is to convert raw data into actionable insights that can inform hypotheses, guide further research, and contribute to the understanding of microbial behavior and resistance mechanisms. In microbiological research, analyzing data through various methods helps in understanding relationships, trends, and patterns. Here's how some common tools and techniques are applied:

i. The chi-square test is a statistical method used to assess the relationship between two categorical variables. It compares observed frequencies in a contingency table to the expected frequencies, which would occur if there were no association between the variables. This test in this study is used for tests of homogeneity, where it compares the distribution of a characteristic across different populations

such as examining whether distribution of *E. coli* in different sources and antibiotic resistance shown by *E. coli* isolates are related. Overall, the chi-square test is a key tool for analyzing categorical data and understanding relationships within that data (McHugh, 2013).

ii. Pearson's correlation is a statistical measure that assesses the strength and direction of the linear relationship between two continuous variables, with the correlation coefficient ranging from -1 to 1. In the context of antibiotic resistance in *E. coli* from different sources such as water, human urine, and chicken fecal samples, Pearson's correlation can be used to determine how resistance levels in these different sources relate to each other (Papageorgiou *et al*., 2022).

iii. A Venn diagram that includes total bacteria, *E. coli*, and ESBL *E. coli* is used for visualizing the relationships between these categories of bacteria. It helps to illustrate the overlap between the total bacterial population, *E. coli*, and ESBL *E. coli*, providing a quick understanding of how many bacteria are specifically *E. coli* and how many of those are ESBL-producing strains (Moktefi & Lemanski).

iv. An Euler diagram is used in identifying shared traits among beta-lactamase genes such as *CTX-M*, *TEM*, and *SHV*. By visually representing the relationships between these genes, the diagram highlights common properties and overlaps, which are crucial for understanding impacts on antibiotic resistance. This clarity is particularly valuable, as it aids in comprehending the distribution and co-occurrence of these enzymes. Overall, the Euler diagram enhances our understanding of how *CTX-M*, *TEM*, and *SHV* beta-lactamase genes relate to one another, offering insights into their roles in antibiotic resistance (Lyalin *et al*., 2020).

CHAPTER 3

3. MATERIALS AND METHODS

All the material and equipment used in the study are enlisted in Appendix I.

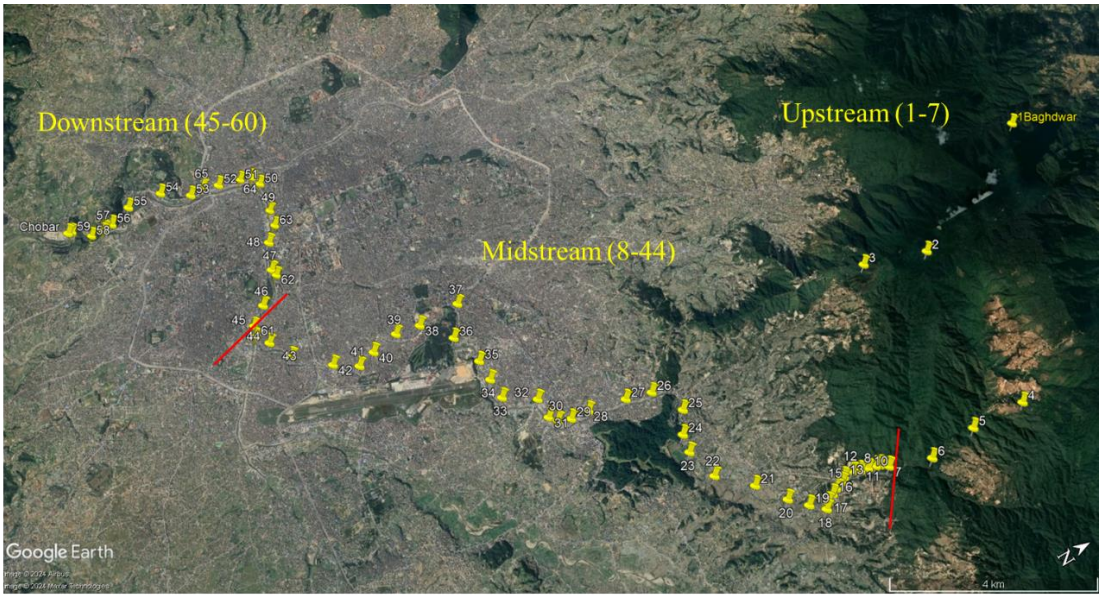
3.1 Study Design

One Health approach based cross-sectional study was conducted from January 2020 to September 2023, focusing on analyzing water samples from the Bagmati river and its tributaries, stone spouts and shallow wells, human urine samples, and poultry fecal samples. The study attempted to evaluate the inter-relation of environment, animal, and human health in understanding the antimicrobial resistance transmission dynamics.

3.2 Study area

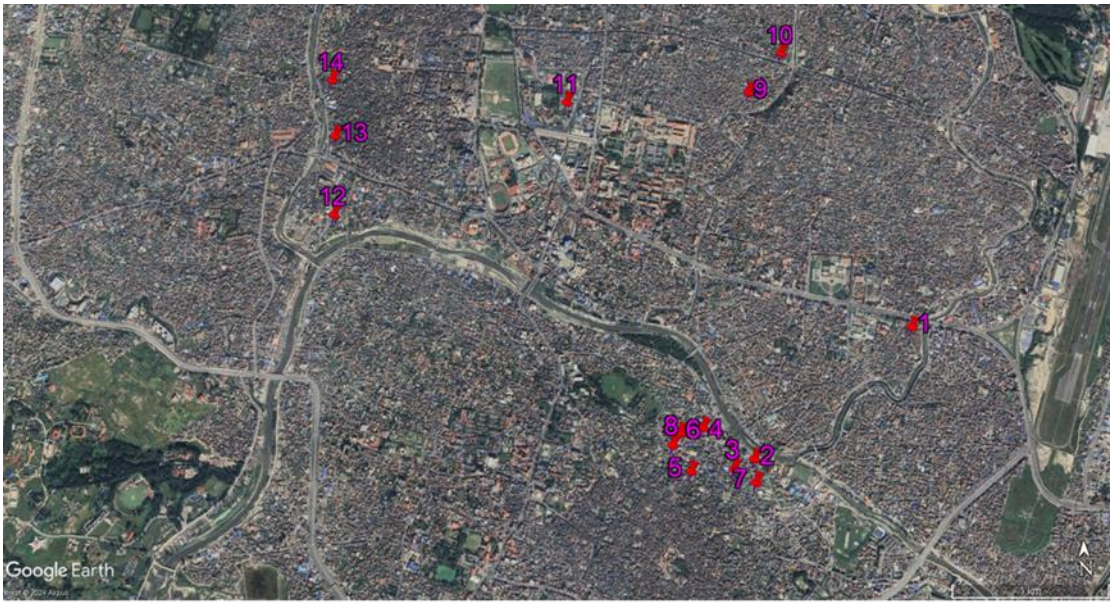
For this study, three areas were identified for data collection. The Bagmati river and its tributaries (Manohara, Dhobikhola, Tukucha, Bishnumati, Balkhu, Nakkhu) along with stone spouts and shallow wells within Kathmandu valley were chosen for environmental sample collection. The National Public Health Laboratory was selected for clinical sample collection. Samples from chickens were collected from poultry farms in the Kathmandu valley that are linked to the river basin of Kathmandu valley. The map of the Bagmati river showing major tributaries, stone spouts, and shallow wells, is presented in Figure 1, Figure 2 and Figure 3. The location map for sample collection from the chicken poultry farms is displayed in Figure 4. Based upon the location of the poultry farms in Kathmandu valley the poultry farms were clustered into A, B, C, D, and E clusters: A- Phutung lying at northwest corner, B- Mulpani lying in northeast corner, E- Sanglaphata lying north corner, C- Suryabinayak- southeast corner, and D- Godawori-south east corner, E- Sanglaphata lying north corner of Kathmandu valley. The coordinates of sample collection points for water samples, poultry feces samples from poultry farms and human urine sample from NPHL laboratory are given in appendix II and appendix III.

Human urine samples received at National Public Health Laboratory, a national reference laboratory for AMR surveillance in Nepal, receives and analyses samples from the priority groups of patients including Kidney Transplant patients, and referral cases from valley hospitals, and referred cases throughout the country, which gives a snap-shot of the country representativeness.



Location map showing water sampling sites along Bagmati river (upstream: 1-7; midstream: 8-44; downstream: 45-60; tributaries: 61, 62, 63, 64, 65, 66)

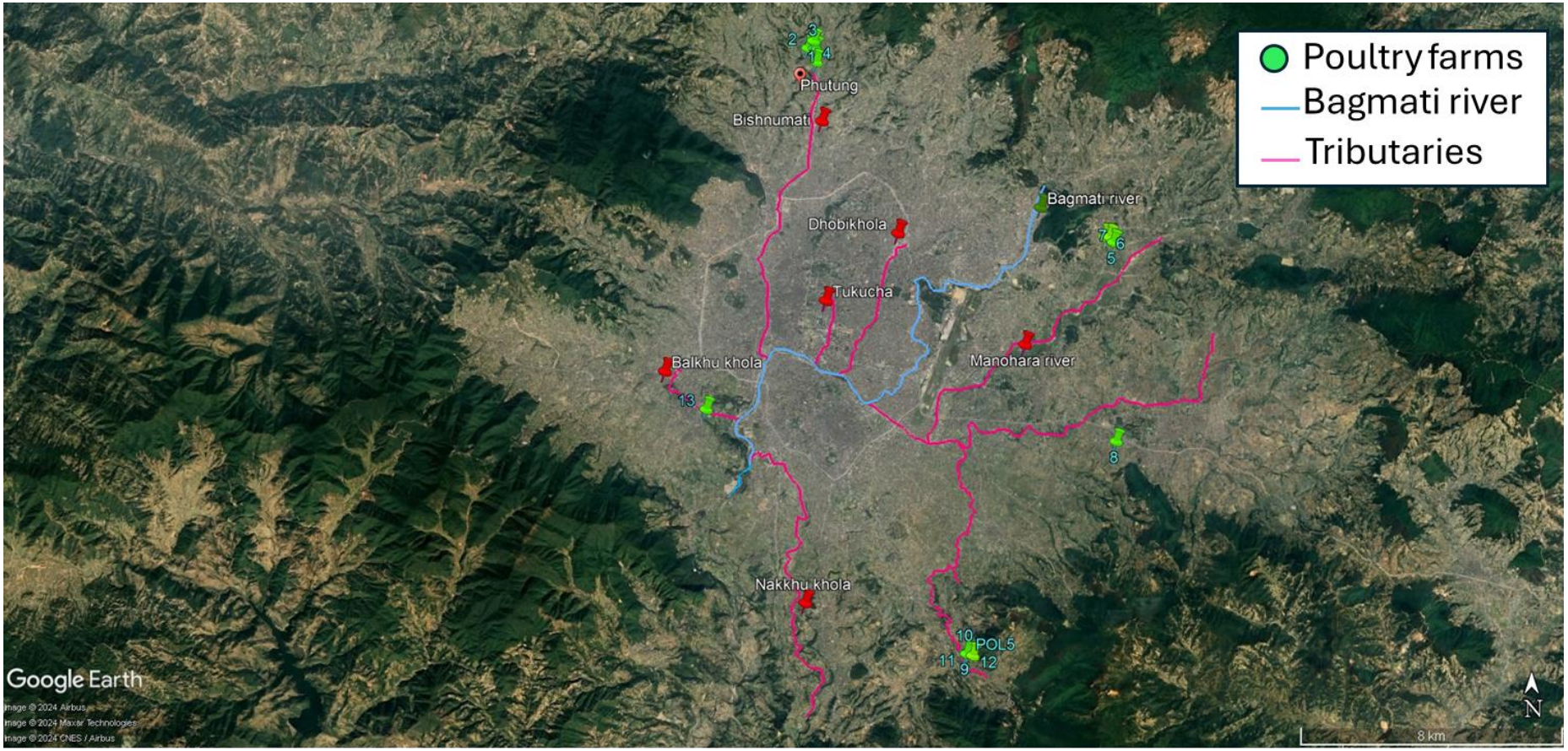
Figure 1: Location map showing sample collection points along Bagmati river and its tributaries



Location map showing distribution of 14 stone spouts

Figure 2: Locations map showing sample collection points for stone spouts along Bagmati river basin

Figure 3: Location map showing sample collection points for shallow well along Bagmati river basin



Location map showing poultry farms within five locations, A: Phutung (1,2,3,4), B: Mulpani (5,6,7), C: Suryavinayak (8), D: Godavari (9, 10, 11, 12), E: Sanglaphanta (13)

Figure 4: Location map showing areas of poultry farms for collection of poultry fecal samples

3.3 Ethical Approval

Before collecting the samples, ethical permission for conducting the research was obtained from the Nepal Health Research Council with protocol number 936/2019P, on January 9, 2020. Administrative approval for upstream sample collection from the Bagmati river was granted by the Department of National Parks and Wildlife Reserve, Government of Nepal on December 7, 2020, with reference number 1572. Written consent was obtained from the poultry farms owners/managers before the sample collection.

3.4 Sample size calculation

The minimum sample size for detecting the prevalence of ESBLs in the given population was calculated using the formula (Equation 1) for one sample with dichotomous outcome (Noordzij *et al*., 2010).

$$N = p(1-p)\left(\frac{Z}{E}\right)^2$$ ……equation 1

Here,

N= sample size

Z=1.96 for 95% confidence interval for unknown population p

E= margin of error= 0.05

When,

Range of p=0 to 1

p (1-p) =0-1

p=0.5 (the value of p that maximizes p (1-p) = 0.5

So,

$$N = p(1-p)\left(\frac{Z}{E}\right)^2$$

N= $0.5(1-0.5)\ (1.96/0.05)^2$

N=384.2

In the study a total of 1,874 samples were collected (water - 264, human urine - 1220, poultry feces - 390)

3.5 Sample

Water samples were collected from the Bagmati river and its tributaries, stone spout and shallow wells. Fresh fecal samples were collected from chicken poultry farms. . The poultry farms were conveniently chosen based on their proximity to tributaries or

sub-tributaries of the Bagmati River and their role in contamination of water bodies with ESBL- *E. coli*, thereby exploring the potential environmental and public health implications within the River Basin within Kathmandu valley.

Urine samples were collected from the patients visiting OPD services at the National Public Health Laboratory (NPHL), Teku, Kathmandu, based on GLASS (2021) guideline for surveillance of ESBL *E. coli.* Stool samples were recommended for *Salmonella/Shigella* and not the priority organisms for detection of ESBL *E coli*.

3.6 Sample collection and transportation

3.6.1 Water collection

i. River water collection: The cap and cover of the sterile sample bottle were removed aseptically, and the mouth of the bottle was positioned opposite to the flow of river water. The neck of the bottle was submerged approximately 30 cm below the water surface, and the neck was tilted slightly upwards with the help of a stick to allow it to fill completely before the cap and cover were carefully replaced (Ewuzie *et al*., 2021).
ii. Stone spout and shallow well water collection: The sterile water bottle was filled from the stone spout, and the bucket used by indigenous people to draw water from the shallow well was used to fill the sterile water bottle for sample collection. The bottle was then labeled with the sample code number, location, and date.

3.6.2 Chicken feces collection

Chicken excreta (fresh fecal material) from the ground inside the poultry farm were collected aseptically with the help of a sterile wooden spatula into wide-mouth containers and kept in an ice box with ice packs during transportation. They were delivered to the laboratory within the day of collection (Ybañez *et al*., 2018).

3.6.3 Human urine collection

The mid-stream urine sample from the patient visiting NPHL was collected and causative bacterial isolates for significant bacteriuria was identified at NPHL by M. Sc. student (Mrs. Zenisha Acharya) of the Central Department of Microbiology as part of collaborative study, during the study period under the supervision of principal investigators. The written informed consent (signed ICD) was obtained from the participants after explaining the objectives, procedures, benefits and discomforts if any during the study.

3.6.4 Sample transportation

All the collected samples from river and poultry, and isolates from human urine were transported to Central Department of Microbiology (CDMi) Tribhuvan University (TU) in cold box (2-8 ^{0}C) using the ice pack within 2-3 hours of sample collection.

3.7 Sample processing

3.7.1 Water sample processing

The water samples were processed following the Most Probable Number count method (APHA, 1999). Briefly the process is as below:

i. Presumptive test (Most Probable Number 5 tubes): Double and single dilution Mac Conkey broths were prepared for three sets with five tubes for each set with Durham's tubes inserted into each broth tube. Double dilution broth was made in volume of 10 mL for first set whereas for second and third set 5mL volume of single dilution broth was prepared. The water sample dilutions were prepared in phosphate buffer solution. The first sets containing 10 mL of double strength lactose broth were used to inoculate 10 mL of water sample in the first set. For the second and third sets, 1 mL and 0.1 mL of water sample was added to 5 mL of single strength broth in five tubes for upstream, stone spouts, and shallow well water samples. Dilution sets were prepared with 0.01 mL and 0.001 mL dilutions to inoculate in 5 mL of single strength broth, for midstream, downstream, and tributary river water samples. All tubes were incubated at 37 °C for 48 hours.

ii. Confirmatory test : All tubes in presumptive tests, showing color change in Mac Conkey broth and gas production in Durham's tube were considered positive for the preliminary test of coliforms. A loopful of samples from each positive tube was inoculated aseptically into 10 mL of brilliant green lactose bile (BGLB) broth with Durham's tube and into tryptone broth. Both tubes were incubated at 37 °C for 48 hours. Additionally, a loopful of water sample from positive tubes was inoculated into 5 ml of tryptone broth and incubated at 44 °C for 24 hours.

iii. Completed test: After incubation for 48 hours, the BGLB broth that showed gas formation in Durham's tube was identified as positive for total coliforms. One loopful of sample from the positive tubes was streaked onto eosin methylene blue (EMB) agar media for isolation of coliforms. One drop of Kovac’s reagent was introduced to the tryptone broths incubated at 44 °C, and a red ring forming near the surface indicated the presence of *E. coli*. The Tryptone broths incubated at 37

°C, which showed a positive red ring test at 44 °C, were streaked onto EMB agar media for isolation of *E. coli.*

The MPN count of total coliforms was calculated using the MPN table values as given in WHO (1997).

3.7.2 Chicken fecal sample processing

Briefly, the samples were suspended in buffered peptone water in 1:10 (w/v) for enrichment (Stromberg *et al*., 2017) and incubated at 37 °C for 24 hours. After 24 hours, a loopful of sample was streaked onto Mac Conkey agar plate and incubated at 37 °C for 24 hours. After 24 hours the colony characteristics were noted either as lactose fermenter or lactose non fermenter. To ensure that bacteria from the same colony were not counted multiple times, several measures were implemented during the isolation process. First, colonies with distinct morphological characteristics such as shape, color, size, and texture were carefully selected. If two colonies appeared identical, only one was chosen for further analysis to avoid redundancy. Each selected colony was then streaked onto a new agar plate to obtain a pure culture, ensuring that only one type of bacterium was further analyzed. Meticulous record-keeping was maintained, with each isolate given a unique identifier number, allowing for accurate tracking and preventing duplicate counts. Biochemical tests were employed to confirm the identity of the isolates, further ensuring that only distinct bacterial strains were counted. Additionally, strict sterile techniques were used throughout the process to avoid cross-contamination between the samples/isolates. These steps collectively minimized the risk of counting the same bacterial isolate more than once (FDA, 1998).

3.7.3 Urine sample processing

The bacteria isolated at NPHL , from the urine samples on Mac Conkey agar media were obtained and transferred to Microbiology laboratory of CDMi, TU, for further characterization as a part of collaborative research work.

3.8 Identification of organism

The bacterial colony isolated on differential media was streaked onto a nutrient agar plate. All the organisms isolated on nutrient agar plates underwent Gram´s staining, catalase, and oxidase tests. Subsequently, a biochemical test was performed using a panel of seven tests, namely, indole, methyl red, Voges-Proskauer, citrate, oxidative/fermentative, triple sugar iron (TSI), and urease Test. The colony

characteristics on Mac Conkey agar media, EMB media, enzymatic, and biochemical tests confirm the presence of *E. coli* along with various Enterobacteriaceae species (Jorgensen *et al*., 2015).

3.9 Antibiotic susceptibility testing

The isolated *E. coli* was subjected for antibiotic susceptibility testing using modified Kirby and Bauer method in Mueller Hinton agar media (Bauer *et al*., 1966)The antibiotics were categorized into 11 different categories for which 17 different antibiotics of HI media were used as below Table 1 (CLSI, 2020; Magiorakos *et al*., 2010). The antibiotic susceptibility testing including ESBL detection was done following CLSI guidelines (CLSI, 2020).

Table 1: Antibiotics used for antibiotic susceptibility testing

S.N.	Antibiotic Category	Test/ Report Group	Antibiotics	Antibiotics abbreviate	Dosage
1	Aminoglycoside	B	Amikacin	AK	30 µg
2	Beta lactam combination	B	Amoxicillin clavulanic acid	AMC	50/10 µg
3	Beta lactam combination	B	Piperacillin tazobactam	PTZ	100/10 µg
4	Carbapenem	B	Imipenem	IPM	10 µg
5	Cephems	O	Cefixime (III)	CFM	5 µg
6	Cephems	B	Cefepime (IV)	CPM	30 µg
7	Cephems	C	Ceftazidime	CAZ	30 µg
8	Cephems	B	Cefotaxime	CTX	30 µg
9	Macrolides	−	Erythromycin	E	15 µg
10	Nitrofuran	U	Nitrofurantoin	NIT	300 µg
11	Penicillin	O	Piperacillin	PI	100 µg
12	Penicillin	A	Ampicillin	AMP	30 µg
13	Phenicol	C	Chloramphenicol	C	30 µg
14	Quinolone	B	Ciprofloxacin	CIP	5 µg
15	Quinolones	O	Nalidixic acid	NA	30 µg
16	Sulfonamides	B	Cotrimoxazole	COT	25µg,1.25/23.75 µg
17	Tetracyclines	O	Tetracycline	TE	3 µg

3.10 Phenotypic ESBL screening and confirmation

All the *E. coli* isolates were further screened for ESBL *E. coli* by susceptibility testing using cefotaxime and ceftazidime antibiotic disk (Overdevest *et al*., 2011). All the *E. coli* showing resistance towards either cefotaxime or ceftazidime antibiotics disk were further assessed for the confirmation of ESBL *E. coli*. The combination disk method was used for phenotypic confirmation of ESBL *E. coli*. Briefly, the cefotaxime (30 µg) and cefotaxime/clavulanic acid (30/10 µg) and ceftazidime (30 µg) and

ceftazidime/clavulanic acid (30/10 µg) combined disks were used. The zone diameter showing greater than 5 mm zone size compared to single and combined disk were confirmed as the phenotypic presence of ESBL in *E. coli* (CLSI, 2020).

3.11 Organism storage

All the identified *E. coli* were cultured on nutrient agar broth for 24 hours. From the overnight-incubated broth, 0.25 mL was inoculated into 0.75 mL of sterile glycerol solution (30 %) and stored at -80°C in three sets for further analysis (Manjana *et al*., 2016).

3.12 DNA extraction

The Spin Star™ Total DNA Kit (ADT, Biotech) was used for DNA extraction (Kuhn *et al*., 2017). The *E. coli* was cultured in Luria Bertani broth and incubated at 37 °C for 24 hours. The pellet was collected by centrifuging at 1000 rpm for 5 minutes. The supernatant was discarded. 20 µL of proteinase K and 2 µL of lysis enhancer buffer were added to 200 µL of CB buffer. This prepared solution was then added to the recovered pellet. It was incubated at 65 °C for 10 minutes and centrifuged for 1 minute. Absolute ethanol (200 µL) was added, and the mixture was vortexed. This mixture was transferred into a clean column and washed with 500 µL of wash buffer 1 and then wash buffer 2. It was centrifuged at 15,000 rpm for 15 minutes. The flow-through was discarded, and 100 µL of elution buffer was added. It was incubated at room temperature for 15-25 minutes and centrifuged at 10,000 rpm for 1 minute. The eluate was stored at -20 °C for further use.

3.13 Polymerase chain reaction

For the detection of ESBL gene types, the Cica Geneus ESBL Gene Detection Kit was used (Akine *et al*., 2021). Two reaction mixtures were prepared for the multiplex PCR. In Mixture 1, Reagents A, B, and C were added in volumes of 4 µL, 7 µL, and 4 µL, respectively. For Mixture 2, Reagents A, B, and D were mixed in volumes of 4 µL, 7 µL, and 4 µL, respectively. Extracted DNA was added at 5 µL to prepare a 20 µL PCR mixture. The details' reaction profiles are shown in Table 2.

Table 2: Reaction profiles of thermal cycler for ESBL gene detection

Type	Target gene	Amplicon size (bp)	Cycle	Denaturation	Annealing	Extension
Reaction mixture 1	*bla CTX M-25* group	522	30	94 ºC for 15 s	63 ºC for 15 s	72 ºC for 40 s
	bla CTX M-chimera	391				
	bla CTX M-1 group	268				
	bla CTX M-8 group	189				
Reaction mixture 2	*bla SHV*	655				
	bla CTX M-2 group	475				
	bla CTX M-9 group	350				
	bla GES (ESBL type)	228				
	bla TEM	132				

3.14 Gel electrophoresis

The PCR product was loaded onto an agarose gel and separated using gel electrophoresis to visualize and determine its size or confirm its presence (Green & Sambrook, 2019). 2 % agarose gel was prepared using 1 % Tris-acetate-EDTA (TAE) buffer. 4 µL of loading dye was added to the PCR reaction mixture. Into the wells of the agarose gel, 6 µL of the mixture was loaded. The positive and negative controls were carefully added to the wells. A 100 bp DNA ladder was used as the molecular weight marker. 5 µL of ethidium bromide was added to the buffer solution. Electrophoresis was performed at 100 V for 30 minutes. The bands were visualized using the Azure Biosystems c200 gel documentation imaging system.

3.15 Data entry, data curation, and analysis

All data obtained in the lab were entered into a logbook, maintaining safety and confidentiality. This data was then filtered and entered into Microsoft Excel 2010. Column diagrams and tables were prepared using Microsoft Excel, 2010. The Chi-square test for association and Pearson's correlation analysis was calculated using the Statistical Package for Social Science version 21.0. The test was considered significant at $p < 0.05$.To understand the presence of different types of coliforms, Venn diagram was created using triple Venn in R software version 4.2.1 To elucidate the relationship in between different beta lactamase gene types. Euler diagram was created using Ellipses Area Proportionality (EdeapAP) similarity map was prepared, and distance matrix was created using Exploratory version 6.10.3.

The coordinates of the sample collection points were recorded using a Gramin, GPS instrument. The location map of the Bagmati river and its tributary, stone spouts, shallow well and the poultry farm was created using Google Earth and ArcGIS pro-3.5.

3.16 Quality control

E. coli ATCC 25922 was employed as the standard strain to ensure the quality and consistency of results in various laboratory tests, including Gram staining, enzymatic analysis, and biochemical assays. In addition to *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 760093 was selected as a control organism for the screening and phenotypic confirmation (combination disk) tests for extended-spectrum beta-lactamase (ESBL).

3.17 Flowchart of the overall methods and methodology

This flowchart (Figure 5) outlines the sequential steps and procedures followed throughout the research process, providing a visual representation of the methodology adopted.

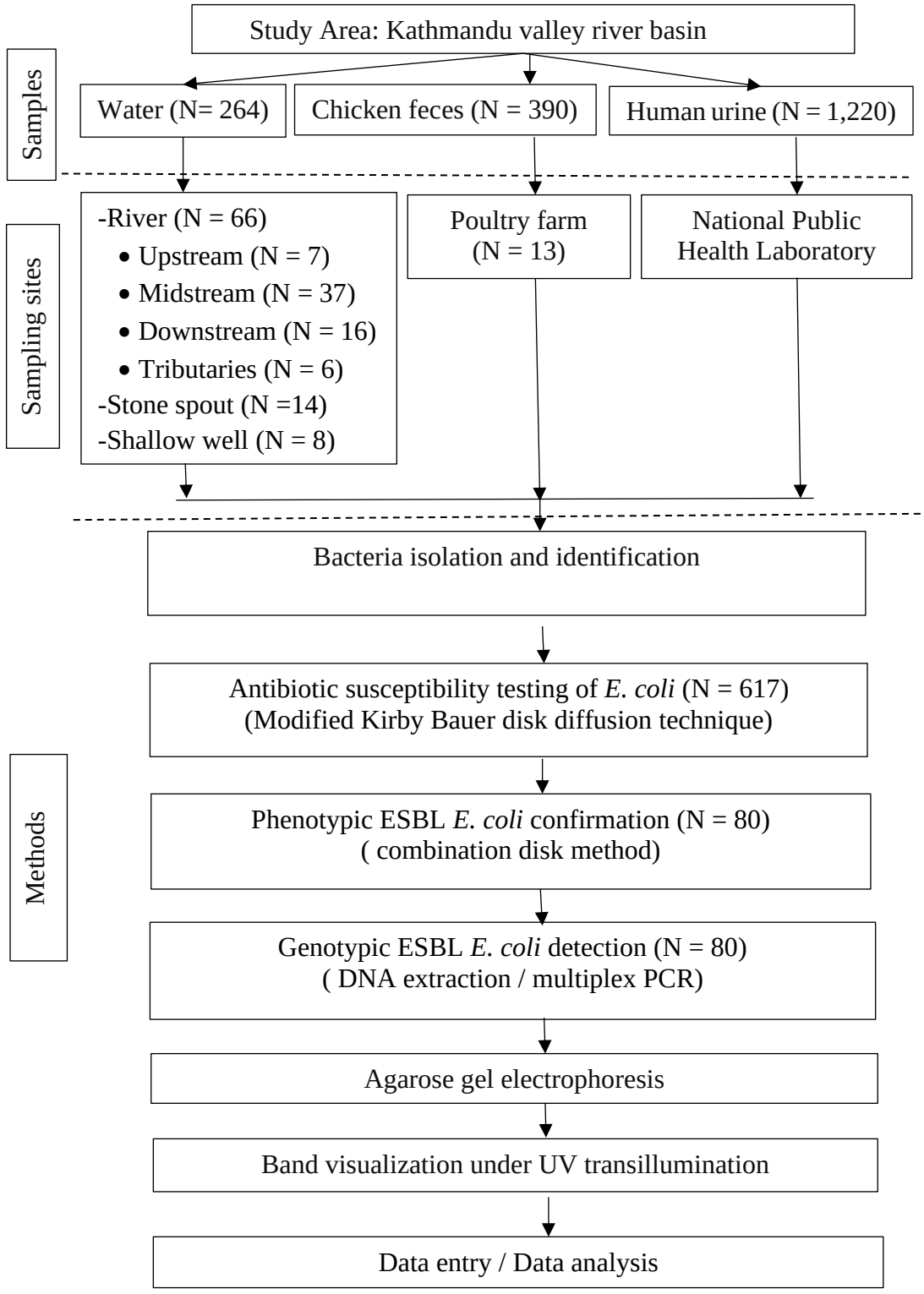


Figure 5: Flowchart of overall methods and methodology employed in the study

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Results

A total of 1,295 bacterial isolates were identified and analyzed from the collected samples. Out of the 1,295 bacterial isolates, 617 (48%) were identified as *E. coli*. Among the 617 *E. coli* isolates, 288 were from water samples, 266 were from poultry (chicken fecal) fecal samples, and 63 were from human urine samples. Analysis revealed that 13 % (80/617) were extended-spectrum beta-lactamase producing *E. coli* (ESBL *E. coli*). ESBL *E. coli* was not detected in upstream, stone spout, and shallow well water sources. The details of distribution of *E. coli* and ESBL *E. coli* in water samples, poultry feces and human urine are shown below in Table 3.

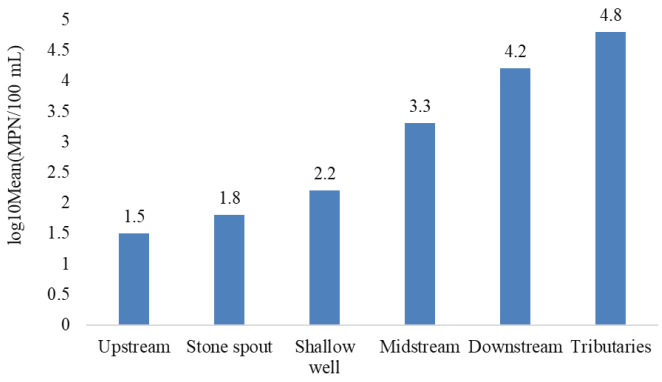
Table 3: Distribution of *E. coli* and ESBL *E. coli* in water sampling sites

S.N.	Location/source	Sample	Total Sites	No. of samples	No. of bacterial isolates	No. of *E. coli*	No. of ESBL *E. coli*	Detection sites of ESBL *E. coli*
1	Bagmati river							
	a. Upstream	**Water**	**7**	**21**	**50**	**7 (1)**	**0 (0)**	**0**
	b. Midstream	Water	37	111	338	140 (23)	15 (19)	12
	c. Downstream	Water	16	48	158	62 (10)	14 (18)	8
	d. Tributaries	Water	6	18	69	32 (5)	11 (14)	6
2	**Shallow well**	**Water**	**8**	**24**	**60**	**30 (5)**	**0 (0)**	**0**
3	**Stone spouts**	**Water**	**14**	**42**	**122**	**17 (3)**	**0 (0)**	**0**
4	Poultry farm	Chicken feces	13	390	402	266 (43)	22 (28)	11
5	NPHL	Human urine	NA	1,220	96	63 (10)	18 (23)	NA
	Total		102	1,874	1,295	617	80	37

4.1.1 Detection of bacterial isolates from water samples

4.1.1.1 Coliform count in water samples from different sources

The mean Most Probable Number (MPN) count per 100 mL revealed increasing trend from upstream till tributaries: upstream (31) harbored lowest number of coliforms followed by stone spout (69), shallow well (160), midstream (2,400), downstream (17,000) and tributaries (70,000) (Figure 6).



Sites	Mean (MPN/100mL)	log10 Mean (MPN/100mL)
Upstream	31.1	1.5
Stone spout	68.52	1.8
Shallow well	159.59	2.2
Midstream	2436.94	3.3
Downstream	16995.83	4.2
Tributaries	69833.33	4.8

Figure 6: MPN count in water samples from different water sources

4.1.1.2 Detection of coliforms in water sampling sites

Among the 88 water sample collection sites, *E. coli* was not detected in 29 % (2/7) of the upstream sites and in 1/14 of the stone spouts. *E. coli* was detected in 97 % (85/88) of the samples from midstream, downstream, tributaries, and shallow wells (Table 4). Six species of coliform bacteria were identified viz., *E. coli in* 97 % (85/88), *Klebsiella oxytoca* in 89 % (78/88), *Citrobacter freundii* in 84 % (74/88), *Citrobacter koseri* in 80% (70/88), *Klebsiella pneumoniae* in 55 % (48/88), and *Enterobacter aerogenes* in 26% (23/88) of sampling sites (Figure 7). From 88 water sampling sites 797 bacteria were isolated among which 288 (40%) were *E. coli* (Table 5). *E. coli* was not detected in water samples from site number 2 and 4 in the upstream and site no. 80, stone spout (Figure 8) (Figure 9).

Table 4: Occurrence of coliforms in water sampling sites

Water source (sampling sites)	Bacterial isolates					
	Enterobacter aerogenes	*Klebiella pneumoniae*	*Citrobacter koseri*	*Citrobacter freundii*	*Klebsiella oxytoca*	*Escherichia coli*
	n %					
Upstream (n = 7)	2 (28.6)	6 (85.7)	3 (42.9)	6 (85.7)	7 (100)	5 (71.4)
Midstream (n = 37)	3 (8.1)	13 (35.1)	30 (81.1)	32 (86.5)	30 (81.1)	37 (100)
Downstream (n = 16)	3 (18.8)	8 (50)	16 (100)	13 (81.3)	13 (81.3)	16 (100)
Tributaries (n = 6)	1 (16.7)	5 (83.3)	5 (83.3)	4 (66.7)	6 (100)	6 (100)
Stone spouts (n = 14)	9 (64.3)	11 (78.6)	12 (85.7)	12 (85.7)	14 (100)	13 (92.9)
Shallow well (n = 8)	5 (62.5)	5 (62.5)	4 (50)	7 (87.5)	8 (100)	8 (100)
Total = 88	23 (26.1)	48 (54.5)	70 (79.5)	74 (84.1)	78 (88.6)	85 (96.59)

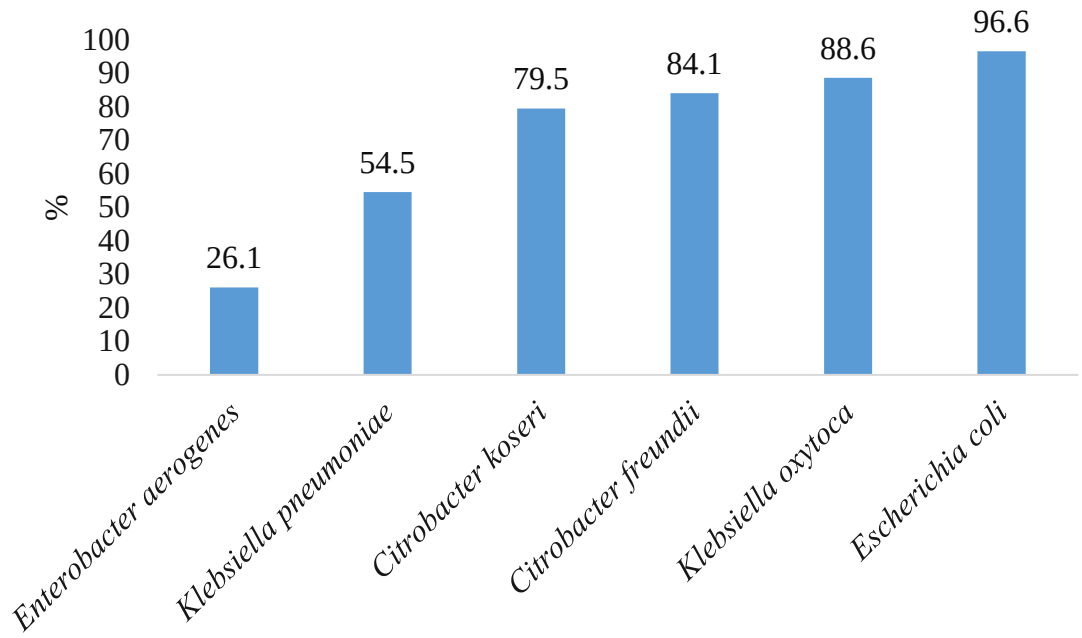


Figure 7: Bacterial isolates in water samples from different sampling sites (N = 88)

Table 5: Bacterial isolates in water samples

Site/ Organisms	*E. aerogenes*	*E. coli*	*C. freundii*	*C. koseri*	*K. Oxytoca*	*K. pneumoniae*	Total
Upstream	5	7	9	3	15	7	46
Midstream	3	140	75	52	47	23	340
Downstream	3	62	36	28	21	8	158
Tributaries	1	32	8	7	12	9	69
Stone spouts	11	30	32	16	20	15	124
Shallow well	10	17	11	4	12	6	60
Total	33	288	171	110	127	68	797
%	4.1	36.1	21.5	13.8	15.9	8.5	100

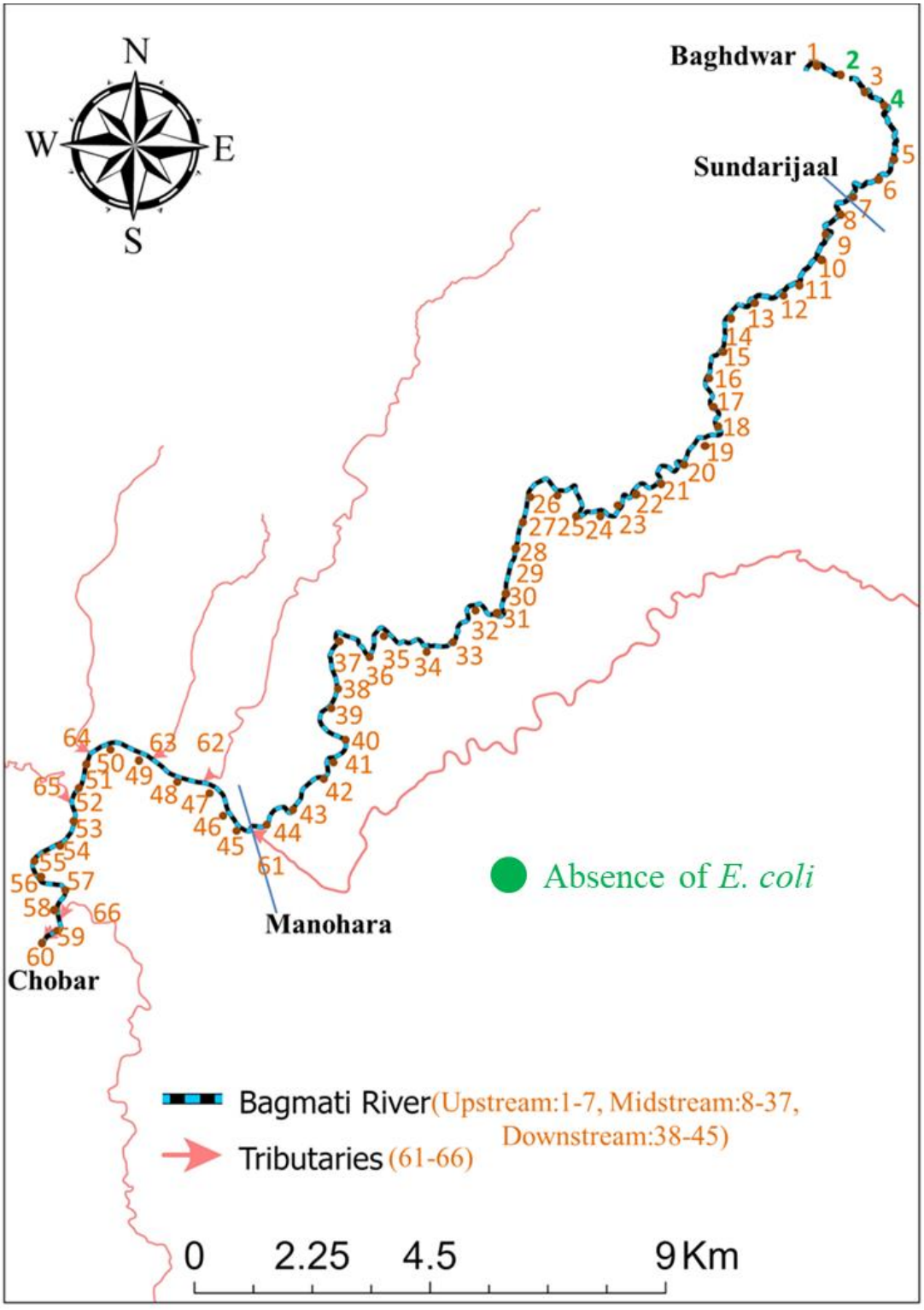


Figure 8: Location map showing distribution of *E. coli* along Bagmati river and its tributaries

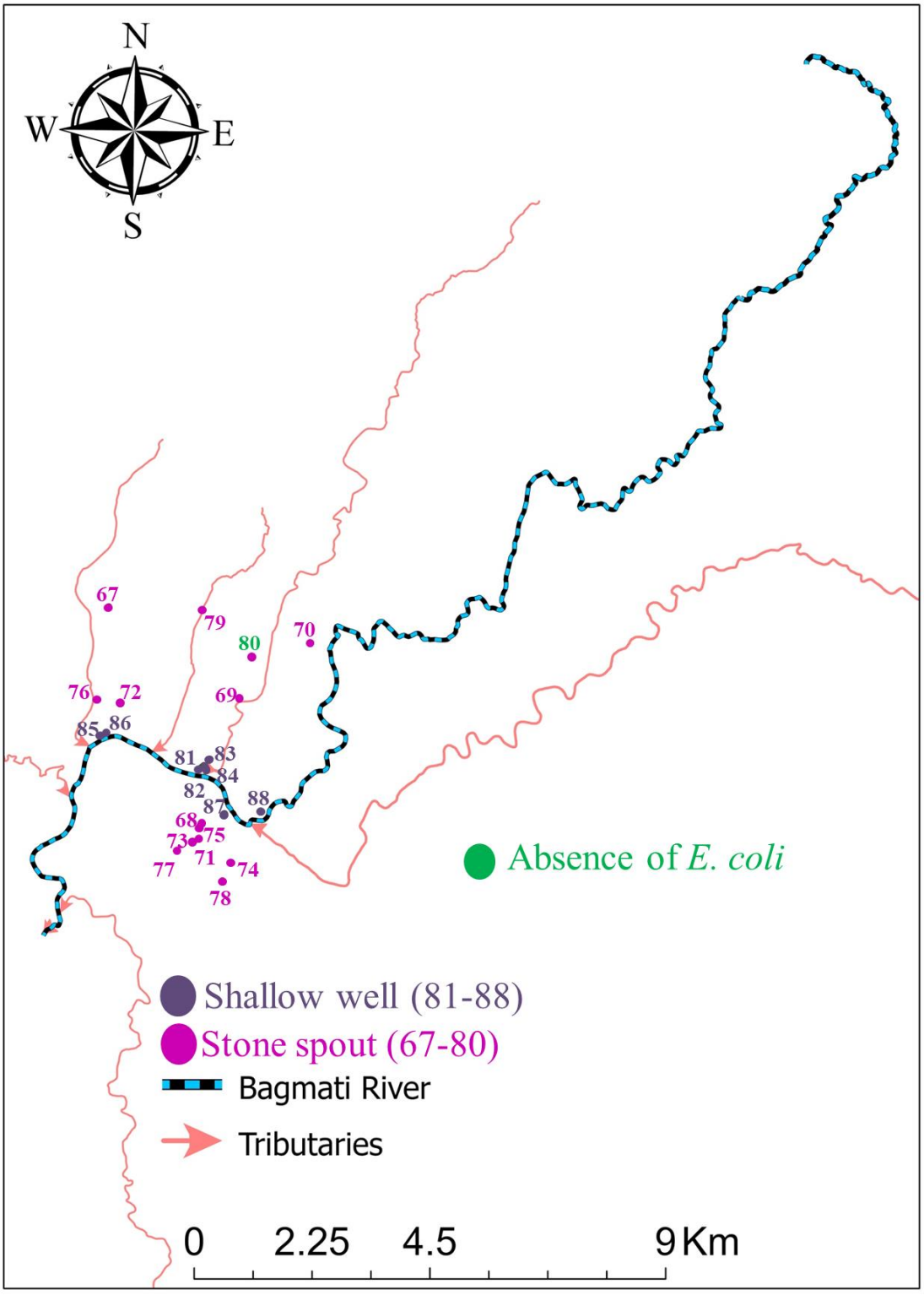


Figure 9: Location map showing distribution of *E. coli* along and stone spouts and shallow well

4.1.1.3 Distribution of *E. coli* and ESBL *E. coli* in water samples

Of a total of 797 bacteria representing Enterobacteriaceae family identified from water samples, 288 (36 %) were *E. coli* among which 40 (14 %) were ESBL *E. coli.* (Figure 10).

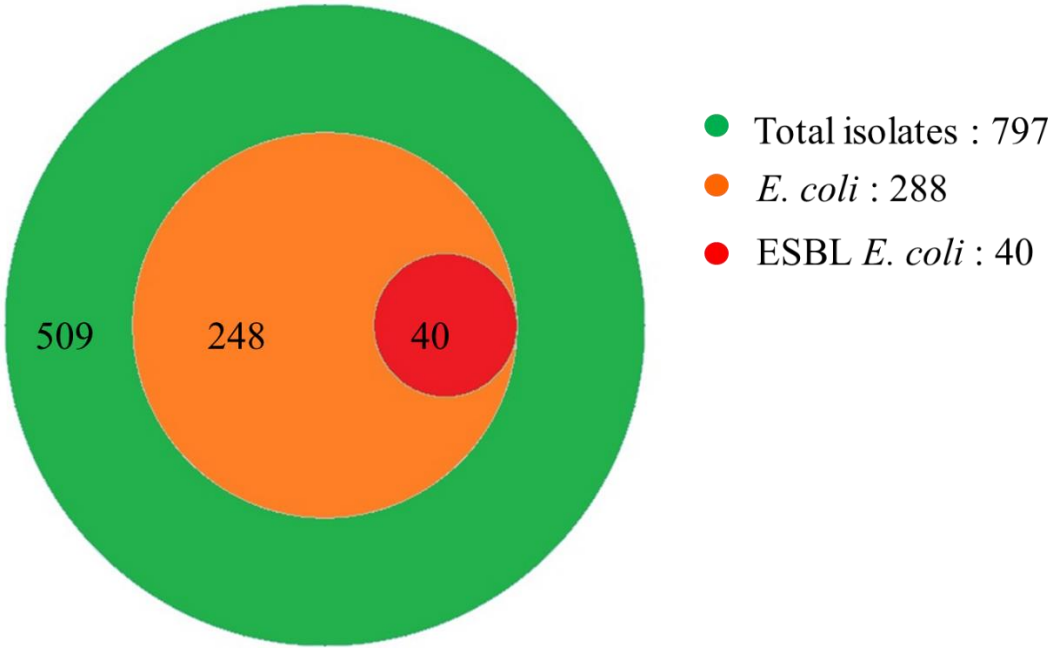


Figure 10: Venn diagram showing distribution of *E. coli* and ESBL *E. coli* in water isolates

4.1.1.4 Detection of ESBL *E. coli* in different water sampling sites

ESBL *E. coli* was not isolated from water samples collected from upstream of the Bagmati river, stone spouts, and shallow wells (Table 2). ESBL *E. coli* was detected in 32 % (12/37) of the water samples from midstream, 50 % (8/16) in the downstream, and in 100 % of all six river tributaries respectively. ESBL *E. coli* was detected in water samples from only two of the 25 midstream sites between Sundarijaal and Guhyeshwori, specifically at sites 23 and 29 (Figure 11).

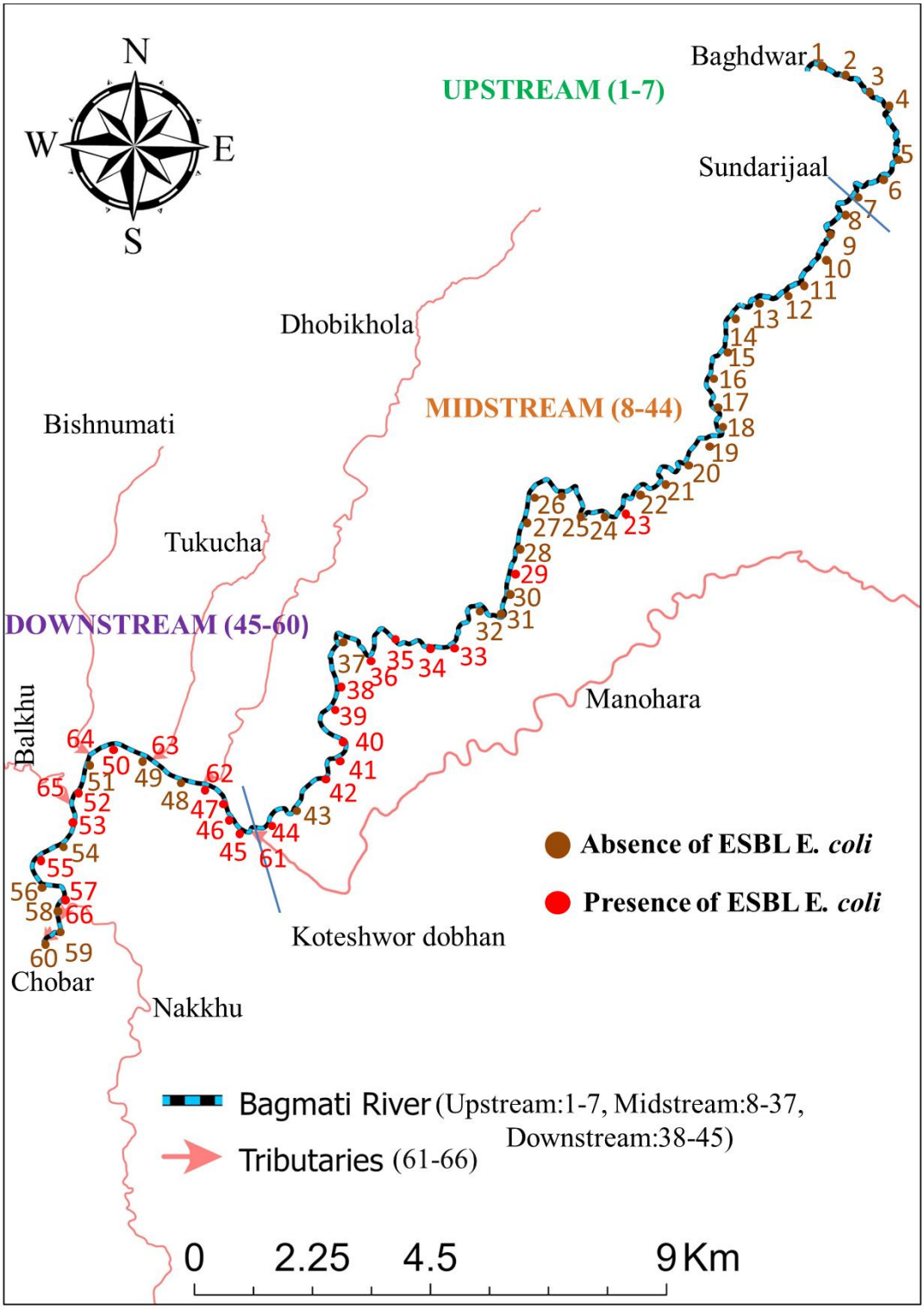


Figure 11: Distribution of ESBL *E. coli* along Bagmati river

4.1.1.5 Antibiotic resistance in non ESBL *E. coli* isolated from water samples

The antibiotic resistance in 248 non ESBL *E. coli* isolated from water samples was analyzed among 11 antimicrobial categories and 5 test/report groups (CLSI, 2020). More than 99 % (246/248) of non ESBL *E. coli* showed resistance towards

erythromycin, macrolide category of an antibiotics. In test/report group A, more than 96 % (239/248) of non ESBL *E. coli* showed resistance towards ampicillin, penicillin category. In test/report group B, altogether six antimicrobial categories were identified. *E. coli* showed the highest resistance of 98 % (244/248) towards amoxiclav antibiotics within beta lactam combination category. In this group, 11 % (28/248) of non ESBL *E. coli* showed the least percentage of resistance towards imipenem within carbapenems category. Meanwhile in test/report group C, non ESBL *E. coli* exhibited resistance of 24 % (59/248) towards chloramphenicol within phenicol category, in contrast to 43 % (106/248) against ceftazidime within cephems category. In test/report group O, within tetracycline category, non ESBL *E. coli* showed resistance of 96 % (237/248) towards tetracycline, similar with piperacillin in penicillin category. Conversely, 34 % (85/248) of non ESBL *E. coli* were resistant towards nalidixic acid within quinolones category. In test/report group U, 29 % (73/248) of non ESBL *E. coli* showed resistance towards nitrofurantoin, furan category. The detailed antibiotic resistance in non ESBL *E. coli* from water samples is shown in Table 6.

Table 6: Antibiotic resistance in non ESBL *E. coli* isolated from water samples

Antimicrobial category	Test/Report Group	Antibiotics	No. of isolates	Resistant %
Macrolides	-	Erythromycin (E)	246	99.2
Beta lactam combination	B	Amoxicillin/Clavulanic acid (AMC)	244	98.4
Penicillin	A	Ampicillin (AMP)	242	97.6
Penicillin	O	Piperacillin (PI)	237	95.6
Tetracycline	O	Tetracycline (TE)	237	95.6
Cephems	O	Cefixime (CFM)	191	77
Cephems	B	Cefepime (CPM)	176	71
Beta lactam combination	B	Piperacillin/Tazobactam (PTZ)	163	65.7
Cephems	C	Ceftazidime (CAZ)	107	43.1
Cephems	B	Cefotaxime (CTX)	105	42.3
Aminoglycosides	B	Amikacin (AK)	103	41.5
Quinolones	O	Nalidixic acid (NA)	85	34.3
Nitrofurans	U	Nitrofurantoin (NIT)	72	29
Folate pathway inhibitors	B	Trimethoprim/Sulfamethoxazole (COT)	65	26.2
Phenicol	C	Chloramphenicol (C)	59	23.8
Quinolones	B	Ciprofloxacin (CIP)	59	23.8
Carbapenem	B	Imipenem (IPM)	28	11.3

Total non ESBL *E. coli* isolates = 248

4.1.1.6 Antibiotic resistance in ESBL *E. coli* isolated from water samples

The antibiotic resistance in 40 ESBL *E. coli* isolates from water samples was analyzed among 11 antimicrobial categories and 5 test/report groups (CLSI, 2020).

In test/report group A, 100 % (40/40) of ESBL *E. coli* showed resistance towards ampicillin, penicillin category. In test/report group B, altogether six antimicrobial categories were identified. ESBL *E. coli* showed the highest resistance percentage of 100 % (40/40) towards amoxiclav, beta lactam combination category in similar with 100% (40/40) resistance towards cefotaxime and ceftazidime within cephems category. In this group, 28 % (11/40) of ESBL *E. coli* showed least resistance percentage towards ciprofloxacin, quinolone category. Altogether 18 % (7/40) of the ESBL *E. coli* were resistant to imipenem antibiotics, carbapenem category. Meanwhile in test/report group C, ESBL *E. coli* exhibited resistance of 20 % (8/40) towards chloramphenicol, phenicol category, whereas 100 % (40/40) resistance against ceftazidime, cephems category. In test/report group O, ESBL *E. coli* showed resistance of 100 % (40/40) to piperacillin, penicillin category followed by 98 % (39/40) towards tetracycline, tetracycline category and 43 % (17/40) resistance to nalidixic acid. In this group the least resistance

percentage of 41 % (16/40) was towards nalidixic acid, quinolones category. In test/report group U, 55 % (22/40) of ESBL *E. coli* were resistant towards nitrofurantoin, furan category. The detailed antibiotic resistance in ESBL *E. coli* isolated from water samples is shown in Table 7.

Table 7: Antibiotic resistance in ESBL *E. coli* isolated from water samples

Antimicrobial category	Test/Report Group	Antibiotics	No. of isolates	Resistant %
Beta lactam combination	B	Amoxicillin/Clavulanic acid (AMC)	40	100
Penicillin	A	Ampicillin (AMP)	40	100
Penicillin	O	Piperacillin (PI)	40	100
Cephems	C	Ceftazidime (CAZ)	40	100
Cephems	B	Cefotaxime (CTX)	40	100
Macrolides	-	Erythromycin (E)	39	97.5
Tetracycline	O	Tetracycline (TE)	39	97.5
Cephems	O	Cefixime (CFM)	36	90
Cephems	B	Cefepime (CPM)	33	82.5
Beta lactam combination	B	Piperacillin/tazobactam (PTZ)	26	65
Nitrofurans	U	Nitrofurantoin (NIT)	22	55
Aminoglycosides	B	Amikacin (AK)	18	45
Quinolones	O	Nalidixic acid (NA)	18	45
Folate pathway inhibitors	B	Trimethoprim/sulfamethoxazole (COT)	13	32.5
Quinolones	B	Ciprofloxacin (CIP)	11	27.5
Phenicol	C	Chloramphenicol (C)	8	20
Carbapenem	B	Imipenem (IPM)	7	17.5
Total ESBL *E. coli* isolates = 40				

4.1.1.7 Multiple drug resistance in non ESBL *E. coli* isolated from water samples

Among the 248 non ESBL *E. coli* isolated from water samples, 94.4% (234/248) of the isolates exhibited multi-drug resistance (MDR). About half of the isolates (50%) of these MDR non ESBL *E. coli* were resistant to five categories of antibiotics: macrolides, penicillin, beta-lactam combinations, tetracyclines, and cephems. Less than 5% of the MDR isolates were resistance to 11 categories with additional 6 categories (aminoglycosides, furans, folate pathway inhibitors, quinolones, carbapenems, and phenicol). The detailed drug resistance pattern is shown in Table 8.

Table 8: Multiple drug resistant non ESBL *E. coli* isolated from water samples

Antibiotics	No. of isolates	Resistant %
E	246	99.2
E + AMC	243	98
E + AMC + AMP	234	94.4
E + AMC + AMP + PI	232	92.3
E + AMC + AMP + PI + TE	232	88.7
E + AMC + AMP + PI + TE + CFM	177	68.5
E + AMC + AMP + PI + TE + CFM + CPM	133	49.2
E + AMC + AMP + PI + TE + CFM + CPM + PTZ	96	30.2
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ	56	12.9
E + AMC + AMP + PI + TE + CFM+ CPM + PTZ + CAZ + CTX	44	7.7
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK	24	6.5
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK + NA	20	4.4
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK + NA + NIT	7	2
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK + NA + NIT + COT	1	0.4
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK + NA + NIT + COT + C	1	0.4
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK + NA + NIT + COT + C + CIP	1	0.4
E + AMC + AMP + PI + TE + CFM + CPM + PTZ + CAZ + CTX + AK + NA + NIT + COT + C + CIP + IPM	0	0

AK : Amikacin, AMC : Amoxicillin clavulanic, AMP : Ampicillin, CPM : Cefepime, CFX : Cefixime, CTX : Cefotaxime, CAZ : Ceftazidime, C : Chloramphenicol, CIP : Ciprofloxacin, COT: Cotrimoxazole, E : Erythromycin, IPM : Imipenem, NA : Nalidixic acid, NIT : Nitrofurantoin, PI : Piperacillin, PTZ : Piperacillin Tazobactam, TE : Tetracycline

Total non ESBL *E. coli* = 248

4.1.1.8 Multiple drug resistant ESBL *E. coli* isolated from water samples

Among the 40 ESBL *E. coli* isolated from water samples, 100 % (40/40) of the isolates exhibited multi-drug resistance (MDR). About half of the isolates (50 %) of these MDR ESBL *E. coli* were resistant to four categories of antibiotics: beta-lactam, penicillin, macrolide and cephems. Less than 5 % of the MDR isolates were resistant o 11 categories with additional 7 categories (aminoglycosides, furans, folate pathway inhibitors, quinolones, carbapenems, and phenicol). Despite being resistant to all 17 antibiotics tested, none of the isolates qualify as pan drugresistant because it fell short of the criteria for resistance across the required groups of antibiotics (Magiorakos *et al*., 2010). The detailed drug resistance is given in Table 9.

Table 9: Multiple drug resistant ESBL *E. coli* isolated from water samples

Antibiotics	No. of isolates	Resistant %
AMC + AMP + CAZ + CTX + E + PI	40	100
AMC + AMP + CAZ + CTX + E + PI + TE	38	95
AMC + AMP + CAZ + CTX + E + PI + TE + CFM	35	87.5
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM	30	75
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ	19	47.5
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ + NIT	8	20
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ + NIT + AK	5	12.5
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ + NIT + AK + NA	4	10
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ + NIT + AK + NA + COT	2	5
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ + NIT + AK + NA + COT + CIP	2	5
AMC + AMP + CAZ + CTX + E + PI + TE + CFM + CPM + PTZ + NIT + AK + NA + COT + CIP + C	2	5
AK : Amikacin, AMC : Amoxiclav, AMP : Ampicillin, CPM : Cefepime, CFX : Cefixime, CTX : Cefotaxime, CAZ : Ceftazidime, C : Chloramphenicol, CIP : Ciprofloxacin, COT: Cotrimoxazole, E : Erythromycin, IPM : Imipenem, NA : Nalidixic acid, NIT : Nitrofurantoin, PI : Piperacillin, PTZ : Piperacillin Tazobactam, TE : Tetracycline		
Total ESBL *E. coli* = 40		

4.1.2 Detection of the bacterial isolates from human urine samples

4.1.2.1 Distribution of bacterial isolates in human urine samples

From 1,220 urine samples analyzed, 96 bacteria were isolated. were detected. From a total of 96 bacterial isolates, *E. coli* (66%, 63/96) was the commonest isolate, followed by *Citrobacter freundii* at 17 % (16/96), *Citrobacter koseri* at 5 % (5/96), *Klebsiella pneumoniae* at 4 % (4/96), *Enterobacter aerogenes* at 3 % (3/96), both *Proteus mirabilis* and *Klebsiella oxytoca* at 2 % (2/96), and *Proteus vulgaris* at 1 % (1/96) (Figure 12).

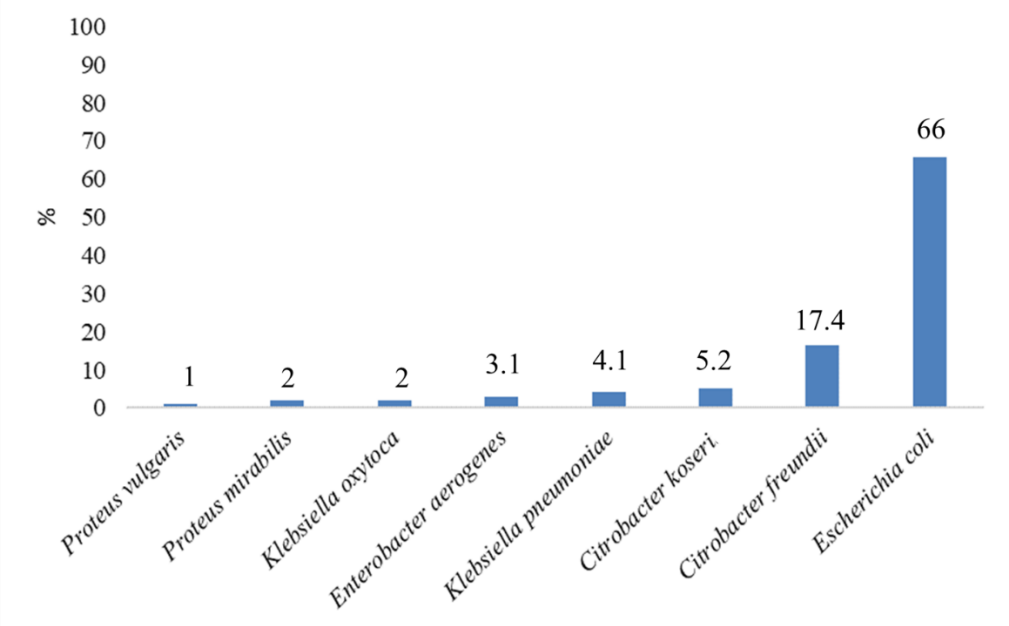


Figure 12: Bacterial isolates in human urine samples

4.1.2.2 Distribution of *E. coli* and ESBL *E. coli* in human urine samples

A total of 96 bacterial isolates were detected from urine samples. Among the total isolates 66 % (63/96) were *E. coli* and 19 % (18/96) were ESBL *E. coli* (Figure 13).

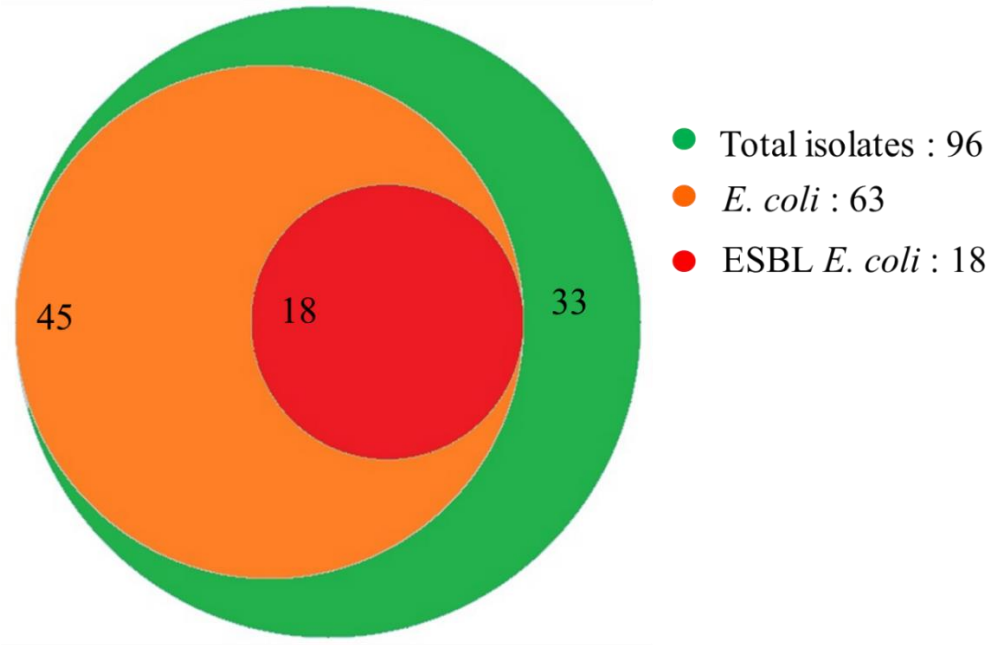


Figure 13: Venn diagram showing distribution of *E. coli* and ESBL *E. coli* in urine isolates

4.1.2.3 Antibiotic resistance in non ESBL *E. coli* isolated from human urine samples

The antibiotic resistance pattern of non ESBL *E. coli* detected from urine samples, were further analyzed for resistance to 17 antibiotics within 11 antimicrobial categories, 5 test/report groups (CLSI, 2020). In test/report group A, non ESBL *E. coli* showed the

highest resistance percentage of 89 % (40/45) towards ampicillin, penicillin category. In test/report group B, non ESBL *E. coli* showed the highest resistance percentage of 69 % (31/45) towards cefepime, and lowest resistance percentage of 26.7 % (12/45) to imipenem, carbapenem category. Meanwhile in test/report group C, non ESBL *E. coli* exhibited resistance percentage of 69 % (31/45) towards chloramphenicol, phenicol category, in contrast to 26.7 % (12/45) against ceftazidime, cephems category. In test/report group O, more than 89 % (40/45) of non ESBL *E. coli* were resistant against penicillin category, piperacillin antibiotics. Within the cephems category, non ESBL *E. coli* showed resistance percentage of 87 % (39/45) towards cefixime, comparable with 84 % (38/45) towards tetracycline, tetracycline category. In test/report group U, nitrofuran category, non ESBL *E. coli* exhibited a resistance percentage of 58 % (26/45), indicating moderate resistance percentage among the tested antibiotics. The detailed antibiotic resistance percentage in non ESBL *E. coli* from urine samples is shown in Table 10.

Table 10: Antibiotic resistance in non ESBL *E. coli* isolated from human urine samples

Antimicrobial category	Test/Report Group	Antibiotics	No. of isolates	Resistant %
Penicillin	A	Ampicillin (AMP)	40	88.9
Penicillin	O	Piperacillin (PI)	40	88.9
Cephems	O	Cefixime (CFM)	39	86.7
Tetracycline	O	Tetracycline (TE)	38	84.4
Macrolides	-	Erythromycin (E)	35	77.8
Quinolones	O	Nalidixic acid (NA)	34	75.6
Cephems	B	Cefepime (CPM)	33	73.3
Phenicol	C	Chloramphenicol (C)	30	66.7
Beta lactam combination	B	Piperacillin/tazobactam (PTZ)	30	66.7
Quinolones	B	Ciprofloxacin (CIP)	30	66.7
Beta lactam combination	B	Amoxicillin/Clavulanic acid (AMC)	29	64.4
Nitrofurans	U	Nitrofurantoin (NIT)	26	57.8
Folate pathway inhibitors	B	Trimethoprim/sulfamethoxazole (COT)	24	53.3
Aminoglycosides	B	Amikacin (AK)	16	35.6
Cephems	C	Ceftazidime (CAZ)	12	26.7
Cephems	B	Cefotaxime (CTX)	12	26.7
Carbapenem	B	Imipenem (IPM)	11	24.4
Total non ESBL *E. coli* = 45				

4.1.2.4 Antibiotic resistance in ESBL *E. coli* isolated from human urine samples

The antibiotic resistance of 18 ESBL *E. coli* detected from urine samples was analyzed among 17 antibiotics within 11 antimicrobial categories, 5 test/report groups (CLSI, 2020). In test/report group A, ESBL *E. coli* showed the highest resistance percentage of 89 % (40/45) towards ampicillin, penicillin category. In test/report group B, 100% (18/18) ESBL *E. coli* were resistant against cefotaxime, ceftazidime, cephems and ciprofloxacin, quinolone category. In this group, 17 % (3/18) of ESBL *E. coli* showed resistance against imipenem, carbapenem category and 22 % (4/18) of ESBL *E. coli* were resistant towards amikacin, aminoglycoside category. Meanwhile in test/report group C, ESBL *E. coli* exhibited resistance of 67 % (12/18) towards chloramphenicol, phenicol category, which is lower in compared to 100 % (18/18) against ceftazidime, cephems category. In test/report group O, 89 % of ESBL *E. coli* were resistance towards cefixime, cephems category. Almost 79 % (14/18) of ESBL *E. coli* were resistant against tetracycline, tetracycline category and piperacillin, penicillin category. In test/report group U, nitrofuran category, ESBL *E. coli* showed a resistance of 39 % (7/18), indicating moderate resistance among the tested antibiotics. The detailed antibiotic resistance in ESBL *E. coli* from urine sample is shown in Table 11.

Table 11: Antibiotic resistance in ESBL *E. coli* isolated from human urine samples

Antimicrobial category	Test/Report Group	Antibiotics	No. of isolates	Resistant %
Cephems	C	Ceftazidime (CAZ)	18	100
Cephems	B	Cefotaxime (CTX)	18	100
Quinolones	B	Ciprofloxacin (CIP)	18	100
Macrolides	–	Erythromycin (E)	17	94.4
Penicillin	A	Ampicillin (AMP)	16	88.9
Cephems	O	Cefixime (CFM)	16	88.9
Beta lactam combination	B	Amoxicillin/Clavulanic acid (AMC)	15	83.3
Cephems	B	Cefepime (CPM)	15	83.3
Penicillin	O	Piperacillin (PI)	14	77.8
Tetracycline	O	Tetracycline (TE)	14	77.8
Phenicol	C	Chloramphenicol (C)	12	66.7
Beta lactam combination	B	Piperacillin/tazobactam (PTZ)	11	61.1
Quinolones	O	Nalidixic acid (NA)	11	61.1
Folate pathway inhibitors	B	Trimethoprim/sulfamethoxazole (COT)	10	55.6
Nitrofurans	U	Nitrofurantoin (NIT)	7	38.9
Aminoglycosides	B	Amikacin (AK)	4	22.2
Carbapenem	B	Imipenem (IPM)	3	16.7
Total ESBL *E. coli* = 18				

4.1.2.5 Multiple drug resistance in non ESBL *E. coli* isolated from human urine samples

Among 45 non ESBL *E. coli* isolated from human urine samples, 67 % (30/45) were multiple drug resistance showing resistance against three categories of antibiotics viz., penicillin, cephems and tetracyclines. About 50 % (22/45) of the non ESBL *E. coli* isolates were resistance towards 4 drug categories viz., penicillin, cephems, tetracycline and macrolides. About 2 % of the isolates were resistant towards seven drug categories with additional 4 drug categories viz: quinolones, beta lactam, phenicol and nitrofurans (Table 12).

Table 12: Multiple drug resistant non ESBL *E. coli* isolated from human urine samples

Antibiotics	No. of isolates	Resistant %
AMP	40	88.9
AMP + PI	37	82.2
AMP + PI + CFM	33	73.3
AMP + PI + CFM + TE	30	66.7
AMP + PI + CFM + TE + E	22	48.9
AMP + P I+ CFM + TE + E + NA	16	35.6
AMP + PI + CFM + TE + E + NA + CPM	13	28.9
AMP + PI + CFM + TE + E + NA + CPM + PTZ	9	20.0
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP	5	11.1
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C	3	6.7
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC	3	6.7
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC + NIT	1	2.2
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC + NIT + COT	0	0.0
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC + NIT + COT + AK	0	0.0
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC + NIT + COT + AK + CAZ	0	0.0
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC + NIT + COT + AK + CAZ + CTX	0	0.0
AMP + PI + CFM + TE + E + NA + CPM + PTZ + CIP + C + AMC + NIT + COT + AK + CAZ + CTX + IPM	0	0.0

AK : Amikacin, AMC : Amoxicillin clavulanic, AMP : Ampicillin, CPM : Cefepime, CFX : Cefixime, CTX : Cefotaxime, CAZ : Ceftazidime, C : Chloramphenicol, CIP : Ciprofloxacin, COT: Cotrimoxazole, E : Erythromycin, IPM : Imipenem, NA : Nalidixic acid, NIT : Nitrofurantoin, PI : Piperacillin, PTZ : Piperacillin Tazobactam, TE : Tetracycline

Total non ESBL *E. coli* = 45

4.1.2.6 Multiple drug resistance in ESBL *E. coli* isolated from human urine samples

Among the 18 ESBL *E. coli* isolated from water samples, 94 % (17/18) of the isolates exhibited multi-drug resistance (MDR) showing resistance against cephems, quinolones and macrolide antibiotic category. About half of the isolates (50 %) among these MDR ESBL *E. coli* were resistant to five categories of antibiotics: cephems,

quinolones, macrolide, beta-lactam and penicillin. And 11 % (2/18) of the MDR isolates were resistant to 11 categories with additional 6 categories (aminoglycosides, furans, folate pathway inhibitors, carbapenems, and phenicol). The details are given in Table 13. Despite being resistant to all 17 antibiotics tested, none of the isolates qualify as pan drug resistant because it fell short of the criteria for resistance across the required groups of antibiotics (Magiorakos *et al*., 2010) .

Table 13: Multiple drug resistant ESBL *E. coli* isolated from human urine samples

Antibiotics	No. of isolates	Resistant %
CTX + CAZ + CIP	18	100
CTX + CAZ + CIP + E	17	94.4
CTX + CAZ + CIP+ E + AMP	15	83.3
CTX + CAZ + CIP+ E + AMP + CFM	14	77.8
CTX + CAZ + CIP+ E + AMP + CFM + AMC	13	72.2
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM	12	66.7
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI	9	50
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE	7	38.9
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE+C	6	33.3
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE + C + NIT	5	27.8
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE + C + NIT + PTZ	4	22.2
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE + C + NIT + PTZ + COT	4	22.2
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE + C + NIT + PTZ + COT + NA	4	22.2
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE + C + NIT + PTZ + COT + NA + AK	2	11.1
CTX + CAZ + CIP+ E + AMP + CFM + AMC + CPM + PI + TE + C + PTZ + COT + NA + NIT + AK + IPM	2	11.1

AK : Amikacin, AMC : Amoxiclav, AMP : Ampicillin, CPM : Cefepime, CFX : Cefixime, CTX : Cefotaxime, CAZ : Ceftazidime, C : Chloramphenicol, CIP : Ciprofloxacin, COT: Cotrimoxazole, E : Erythromycin, IPM : Imipenem, NA : Nalidixic acid, NIT : Nitrofurantoin, PI : Piperacillin, PTZ : Piperacillin Tazobactam, TE : Tetracycline

Total ESBL *E. coli* = 18

4.1.3 Detection of the bacterial isolates from poultry feces

4.1.3.1 Detection of bacterial isolates in poultry feces

E. coli isolates were detected from all poultry farms. From 13 poultry farms, 390 poultry fecal samples were collected. Altogether 402 bacterial isolates were detected from 390 fecal samples. *E. coli* was the most common isolate 68% (266/402). Apart from *E. coli*, *Citrobacter freundii* and *Citrobacter koseri* were detected in 18 % (72/402) and 8 % (33/402) of total isolates respectively. *Proteus* spp., *Salmonella* spp., *Shigella* spp. and *Acinetobacter* spp. comprised less than 3 % of the total isolates (Figure 14).

E. coli was isolated from 68 % (266/390) of the samples. *E. coli* was detected in 82 % (90/110) of the samples from cluster A, followed by cluster D-74 % (81/100), cluster B-59 % (29/40), cluster C- 54 % (54/100), and cluster E- 40 % (12/30) (Table 14).

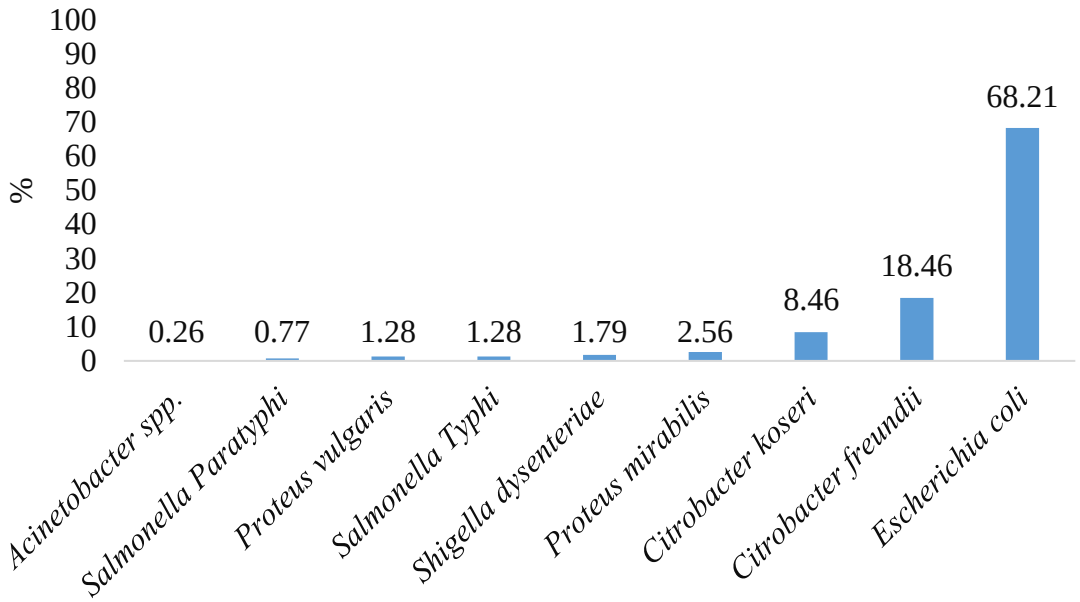


Figure 14: Bacterial isolates in poultry fecal samples

Table 14: Bacterial isolates in poultry fecal samples from different poultry farms

Poultry farm	Cluster	No. of Sample	*E. coli*	*C. freundii*	*C. koseri*	*P. mirabilis*	*P. vulgaris*	*S.* Typhi	*S.* Paratyphi	*S. dysenteriae*	*Acinetobacter* spp.	Total isolates
			n (%)									
1	A	30	22 (72)	2 (7)	4 (13)	2 (7)		2 (7)	3 (10)			35
2	A	30	27 (90)	6 (20)	1 (3)		1 (3)			4 (13)		39
3	A	40	31 (78)	8 (20)	3 (8)			1 (3)				43
4	A	10	10 (100)	3 (30)	1 (10)							14
5	B	40	29 (73)	5 (13)	5 (13)	1 (3)						40
6	B	40	23 (58)	7 (18)	4 (10)	3 (8)				2 (5)		39
7	B	20	13 (65)	5 (25)				2 (10)				20
8	C	40	18 (45)	2 (5)						1 (3)		21
9	D	30	26 (87)	6 (20)	5 (17)						1 (3)	38
10	D	30	15 (50)	4 (13)	5 (17)	2 (7)	1 (3)					27
11	D	40	32 (80)	8 (20)	3 (8)	2 (5)	3 (8)					48
12	D	10	8 (80)	4 (40)	2 (20)							14
13	E	30	12 (40)	12 (40)								24
	Total		266	72	33	10	5	5	3	7	1	402
	%		68.2	18.5	8.5	2.6	1.3	1.3	0.8	1.8	0.3	

4.1.3.2 Distribution of *E. coli* and ESBL *E. coli* in poultry feces

Of a total 402 bacterial isolates, 66 % (266/402) were *E. coli* isolates and 8 % (22/266) were ESBL *E. coli* (Figure 15). ESBL *E. coli* was not detected in 15 % (2/13) of the poultry feces from the farms viz., farm 8 in cluster C and farm13 in cluster E (Figure 16). Among total *E. coli* 8 % (22/266) were ESBL *E. coli* (Table 15).

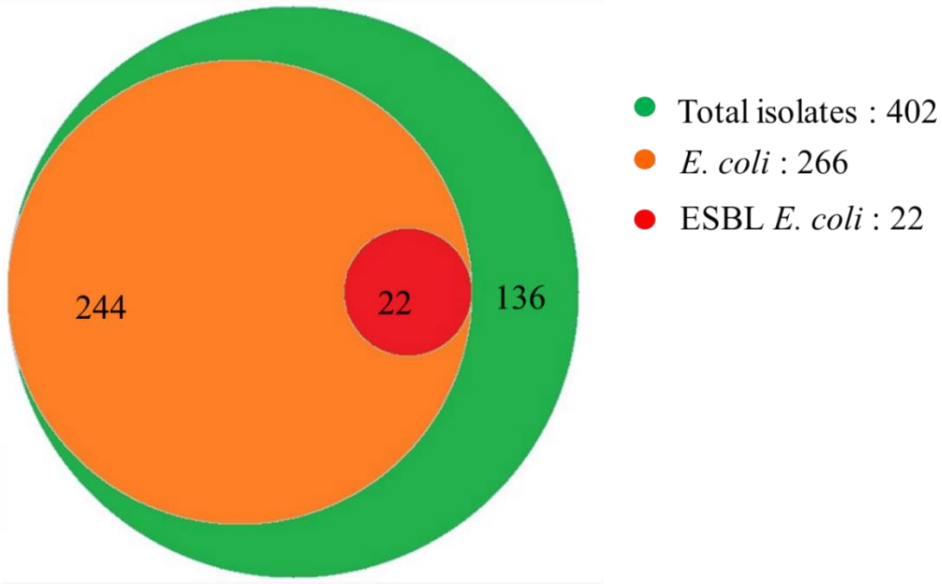
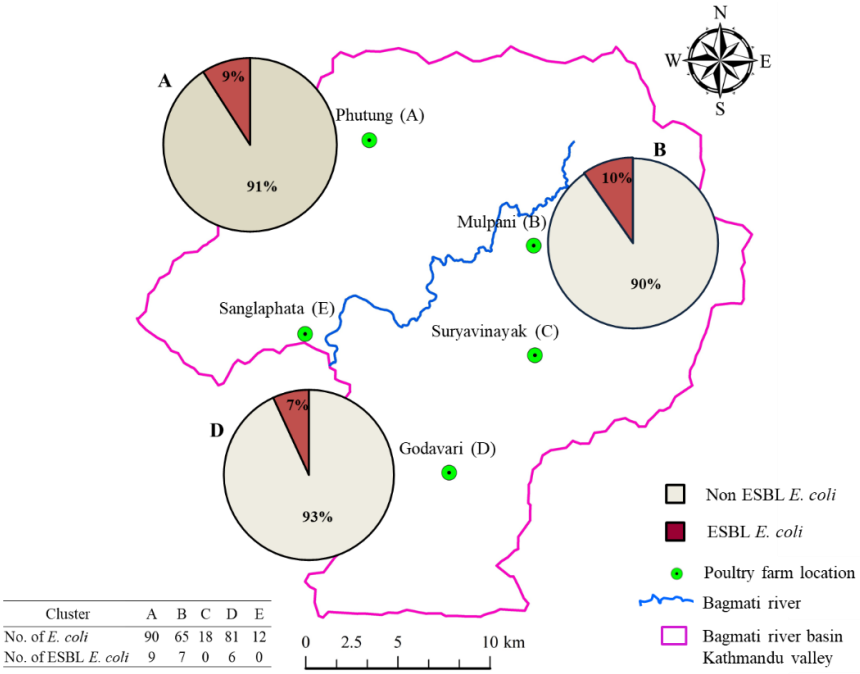


Figure 15: Distribution of *E. coli* and ESBL *E. coli* in poultry fecal isolates



Cluster	A	B	C	D	E
No. of *E. coli*	90	65	18	81	12
No. of ESBL *E. coli*	9	7	0	6	0

Figure 16: Location map showing distribution of ESBL *E. coli* among poultry farms within five clustered areas

Table 15: Distribution of *E. coli* and ESBL *E. coli* in different clusters of poultry farm

Poultry farm	Cluster	Total sample	Location	*E. coli*		ESBL *E. coli*	
				No. of isolates	%	No. of isolates	%
1	A	30	Phutung	22	73.3	2	9.09
2	A	30	Phutung	27	90	3	11.1
3	A	40	Phutung	31	77.5	2	6.5
4	A	10	Phutung	10	100	2	20
5	B	40	Mulpnai	29	72.5	3	10.3
6	B	40	Mulpani	23	57.5	3	13.1
7	B	20	Mulpani	13	65	1	7.7
8	C	40	Suryavanayak	18	45	0	0
9	D	30	Godavari	26	86.6	2	7.7
10	D	30	Godavari	15	50	2	13.3
11	D	40	Godavari	32	80	1	3.1
12	D	10	Godavari	8	80	1	12.5
13	E	30	Sanglaphanta	12	40	0	0
Total				266	68.21	22	8.27

4.1.3.3 Antibiotic resistance in non ESBL *E. coli* isolated from poultry feces

The antibiotic resistance of 244 non ESBL *E. coli* from poultry fecal samples was distributed among 17 antibiotics within 11 antimicrobial categories and 5 test/report groups (CLSI, 2020).

In macrolide category, 95 % (232/244) of *E. coli* were resistant to erythromycin. In test/report group A, *E. coli* showed the highest resistance percentage of 93 % (227/244) towards ampicillin within penicillin category. In test/report group B, altogether six antimicrobial categories were identified. Non ESBL *E. coli* showed the highest resistance percentage of 88 % (214/244) towards amoxicillin clavulanic acid, beta lactamase category. In this group, almost 2 % (4/244) non ESBL *E. coli* were resistant against imipenem, carbapenem category. Meanwhile in test/report group C, non ESBL *E. coli* exhibited resistance of 61 % (163/244) towards chloramphenicol, phenicol category, in contrast to 9 % (23/244) against ceftazidime, cephems category. In test/report group O, within penicillin category, 86 % (209/244) of the non ESBL *E. coli* were resistant against piperacillin, penicillin category in similar with 83 % (203/244) towards cefixime, cephems category. Non ESBL *E. coli* showed 78.3 % (191/244) resistance against tetracycline, tetracycline category and 77 % (188/244) resistance against nalidixic acid within quinolone category. In test/report group U, 63 % (153/244) of *E. coli* were resistant to nitrofurantoin, nitrofuran category.

The details of antibiotic resistance in *E. coli* from poultry feces are shown in Table 16.

Table 16: Antibiotic resistance in non ESBL *E. coli* isolated from poultry feces

Antimicrobial category	Test/Report Group	Antibiotics	No. of isolates	Resistant %
Macrolides	-	Erythromycin (E)	232	95.1
Penicillin	A	Ampicillin (AMP)	227	93
Beta lactam combination	B	Amoxicillin/Clavulanic acid (AMC)	214	87.7
Cephems	B	Cefepime (CPM)	213	87.3
Penicillin	O	Piperacillin (PI)	209	85.7
Beta lactam combination	B	Piperacillin/tazobactam (PTZ)	207	84.8
Cephems	O	Cefixime (CFM)	203	83.2
Tetracycline	O	Tetracycline (TE)	191	78.3
Quinolones	O	Nalidixic acid (NA)	188	77
Quinolones	B	Ciprofloxacin (CIP)	171	70.1
Folate pathway inhibitors	B	Trimethoprim/sulfamethoxazole (COT)	170	69.7
Nitrofurans	U	Nitrofurantoin (NIT)	152	62.7
Phenicol	C	Chloramphenicol (C)	148	60.7
Aminoglycosides	B	Amikacin (AK)	134	55.1
Cephems	B	Cefotaxime (CTX)	23	9
Cephems	C	Ceftazidime (CAZ)	21	8.6
Carbapenem	B	Imipenem (IPM)	4	1.6
Total non ESBL *E. coli* = 244				

4.1.3.4 Antibiotic resistance in ESBL *E. coli* isolated from poultry feces

The antibiotic resistance of 22 ESBL *E. coli* detected from poultry feces was distributed among 17 antibiotics within 11 antimicrobial categories, 5 test/report groups (CLSI, 2020). In test/report group A, ESBL *E. coli* showed resistance of 96 % (21/22) towards ampicillin, penicillin category. In test/report group B, altogether six antimicrobial categories were identified. The ESBL *E. coli* isolates were 100 % (18/18) resistant against amoxicillin clavulanic acid and piperacillin tazobactam, beta lactam category; and ceftazidime, cephems category. ESBL *E. coli* isolates were 96 % (21/22) resistant to cefotaxime and cefepime, cephems category and 91 % (20/22) resistant towards ciprofloxacin, quinolone category. In this group, ESBL *E. coli* had the least resistance percentage against imipenem 23 % (5/22) within carbapenem category followed by amikacin 59 % (13/22). Meanwhile in test/report group C,ESBL *E. coli* exhibited resistance of 69 % (15/22) towards chloramphenicol, phenicol category, which is lower in compared to 100 % (22/22) resistant against ceftazidime, cephems category. In test/report group O, 96 % (21/22) of ESBL *E. coli* were resistant towards cefixime, cephems category, piperacillin in penicillin category and tetracycline, tetracycline

category. Also 82 % (18/22) of ESBL *E. coli* were resistant towards nalidixic acid. In test/report group U, nitrofuran category, 73 % (16/22) of ESBL *E. coli* showed resistance against nitrofurantoin, nitrofuran category. The detailed antibiotic resistance in ESBL *E. coli* from poultry feces is shown in Table 17.

Table 17: Antibiotic resistance in ESBL *E. coli* isolated from poultry feces

Antimicrobial category	Test/Report Group	Antibiotics	No. of isolates	Resistant %
Beta lactam combination	B	Amoxicillin/Clavulanic acid (AMC)	22	100
Cephems	C	Ceftazidime (CAZ)	22	100
Cephems	B	Cefepime (CPM)	22	100
Beta lactam combination	B	Piperacillin/tazobactam (PTZ)	22	100
Macrolides	_	Erythromycin (E)	22	100
Cephems	O	Cefixime (CFM)	21	95.5
Penicillin	O	Piperacillin (PI)	21	95.5
Tetracycline	O	Tetracycline (TE)	21	95.5
Cephems	B	Cefotaxime (CTX)	21	95.5
Penicillin	A	Ampicillin (AMP)	21	95.5
Quinolones	B	Ciprofloxacin (CIP)	20	90.9
Folate pathway inhibitors	B	Trimethoprim/sulfamethoxazole (COT)	19	86.4
Quinolones	O	Nalidixic acid (NA)	18	81.8
Nitrofurans	U	Nitrofurantoin (NIT)	16	72.7
Phenicol	C	Chloramphenicol (C)	15	68.5
Aminoglycosides	B	Amikacin (AK)	13	59.1
Carbapenem	B	Imipenem (IPM)	5	22.7
Total ESBL *E. coli* isolates = 22				

4.1.3.5 Multiple drug resistance in non ESBL *E. coli* isolated from poultry feces

Among the total 244 *E. coli* isolated from poultry feces, 81 % (197/244) were multiple drug resistance showing resistance against three categories of antibiotics viz., macrolides, penicillin and beta lactam combination. About half (50 %, 129/266) of the MDR non ESBL *E. coli* were resistant to 5 antibiotic categories: macrolides, penicillin, beta lactam combination, cephems, tetracycline and quinolones. Whereas less than 5 % of MDR non ESBL *E. coli* were resistant against 10 categories with additional 5 categories (quinolones, folic acid inhibitors, furans, phenicol and aminoglycosides). The detailed drug resistance pattern is shown in Table 18.

Table 18: Multiple drug resistant non ESBL *E. coli* isolated from poultry feces

Antibiotics	No. of isolates	Resistant %
E	232	95.1
E + AMP	215	88.1
E + AMP + AMC	200	82.0
E + AMP + AMC + CPM	177	72.5
E + AMP + AMC + CPM + PI	168	68.9
E + AMP + AMC + CPM + PI + PTZ	165	67.6
E + AMP + AMC + CPM + PI + PTZ + CFM	139	57.0
E + AMP + AMC + CPM + PI + PTZ + CFM + TE	111	45.5
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA	98	40.2
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP	74	30.3
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT	69	28.3
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT + NIT	51	20.9
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT + NIT + C	38	15.6
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT + NIT + C + AK	34	13.9
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT + NIT + C + AK + CTX	3	1.2
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT + NIT + C + AK + CTX + CAZ	1	0.4
E + AMP + AMC + CPM + PI + PTZ + CFM + TE + NA + CIP + COT + NIT + C + AK + CTX + CAZ + IPM	0	0.0
AK : Amikacin, AMC : Amoxiclav, AMP : Ampicillin, CPM : Cefepime, CFX : Cefixime, CTX : Cefotaxime, CAZ : Ceftazidime, C : Chloramphenicol, CIP : Ciprofloxacin, COT: Cotrimoxazole, E : Erythromycin, IPM : Imipenem, NA : Nalidixic acid, NIT : Nitrofurantoin, PI : Piperacillin, PTZ : Piperacillin Tazobactam, TE : Tetracycline		
Total non ESBL *E. coli* = 244		

4.1.3.6 Multiple drug resistance in ESBL *E. coli* isolated from poultry feces

Among 22 ESBL *E. coli* isolated from poultry feces, 100 % (22/22) were multiple drug resistant showing resistance against three categories of antibiotics viz., beta lactam, cephems and macrolides. About 40 % of the ESBL *E. coli* isolates were resistant towards 8 drug categories with an additional 5 categories viz., penicillin, tetracycline, quinolones, folic acid inhibitors and phenicol category. The detailed antibiotic resistance pattern is given in Table 19. Despite being resistant to all 17 antibiotics tested, none of the isolates qualify as pan drug resistant because it fell short of the criteria for resistance across the required groups of antibiotics (Magiorakos *et al*., 2010).

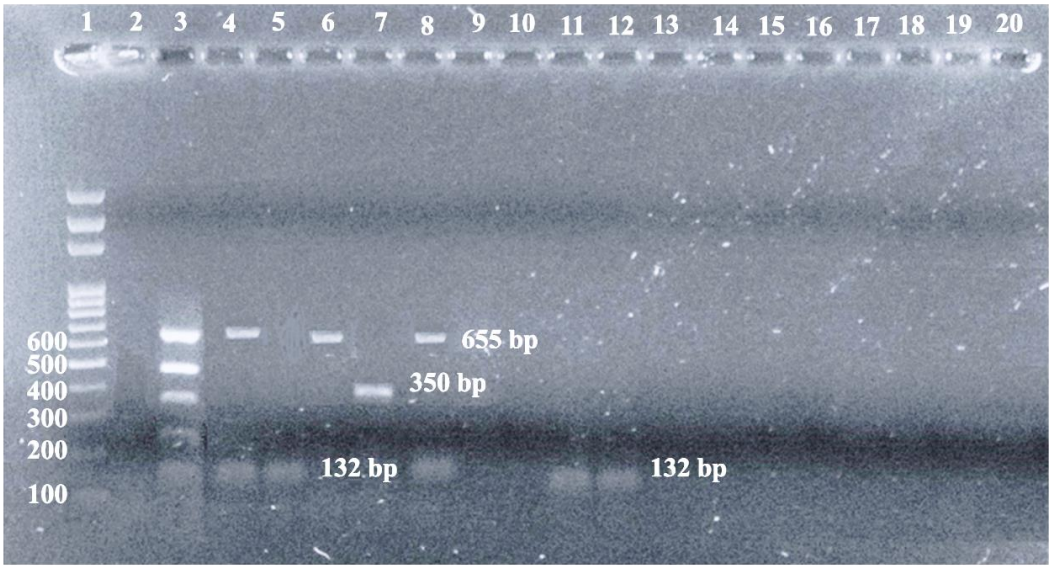
Table 19: Multiple drug resistant ESBL *E. coli* isolated from poultry feces

Antibiotics	No. of isolates	Resistant %
AMC + CAZ + CPM + E + PTZ	22	100
AMC + CAZ + CPM + E + PTZ + CFM	21	95.5
AMC + CAZ + CPM + E + PTZ + CFM + AMP	20	90.9
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX	20	90.9
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI	20	90.9
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE	19	86.4
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP	18	81.8
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E	17	77.3
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E + COT	14	63.6
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E + COT + NA	12	54.6
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E + COT + NA + NIT	9	40.9
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E + COT + NA + NIT + C	7	31.8
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E + COT + NA + NIT + C + AK	6	27.3
AMC + CAZ + CPM + E + PTZ + CFM + AMP + CTX + PI + TE + CIP + E + COT + NA + NIT + C + AK + IPM	1	4.6
AK : Amikacin, AMC : Amoxiclav, AMP : Ampicillin, CPM : Cefepime, CFX : Cefixime, CTX : Cefotaxime, CAZ : Ceftazidime, C : Chloramphenicol, CIP : Ciprofloxacin, COT: Cotrimoxazole, E : Erythromycin, IPM : Imipenem, NA : Nalidixic acid, NIT : Nitrofurantoin, PI : Piperacillin, PTZ : Piperacillin Tazobactam, TE : Tetracycline		
Total ESBL *E. coli* = 22		

4.1.4 Detection of targeted genes conferring ESBL mediated resistance in *E. coli* isolates from water, urine and fecal samples

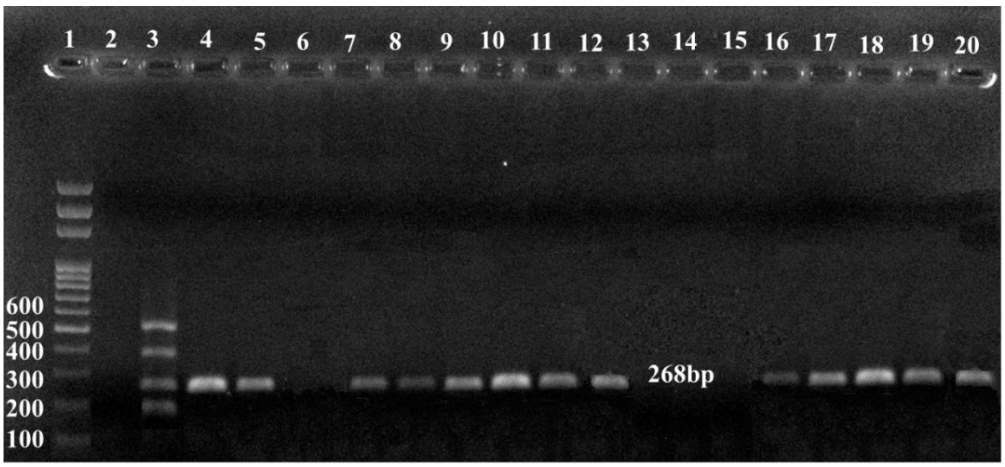
4.1.4.1 Genotypic detection of ESBL *E. coli* gene isolated from water, urine and fecal samples

Beta lactamase gene types *bla CTX M-1* (268 bp), *bla CTX M-9* (350 bp), *bla TEM* (132 bp), *bla SHV* (655 bp) were detected from the ESBL *E. coli* isolates (Figure 17; Figure 18). Of the total 80, ESBL *E. coli* isolates, *bla CTX M-1* gene was detected in 90 % (72/80), bla-*TEM* gene was detected in 40 % (32/80) and *bla SHV* was detected in 15 % (12/80) of the ESBL *E. coli*. Beta lactamase gene type *bla SHV* was not detected in ESBL *E. coli* isolated from human urine samples. *bla CTX M-9* gene was detected in only one ESBL *E. coli* isolate from river water. Extended spectrum beta lactamase gene types: *bla GES*, *bla CTX M-2*, *bla CTX M-8*, *bla CTX M-25* and *bla CTX M (chimera)* were not detected in ESBL *E. coli* isolates from water, human urine and poultry fecal sample. The presence of different gene types in water, urine and feces is shown in Table 20.



Lane 1 : 100 bp DNA ladder
Lane 2 : Negative control (nuclease free water)
Lane 3 : ESBL *E. coli bla TEM* (132 bp), *bla GES* (228 bp), bla *CTX M-9* (350 bp), *bla CTX M-2* (475 bp), *bla SHV* (655 bp) positive control
Lane 4,5,8,11,12 : ***bla TEM* (132 bp)**, ESBL *E. coli* positive (Sample; lane 4,5: water, lane 8,11: feces, lane 12: urine)
Lane 4, 6, 8 : ***bla SHV* (655 bp)**, ESBL *E. coli* positive (Sample; lane 4, 6 : water, lane 8 : feces)
Lane 7 : ***bla CTX M-9* (350 bp)**, ESBL *E. coli* positive (Sample; lane 7 : water)
Lane 9,10, 13,14,15,16,17,18,19,20 : ESBL *E. coli bla TEM*, *bla GES*, *bla CTX M-9, bla CTXM-2, bla SHV* negative

Figure 17 : Gel electrophoresis image showing amplified ESBL genes (*bla CTX M-9*, *bla SHV*, *bla TEM*)



Lane 1 : 100 bp DNA ladder
Lane 2: Negative control (nuclease free water)
Lane 3: ESBL *E. coli bla CTX M-8* (189 bp), *bla CTX M-1* (268 bp), *bla CTX M chimera* (391 bp), *bla CTX M-25* (522bp) positive control
Lane 4, 5, 8, 9, 10, 11, 12, 13, 16, 17, 18, 19, 20 : 268 bp, ***bla CTX M-1***, ESBL *E. coli* positive (Sample- lane 4 , 5 , 8 , 9 , 10 : water; lane 11 , 12, 13: feces; lane 16 ,17 ,18 ,19 , 20 : urine)
Lane 6, 7, 14, 15: ESBL *E. coli bla CTX M-8*, *bla CTX M-1*, *bla CTX M chimera*, *bla CTX M*-25 negative sample

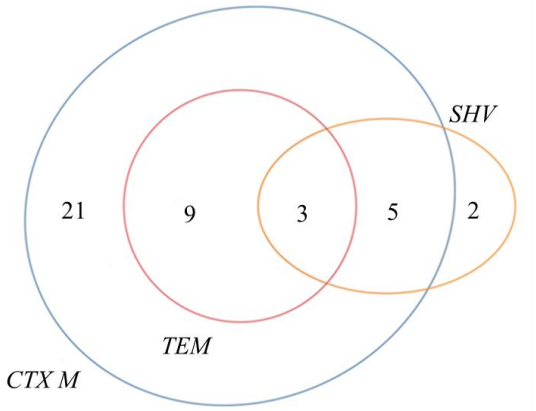
Figure 18: Gel electrophoresis image showing amplified ESBL gene (*bla CTX M-1*)

Table 20: Distribution of ESBL gene in ESBL *E. coli* isolated from water, human urine and poultry fecal sample

Source	Sample	No. of isolates	*bla CTX-M*		*bla TEM*	*bla SHV*
			bla CTX-M 1	*bla CTX-M 9*		
			n (%)			
River	Water	40	38 (92.5)	1 (2.5)	12 (30)	10 (25)
Human	Urine	18	18 (100)	0	8 (44.4)	0
Chicken	Feces	22	16 (72.7)	0	12 (54.5)	2 (9.1)
Total		80	72 (90)	0	32 (40)	12 (15)

4.1.4.2 Detection of ESBL gene types in ESBL *E. coli* isolated from water samples

In 40 ESBL *E. coli* isolates from river water, *bla CTX M* (*bla CTX M-1* and *bla CTX M-9*) genes were detected in 95 % (n=38/40) of the isolates. Both *bla CTX-M* and *bla TEM* were detected in 30 % (12/40) of the isolates, while all three genes *bla CTX-M*, *bla TEM* and *bla SHV* were detected in 7.5 % (3/40) of the isolates (Figure 19).



bla gene (N=40)	**n %**
CTX M	21 (52.5)
TEM	0 (0)
SHV	2 (5)
CTX M + TEM	12 (30)
CTX M + SHV	8 (20)
TEM + SHV	3 (7.5)
CTX M + TEM + SHV	3 (7.5)

Figure 19: Euler diagram showing distribution of ESBL gene types in *E. coli* isolated from river water samples

4.1.4.3 Distribution of ESBL gene in ESBL *E. coli* isolated from river water sampling sites

ESBL genes were not detected in *E. coli* isolates from upstream (1-7) locations, stone spouts and shallow well water samples (Table 2). Out of 66 river sampling sites, beta lactamase gene types were detected in 39.3 % (26/88) of the total sites. The beta-lactamase gene *bla CTX M-1* was present at all 26 sites.; whereas *bla TEM and bla SHV* were present at 30 % (8/26) of the water sampling sites distributed within midstream, downstream and tributaries. The *bla CTX M-9* gene was found in one sampling site from the tributary (Figure 20).

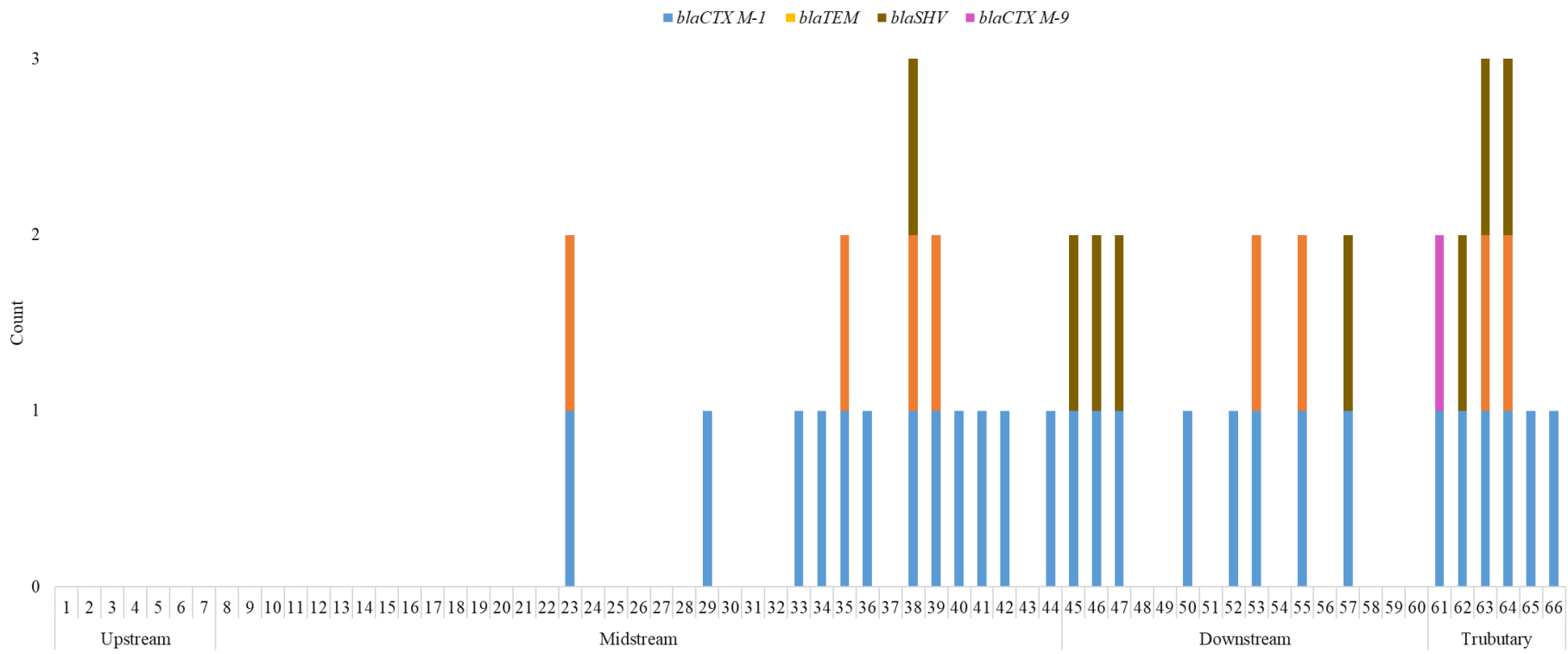
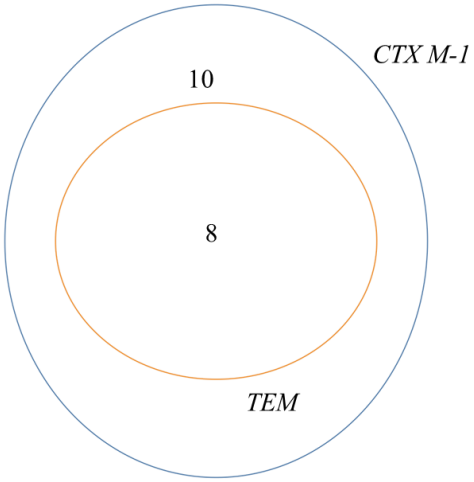


Figure 20: Detection of targeted genes responsible for ESBL in *E. coli* isolated from river water sampling sites

4.1.4.4 Detection of ESBL gene in ESBL *E. coli* isolated from urine samples

Only in 18 ESBL *E. coli* isolated from urine samples, ESBL gene types *bla CTX M-1* was detected in 100 % (18) and *bla CTX M-1* with *bla TEM* were detected in 44 % (8) of the total ESBL *E. coli* isolates (Figure 21).



bla gene (N=18)	n %
CTX M-1	10 (55.5)
TEM	0 (0)
CTX M-1 + TEM	8 (44.4)

Figure 21: Euler diagram showing distribution of ESBL gene types in *E. coli* isolated from human urine samples

4.1.4.5 Distribution of ESBL *E. coli* gene in human urine samples

Among the 18 samples, all are confirmed to have the ESBL gene. Specifically, all 10 *E. coli* isolates were found to possess the *bla CTX M-1* gene type. Furthermore, among these *E. coli* isolates, eight were identified to carry both the *bla CTX M-1* and *TEM* types of the ESBL gene (Figure 22).

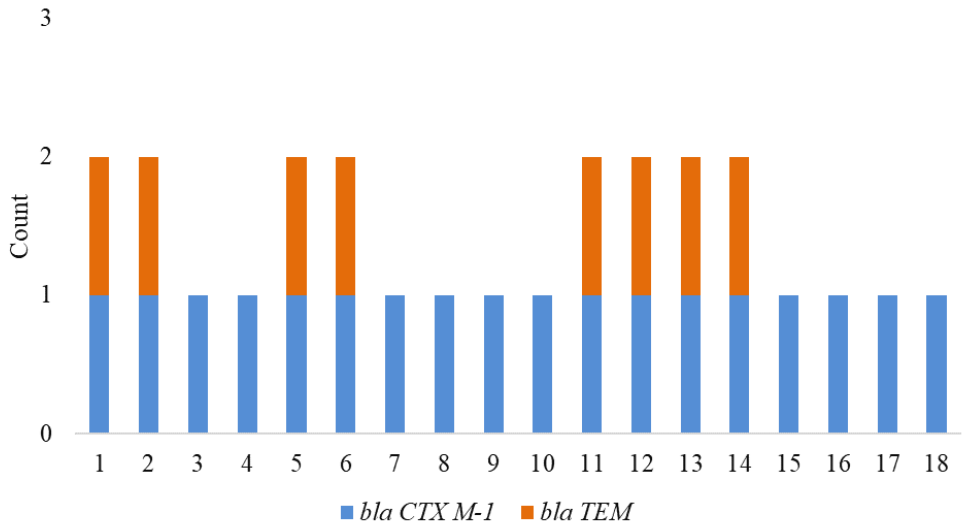
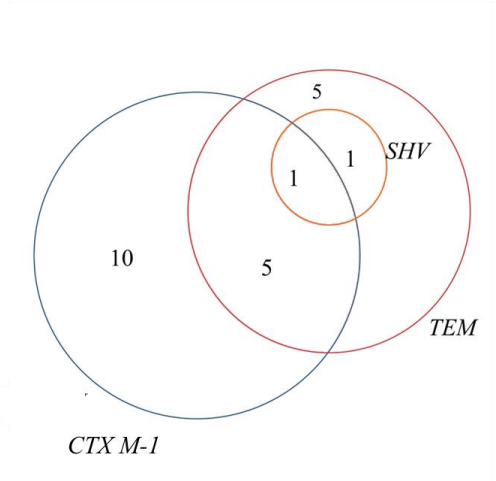


Figure 22: Distribution of ESBL *E. coli* gene types in human urine samples

4.1.4.6 Distribution of ESBL genes in ESBL *E. coli* isolated from poultry feces

In 22 ESBL *E. coli* isolates, *bla CTX M-1* was detected in 73 % (16/22) of the isolates whereas *bla TEM* and *bla SHV* were present in 55 % (12/22) and 9 % (2/22) of the isolates respectively. All three genes *bla CTX M-1*, *bla TEM* and *bla SHV* were detected in 4.5 % (1/22) of the isolates (Figure 23).



bla gene (N=22)	n %
CTX M-1	10 (45)
TEM	5 (22.7)
SHV	0 (0)
CTX M-1 + TEM	6 (22.7)
CTX M-1 + SHV	1 (4.5)
TEM + SHV	2 (9)
CTX M-1 + TEM + SHV	1 (4.5)

Figure 23: Euler diagram showing distribution of ESBL gene types in *E. coli* isolated from poultry feces

4.1.4.7 Distribution of ESBL gene in ESBL *E. coli* isolated from chicken feces in different poultry farms

The ESBL *E. coli* was not detected from fecal samples collected from poultry farm located at Suryavinayak, cluster C and Sanglaphanta, cluster E. ESBL *E. coli* was detected from fecal samples collected from 11 farms located at Phutung (A1, A2, A3, A4), Mulpani (B1, B2, B3) and Godavari (D1, D2, D3, D4). The beta lactamase gene type *bla CTX M-1* was detected from ESBL *E. coli* isolates from poultry farms located at cluster A, B and D. Beta lactamase *TEM* gene was detected from ESBL *E. coli* isolated from fecal samples collected from 3 poultry farms in cluster A (A1, A2, A3) and 2 in cluster B (B5, B6) whereas *bla TEM* gene was detected from only one farm within cluster D group (D 9). Three beta lactamase gene types *bla CTX M-1*, *bla TEM* and *bla SHV* were detected from ESBL *E. coli* isolates from fecal samples collected from farm A1 (Phutung) and farm B6 (Mulpani) whereas *bla SHV* gene type was detected from poultry fecal isolate from farm A1 and A4 located at Phutung and B6 farm from Mulpani (Figure 24).

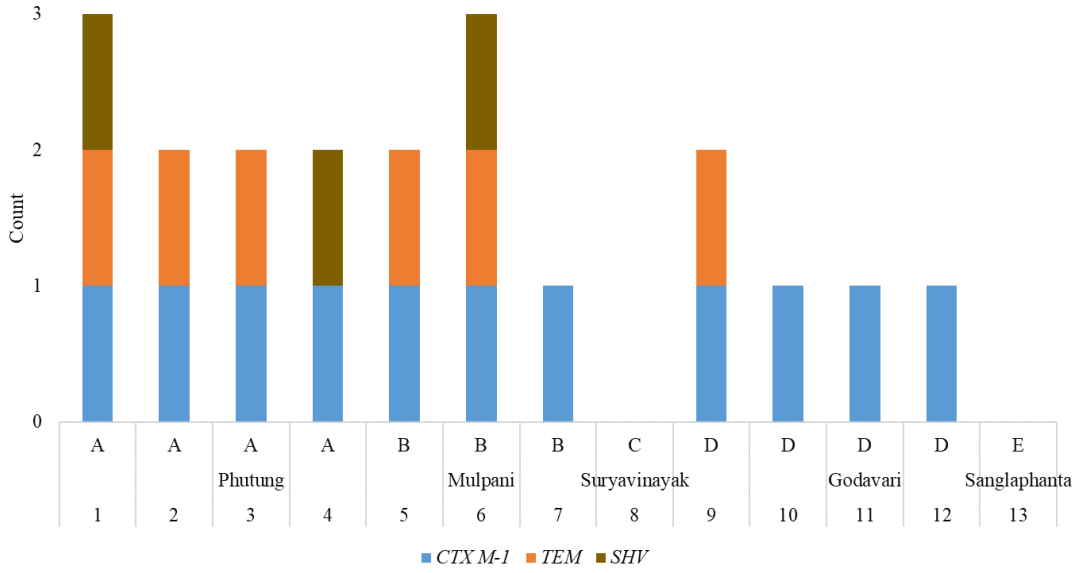


Figure 24: ESBL gene types in ESBL *E. coli* isolated from poultry feces in different clustered locations

4.1.5 Possible linkage between *E. coli* and ESBL *E. coli* isolated from water, clinical, and poultry samples

4.1.5.1 Association of antibiotic resistance in non ESBL *E. coli* isolated from water samples

The chi-square test was applied at a significance level of p = 0.05 to analyze the association of antibiotic resistance patterns of non ESBL *E. coli* isolates from various water sources (upstream, midstream, downstream, tributaries, shallow wells, and stone spouts). Resistance to six antibiotics, namely amoxicillin-clavulanic acid, ampicillin, piperacillin, tetracycline, cefixime, and piperacillin-tazobactam, among non ESBL *E. coli* isolates from various sources, was statistically significantly associated, suggesting that factors such as local antibiotic usage, environmental conditions, or contamination sources may be influencing resistance levels in specific areas. The resistance ranged from about 71 % to 100 % for these antibiotics across upstream, midstream, downstream and tributary. In contrast, resistance to the other 11 antibiotics (erythromycin, cefepime, ceftazidime, cefotaxime, amikacin, nalidixic acid, cotrimoxazole, chloramphenicol, ciprofloxacin, and imipenem) among non ESBL *E. coli* isolates from various sources were statistically in-significantly associated, showing diverse resistance patterns of isolates across different sources (Table 21).

Table 21: Association of antibiotic resistance in non ESBL *E. coli* isolated from water sources

Source/ Antibiotics	Upstream (n=7)		Midstream (n=125)		Downstream (n=48)		Tributary (n=21)		Stone spout (n=30)		Shallow well (n=17)		Total (N=248)		p value
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	
E	6	85.7	125	100	47	97.9	21	100	30	100	17	100	246	99.2	0.305
AMC	7	100	125	100	47	97.9	21	100	29	96.7	15	88.2	244	98.4	**0.022**
AMP	7	100	125	100	46	95.8	21	100	26	86.7	17	100	242	97.6	**0.012**
PI	7	100	125	100	46	95.8	21	100	26	86.7	12	70.6	237	95.6	**<0.001**
TE	7	100	125	100	46	95.8	20	95.2	23	76.7	16	94.1	237	95.6	**<0.001**
CFM	3	42.9	103	82.4	41	85.4	17	81	18	60	9	52.9	191	77	**0.008**
CPM	3	42.9	92	73.6	38	79.2	16	76.2	20	66.7	7	41.2	176	71	0.095
PTZ	5	71.4	88	70.4	35	72.9	15	71.4	12	40	8	47.1	163	65.7	**0.002**
CAZ	2	28.6	53	42.4	31	64.6	10	47.6	6	20	5	29.4	107	43.1	0.106
CTX	2	28.6	53	42.4	28	58.3	11	52.4	7	23.3	4	23.5	105	42.5	0.104
AK	1	14.3	42	33.6	29	60.4	16	76.2	9	30	6	35.3	103	41.5	0.296
NA	1	14.3	36	28	26	54.2	10	47.6	7	23.3	5	29.4	85	34.3	0.735
NIT	2	28.6	42	33.6	11	22.9	4	19	6	20	7	41.2	72	29	0.469
COT	3	42.9	31	24.8	19	39.6	4	16	4	13.3	4	23.5	65	26.2	0.231
C	2	28.6	26	20.8	13	27.1	4	19	8	26.7	6	35.3	59	23.8	0.284
CIP	2	28.6	27	21.6	15	31.3	7	33.3	3	10	5	29.4	59	23.8	0.882
IPM	1	14.3	13	10.4	3	6.3	5	23.8	2	6.7	4	23.5	28	11.3	0.311

AK: Amikacin, AMP: Ampicillin, AMC: Amoxicillin/Clavulanic acid, AMP: Ampicillin, C: Chloramphenicol, CAZ: Ceftazidime, CFM: Cefixime, CIP: Ciprofloxacin, COT: Trimethoprim/Sulfamethoxazole, CPM: Cefepime, CTX: Cefotaxime, E: Erythromycin, NA: Nalidixic, NIT: Nitrofurantoin, PI: Piperacillin, PTZ: Piperacillin Tazobactam, TE: Tetracycline ; n: count, %: resistance %

4.1.5.2 Association of antibiotic resistance in ESBL *E. coli* isolated from water samples

Table 22 presents the antibiotic resistance patterns of ESBL *E. coli* isolated from midstream, downstream and tributaries of a river (Figure 11), tested resistance against 17 different antibiotics. The chi-square test was applied at a significant level of p = 0.05, to analyze the association of antibiotic resistance pattern of ESBL *E. coli* isolates from midstream, downstream and tributary as ESBL *E. coli* was not detected from upstream, stone spouts and shallow well water samples. Resistance to five antibiotics viz., amoxicillin clavulanic acid, ampicillin, ceftazidime, cefotaxime and piperacillin were statistically significantly associated indicating a strong relationship between the resistance to these antibiotics. The chi square test result indicates that the resistance to the five antibiotics is highly associated, suggesting that ESBL production in *E. coli* is likely a contributing factor to this resistance pattern. ESBL *E. coli* showed 100% resistance towards these antibiotics. In contrast, resistance to 12 antibiotics viz., erythromycin, tetracycline, cefixime, cefepime, piperacillin tazobactam, nitrofurantoin, amikacin, nalidixic acid, cotrimoxazole, ciprofloxacin, chloramphenicol and imipenem among isolates from various sources were statistically in-significantly associated. The lack of significant association suggests that the resistance to these antibiotics varies widely across different sources. There isn’t a strong, consistent pattern of co-resistance among the isolates, implying that multiple factors may influence resistance rather than a single, overarching factor. The result indicates diverse resistance patterns among the isolates. This suggests that factors other than ESBL production are influencing resistance to these antibiotics, as ESBLs mainly affect beta-lactam antibiotics.

Table 22: Association of antibiotic resistance in ESBL *E. coli* from water sources

Source/ Antibiotics	Midstream (n=15)		Downstream (n=14)		Tributary (n=11)		Total (N=40)		p value
	n	%	n	%	n	%	N	%	
AMC	15	100	14	100	11	100	40	100	
AMP	15	100	14	100	11	100	40	100	
CAZ	15	100	14	100	11	100	40	100	
CTX	15	100	14	100	11	100	40	100	
PI	15	100	14	100	11	100	40	100	
E	15	100	13	92.9	11	100	39	97.5	0.901
TE	15	100	13	92.9	11	100	39	97.5	0.901
CFM	14	93.3	10	85.7	10	90.9	36	90	0.745
CPM	14	93.3	10	71.4	9	81.8	33	82.5	0.383
PTZ	7	46.7	8	57.1	11	100	26	65	0.341
NIT	9	60	9	64.3	4	36.4	22	55	0.272
AK	5	33.3	8	57.1	5	45.5	18	45	0.480
NA	5	33.3	7	50	6	54.5	18	40.5	0.272
COT	2	11.3	8	57.1	3	27.3	13	32.5	0.338
CIP	3	20	4	28.6	4	36.4	11	27.5	0.359
C	3	20	2	14.3	4	36.4	8	20	0.423
IPM	3	20	2	14.3	2	18.2	7	17.5	0.878

AK: Amikacin, AMP: Ampicillin, AMC: Amoxicillin Clavulanic acid, AMP: Ampicillin, C: Chloramphenicol, CAZ: Ceftazidime, CFM: Cefixime, CIP: Ciprofloxacin, COT: Trimethoprim/Sulfamethoxazole, CPM: Cefepime, CTX: Cefotaxime, E:Erythromycin, NA: Nalidixic, NIT: Nitrofurantoin, PI: Piperacillin, PTZ: Piperacillin Tazobactam, TE: Tetracycline; n: count, %: resistance %

4.1.5.3 Association of *E. coli* isolates from water, feces and urine

Table 23 presents the antibiotic resistance patterns of non ESBL *E. coli* and ESBL *E. coli* isolated from water, human urine, and chicken fecal samples, with resistance tested against 17 different antibiotics. The chi-square test was applied at significance level of $p = 0.05$ to analyze the association of antibiotic resistance patterns of *E. coli* isolates from water, human urine and poultry fecal samples. Resistance to all 17 antibiotics was statistically not significant. The lack of statistically significant associations in resistance patterns across water, human urine, and poultry fecal samples indicates that *E. coli* isolates from these sources demonstrate diverse resistance profiles. Non ESBL *E. coli* showed the resistance ranging between 60 % - 99 % for amoxicillin clavulanic acid, ampicillin, cefixime, cefepime, erythromycin, piperacillin, piperacillin tazobactam and tetracycline, between 20 % - 80 % for amikacin, chloramphenicol, ciprofloxacin, cotrimoxazole, cefotaxime, nalidixic acid and nitrofurantoin. Resistance ranged from 1 % - 11 % for imipenem, 9 % - 40 % for cefotaxime and ceftazidime antibiotics. For ESBL *E. coli*, resistance ranged from 60 % - 100% for amoxicillin clavulanic acid,

ampicillin, ceftazidime, cefixime, cefepime, cefotaxime, erythromycin, piperacillin, piperacillin tazobactam and tetracycline; 20 % - 80 % for chloramphenicol, cotrimoxazole, nalidixic acid and nitrofurantoin and 15 % - 100 %. The resistance ranged from 20 % - 90 % for ciprofloxacin and 17 % - 23 % for imipenem antibiotics. This underscores the complex and varied nature of antibiotic resistance dynamics influenced by environmental, human, and animal factors.

Table 23: Association of antibiotic resistance in *E. coli* isolated from water, urine and fecal samples

Antibiotics	Non ESBL *E. coli* (resistance %)			Total (%)	p value	ESBL *E. coli* (resistance %)			Total (%)	p value	Total (%)
	water (n=248)	urine (n=45)	feces (n=244)	N = 537		water (n=40)	urine (n=18)	feces (n=22)	N = 80		N = 617
Amikacin (AK)	41.5	35.6	55.1	44.1	0.336	45	22.2	59.1	42.10	0.592	43.1
Amoxicillin clavulanic acid (AMC)	98.4	64.4	87.7	83.5	0.663	100	83.3	100	94.4	1	88.9
Ampicillin (AMP)	97.6	88.9	93	93.2	0.455	100	88.9	95.5	94.8	0.569	93.9
Chloramphenicol (C)	23.8	66.7	60.7	50.4	0.261	20	66.7	68.5	51.7	0.212	51.1
Ceftazidime (CAZ)	43.1	26.7	8.6	26.1	0.157	100	100	100	100	-	63.1
Cefixime (CFM)	77	86.7	83.2	82.3	0.372	90	88.9	95.9	91.60	0.286	86.9
Ciprofloxacin (CIP)	23.8	66.7	70.1	53.5	0.205	27.5	100	90.9	72.80	0.256	63.2
Cotrimoxazole (COT)	26.2	53.3	69.7	49.7	0.161	32.5	55.6	86.4	58.2	0.159	53.9
Cefepime (CPM)	71	73.3	87.3	77.2	0.191	82.5	83.3	100	88.6	0.211	82.9
Cefotaxime (CTX)	42.5	26.7	9	26.1	0.158	100	100	95.5	98.5	0.221	62.3
Erythromycin (E)	99.2	77.8	95.1	90.7	0.799	97.5	94.4	90.9	94.3	0.158	92.5
Imipenem (IPM)	11.3	24.4	1.6	12.4	0.549	17.5	16.7	22.7	18.9	0.259	15.7
Nalidixic acid (NA)	34.3	75.6	77	57.5	0.158	40.5	61.1	81.8	62.6	0.234	60.1
Nitrofurantoin (NIT)	29	57.8	62.7	49.8	0.322	55	38.9	72.7	55.5	0.164	52.7
Piperacillin (PI)	95.6	88.9	85.7	90.1	0.166	100	77.8	95.5	91.1	0.786	90.6
Piperacillin tazobactam (PTZ)	65.7	66.7	84.8	72.4	0.209	65	61.1	100	75.4	0.248	73.9
Tetracycline (TE)	95.6	84.4	78.3	86.1	0.163	97.5	77.8	95.5	90.3	0.896	88.2

4.1.5.4 Correlation between non ESBL *E. coli* isolated from water, feces and urine samples

The correlation coefficient was calculated based on the zone of inhibition values of non ESBL *E. coli* isolates from water, fecal, and urine samples. The Pearsons correlation coefficient between non ESBL *E. coli* isolates from chicken fecal and water sample showed low degree of significant positive correlation (r = 0.27) indicating a slight relationship between non ESBL *E. coli* in feces and water. The Pearson correlation coefficient between non ESBL *E. coli* isolates from chicken feces and human urine sample is r = 0.33, indicating a weak to moderate positive correlation. This suggests some degree of relationship between the presence or abundance of non ESBL *E. coli* in chicken feces and human urine samples. Similarly, the correlation between non ESBL *E. coli* isolates from urine and water is r = 0.25, also indicating a weak positive correlation and suggesting a slight relationship between non ESBL *E. coli* in human urine and water sample (Table 24).

Table 24: Pearsons's correlation coefficient between non ESBL *E. coli* isolated from water, urine and fecal samples

Sample		R	p
Feces	Urine	0.33	< 0.05
Feces	Water	0.27	< 0.05
Urine	Water	0.25	< 0.05

4.1.5.5 Correlation between ESBL *E. coli* isolates from water, feces and urine samples

The Pearson correlation coefficients for ESBL *E. coli* isolates indicated the following relationships: a weak positive correlation between water and urine (r = 0.3), suggesting a slight relationship in their presence or abundance; a very weak positive correlation between water and feces (r = 0.17), indicating minimal relationship; and a very weak positive correlation between urine and feces (r = 0.13), also suggesting minimal relationship (Table 25).

Table 25: Pearsons's correlation coefficient between ESBL *E. coli* isolated from water, urine and fecal samples

Sample		R	p
Water	Urine	0.3	< 0.05
Water	Feces	0.17	0.00087
Urine	Feces	0.13	0.019

4.1.5.6 Comparative analysis of antibiotic resistance patterns in non ESBL *E. coli* isolated from water sources

The similarity map, based on the antibiotic resistance patterns of non ESBL *E. coli* isolated from various water sources (Figure 25), reveals notable insights into their resistance profiles. The relatively low distance between downstream and tributaries (40.498) suggests a high degree of similarity, indicating that these water sources likely experience similar contamination or antibiotic exposure. The moderate distance between downstream and midstream (52.372) and between midstream and tributaries (54.489) reflects some level of similarity, implying shared environmental conditions or contamination pathways affecting these areas. A similar moderate distance is observed between shallow wells and stone spouts (54.790), suggesting that these sources might have common local environmental conditions or contamination sources. In contrast, the significant distances between downstream and upstream (96.667) and between tributaries and upstream (98.368) indicate substantial differences in their antibiotic resistance patterns, likely due to varying levels of contamination and distinct environmental influences, with upstream areas generally being less exposed to pollutants. The detailed distance between the similar pairs is given in Table 26.

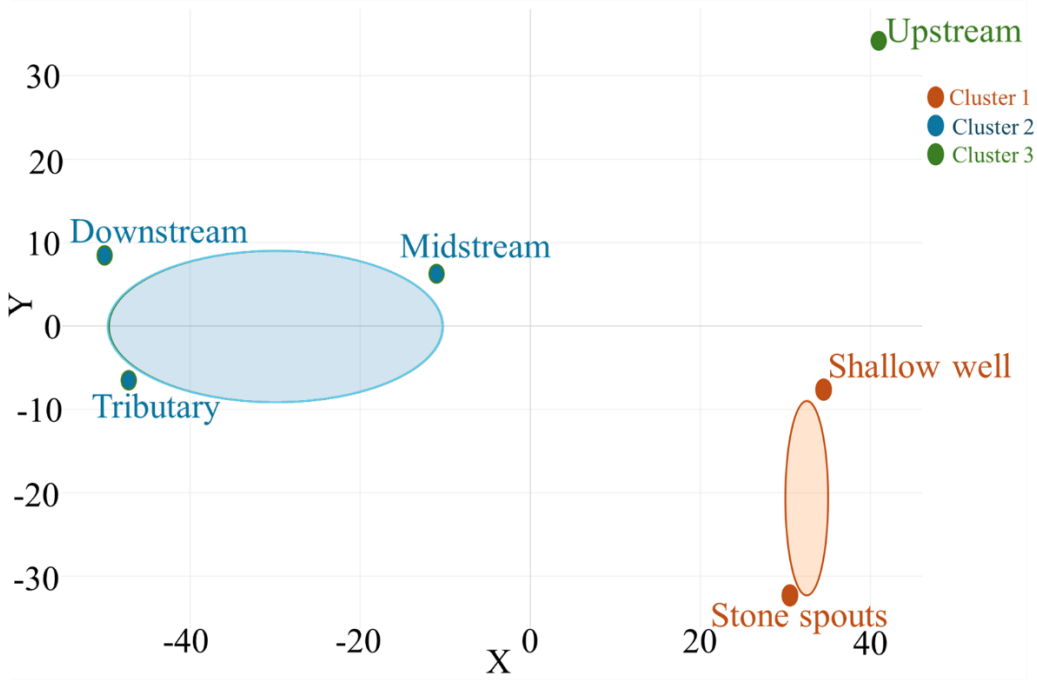


Figure 25: Similarity map based upon the antibiotic resistance pattern of non ESBL *E. coli* in water sources

Table 26: Distance matrix based upon the antibiotic resistance pattern of non ESBL *E. coli* in water sources

Column 1	Column 2	Distance
Downstream	Tributary	40.4982716
Downstream	Midstream	52.3717481
Midstream	Tributary	54.4886227
Shallow well	Stone spout	54.7897801
Downstream	Upstream	96.6674195
Tributary	Upstream	98.3678809

4.1.5.7 Comparative analysis of antibiotic resistance patterns in ESBL *E. coli* isolated from water sources

The similarity map (Figure 26), based on the antibiotic resistance patterns of ESBL *E. coli* isolated from downstream, midstream, and tributary water sources, revealed insights into the degree of similarity in their resistance profiles. The distance between downstream and midstream is 61.614, indicating a moderate degree of similarity in the antibiotic resistance patterns of ESBL *E. coli* from these sources. This suggests that both downstream and midstream areas might be influenced by similar contamination sources or environmental conditions, leading to comparable resistance profiles. The distance between downstream and tributary is slightly higher at 66.421, suggesting that while there is still a moderate similarity, there are more differences compared to the downstream and midstream pair. This could imply some unique contamination sources or environmental influences affecting the tributaries that are not as prevalent in downstream areas. The highest distance is observed between midstream and tributary at 70.223, indicating the greatest degree of dissimilarity among the compared pairs. This suggests that the ESBL *E. coli* isolated from midstream and tributary sources have the most distinct antibiotic resistance patterns, likely due to differing contamination levels or environmental factors specific to each water source. The comparative analysis highlights that ESBL *E. coli* isolated from downstream and midstream sources have more similar antibiotic resistance patterns compared to those from tributary sources. The detailed distance between the similar pairs is given in Table 27.

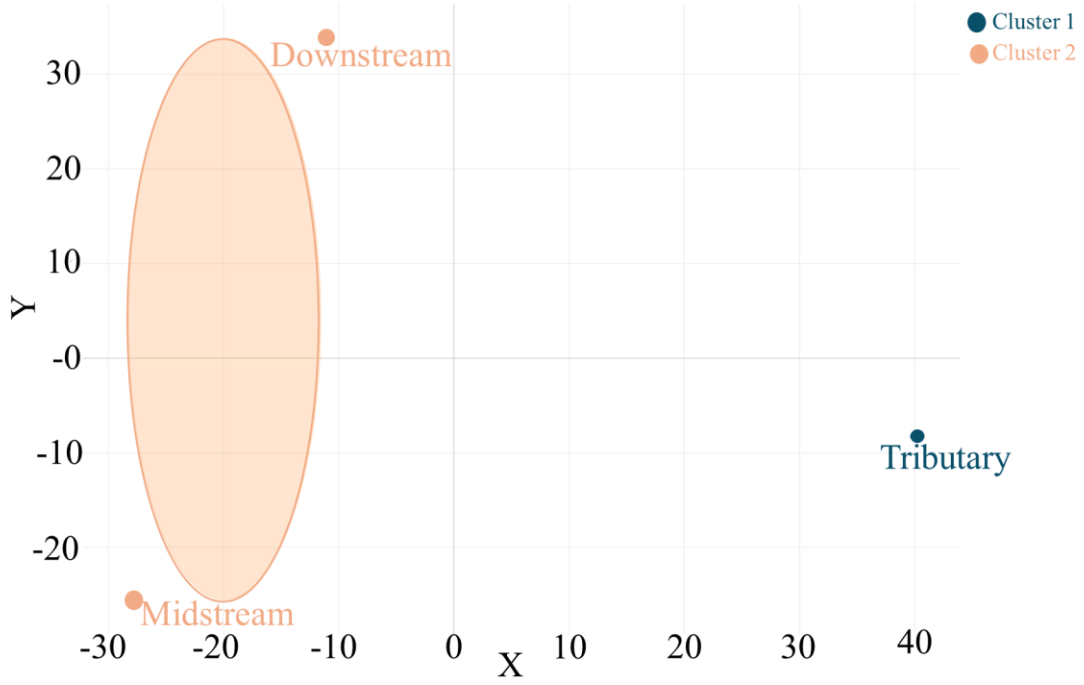


Figure 26: Similarity map based upon the antibiotic resistance pattern of ESBL *E. coli* in water sources

Table 27: Distance matrix based upon the antibiotic resistance pattern of ESBL *E. coli* in water sources

Column 1	Column 2	Distance
Downstream	Midstream	61.6144464
Downstream	Tributary	66.4209304
Midstream	Tributary	70.2232155

4.1.5.8 Comparative analysis of antibiotic resistance patterns in ESBL *E. coli* isolated from water, urine, and feces

The similarity map (Figure 27), showing the distances between urine and water (99.200) and between feces and water (114.878) (Table 28), is based on the antibiotic resistance patterns of ESBL producing *E. coli* isolated from these sources. The relatively lower distance between urine and water suggests that the antibiotic resistance patterns of ESBL *E. coli* from these sources are somewhat similar. The higher distance between feces and water indicates greater dissimilarity in the antibiotic resistance patterns of ESBL *E. coli* from these sources. The comparative analysis of the distance matrix data reveals that while there is a notable similarity in antibiotic resistance patterns between ESBL *E. coli* from urine and water, the resistance profiles in feces are more distinct from those in water.

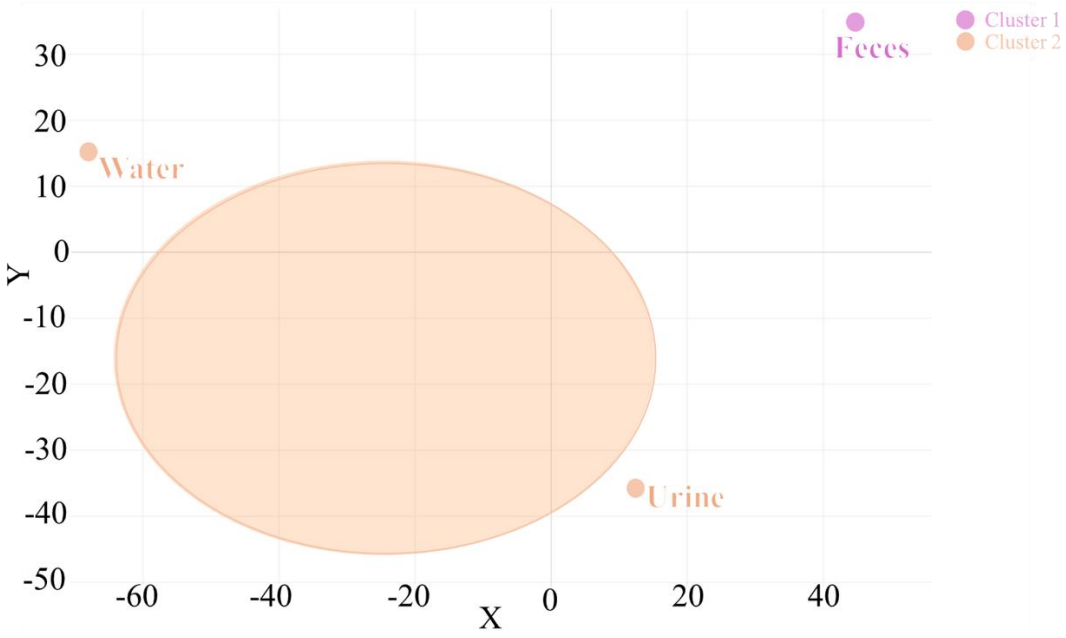


Figure 27: Similarity map based upon the antibiotic resistance pattern of ESBL *E. coli* in water, urine and fecal samples

Table 28: Distance matrix based upon the antibiotic resistance pattern of ESBL *E. coli* in water, urine and fecal samples

Column 1	Column 2	Distance
Water	Urine	99.200
Water	Feces	114.878

4.2 Discussions

This study analyzed the presence and antimicrobial resistance in *E. coli* isolated from water, poultry feces, and human urine specimens in Kathmandu valley and evaluated their relatedness in terms of antimicrobial resistance, antimicrobial resistance conferring genes. The findings have revealed valuable insights into the dynamics of antimicrobial resistance within the Kathmandu valley river basin .

The study revealed nearly half of the *E. coli* isolates among the total bacterial isolates in water, human urine, and chicken fecal samples. Apart from *E. coli*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella* Typhi, *Salmonella* Paratyphi, *Shigella dysenteriae* and *Acinetobacter* spp. were also detected in the analyzed specimens. Detection of Enterobacteriaceae in water, urine, and fecal samples is largely due to their origin in the intestines of humans and animals, fecal contamination of water sources, infections in the urinary tract, their ability to survive

in various environments, and poor sanitation practices. These bacteria can be introduced into these samples through direct contamination, environmental persistence, and infections in the human population (Cabral., 2010). Primarily, *E. coli* is a dominant member of the intestinal flora in humans and many animals, including chickens. As such, it is commonly present in fecal matter, which often contaminates water sources through runoff, sewage discharge, or direct defecation (GoN, MoHP, 2013). In water testing, *E. coli* is frequently used as an indicator organism to assess fecal contamination, which explains its high prevalence in such samples (Martinson *et al*., 2020). Additionally, *E. coli* is a leading cause of urinary tract infections (UTIs) in humans, making it a frequent isolation in urine samples. Its ability to survive and thrive in diverse environments further contributes to its widespread presence (Mancuso *et al*., 2023). Apart from *E. coli*, *Klebsiella oxytoca* was isolated from about 90% of the water samples. *K. oxytoca* primarily comes from the gastrointestinal tracts of humans and animals, spreading to the environment through fecal contamination through sewage, wastewater, and agricultural runoff. Healthcare facilities also contribute through improper waste disposal. It can also be found in soil and plants, usually due to contamination from fecal matter or organic waste. Its presence in water bodies often results from runoff and poor sanitation practices (Essert *et al*., 2023; Loudermilk *et al*., 2022).

In poultry, *E. coli* is a normal inhabitant of the gut, leading to its high numbers in poultry fecal matter. Developing nations face a risk of developing antimicrobial resistance in commensal in poultry farms due to limited economic resources for maintaining good livestock practices in and around the farm areas (Parlasca *et al*., 2023).

In the study, the mean Most Probable Number (MPN) count revealed increasing trend from upstream till tributaries: upstream (31) harbored lowest number of coliforms followed by stone spout (69), shallow well (160), midstream (2,400), downstream (17,000), and tributaries (70,000) (Figure 6). Tributaries are smaller water bodies that fed into larger rivers and often display elevated levels of coliform bacteria compared to upstream river sources. Firstly, tributaries receive runoff from various sources, including agricultural lands, urban areas, and industrial sites, which can introduce fecal matter from livestock, human waste, and other contaminants into the water (Luna Juncal *et al*., 2023). Secondly, the slower flow rates in tributaries provide more opportunity for these contaminants to accumulate and persist, leading to higher concentrations of coliform bacteria (Liu *et al*., 2024). Additionally, tributaries may lack

the dilution capacity of larger rivers, allowing pollutants to remain more concentrated. Furthermore, tributaries are often situated closer to potential point sources of pollution resulting in direct inputs of untreated or partially treated wastewater that can significantly elevate coliform levels in these water bodies (Yao *et al*., 2023).

In this study, *E. coli* was detected at almost all (85 out of 88) of the sites from where water samples were collected and analyzed. *E. coli* was not detected in one fourth of the upstream sites, from where Kathmandu valley receives its municipal water supply, which is an indication that if water safety plans are executed properly the municipal supply of water could be made potable. *E. coli* was also not detected in one of stone spouts, which is also used by some communities for domestic purposes (Shrestha *et al*., 2022). The detection of *E. coli* in water sources indicates fecal contamination, as *E. coli* primarily resides in the intestines of warm-blooded animals. This contamination suggests the potential presence of a diverse range of pathogenic viruses and bacteria in the water system (Pandey *et al*., 2014). The upstream areas of the Bagmati River in the Kathmandu valley lie within the Shivpuri Nagarjun National Park, experiencing minimal human activity (DNPWC, 2024). This results in fewer pollutants entering the river compared to downstream sections, as there is minimal agricultural runoff, industrial discharge, and urban wastewater. Additionally, lower population densities contribute to lesser coliform pollution, resulting in less sewage and waste (Shrestha *et al*., 2023). The natural landscape acts as a filter, trapping and breaking down contaminants before they reach the river, reducing coliform bacteria concentrations (Philippot *et al*., 2024). Higher flow rates in upstream sections, due to steeper gradients, help dilute and carry away contaminants, reducing coliform bacteria accumulation (Chen *et al*., 2021). When the source of the water, such as a spring or an underground aquifer, is naturally clean and free from contamination, the water in stone spouts can be free of *E. coli*. These sources are often protected from direct contamination by human or animal waste (Aranguren-Díaz *et al*., 2024).

In this study, ESBL *E. coli* has been detected in all three sample types viz., urine, water and poultry feces with highest number in urine as compared to water and poultry feces. The detection of ESBL *E. coli* in samples from humans, animals, and the environment indicates its widespread transmission across these three contexts (Husna *et al*., 2023). Antibiotic-resistant bacteria have a propensity to move from one area to another as a result of rapid globalization (Aghamohammad & Shahcheraghi, 2023). Antibiotic usage plays a pivotal role in the development and dissemination of ESBL *E. coli*. The

overuse or misuse of these antibiotics can create selective pressures that favor the survival and proliferation of resistant bacteria like ESBL-producing *E. coli* (Ramatla *et al*., 2023). Moreover, in agriculture, antibiotics are routinely administered to livestock, including chickens, to prevent disease and promote growth (Koirala *et al*., 2021). Consequently, ESBL *E. coli* can develop in the gut flora of these animals, leading to its presence in chicken feces. Intensive farming methods, characterized by overcrowding and unsanitary conditions, create environments conducive to the transmission and proliferation of antibiotic-resistant bacteria (Salam *et al*., 2024). Polluted river water sources serve as reservoirs for antibiotic-resistant bacteria due to the discharge of untreated sewage, agricultural runoff, and industrial effluents. ESBL *E. coli* can persist in these environments, facilitated by factors like nutrient availability, temperature, and pH. Water samples from polluted river water can contain ESBL-producing *E. coli* due to various sources of contamination (Singh *et al*., 2022). One major contributor is agricultural runoff from poultry farms and other livestock operations. Feces from these animals can contain ESBL-producing *E. coli*, which may then be washed into nearby water bodies through runoff during rainfall or irrigation (Bisi-Johnson *et al*., 2023). Additionally, untreated, or inadequately treated sewage from urban areas can introduce ESBL producing *E. coli* into river water, further contributing to contamination. Pollution introduces selective pressures that favor the survival of bacteria capable of withstanding adverse conditions, such as exposure to pollutants and fluctuations in environmental parameters. ESBL producing *E. coli* may adapt to polluted water environments by acquiring genetic mutations or expressing genes that confer resistance to antimicrobial agents and environmental stressors (Wei *et al*., 2024). Moreover, polluted water bodies serve as hotspots for horizontal gene transfer, facilitating the exchange of genetic material between bacteria and promoting the spread of antibiotic resistance genes, including those encoding ESBL production. As a result, ESBL-producing *E. coli* can thrive in polluted water environments, posing risks to human health and environmental integrity (Ligouri *et al*., 2020).

In this study, ESBL *E. coli* was not detected in water samples collected from upstream, stone spout and shallow well. ESBL *E. coli* was only detected in water samples of only two sites from the upper segment (8 - 31) of the mid-streams, whereas frequent ESBL *E. coli* was detected from downstream segment (32 - 44) of midstream. ESBL *E. coli* was detected from almost half of the sampling sites in the downstream and all the tributaries (Figure 10). As the Bagmati river flows through human settlements, the

levels of organic and inorganic pollutants rise (Giri *et al*., 2022). Human activities in urban areas often lead to increased pollution, which can contribute to the presence of ESBL *E. coli* in water systems through direct sewage discharge. Additionally, the high-pressure environment promotes the development of antibiotic resistance genes and bacteria. Polluted water provides a conducive environment for horizontal gene transmission among bacteria of similar genera (Mancuso *et al*., 2021).

In this study ESBL *E. coli* was not detected from fecal samples collected from Sanglaphanta and Suryavinayak. Effective biosecurity practices encompass stringent hygiene measures, controlled access protocols, and disease prevention strategies. The poultry farms implementing adequate biosecurity practices contribute to safeguarding poultry health and minimizing the emergence and spread of antimicrobial resistance (Subasinghe *et al*., 2022).

In the study, ESBL *E. coli* detection in higher number in clinical cases compared to animal and environmental samples can be attributed to several factors related to healthcare settings. Antibiotics are frequently used to treat bacterial infections. The selective pressure exerted by antibiotic usage promotes the emergence and proliferation of antibiotic-resistant bacteria, including ESBL *E. coli*. NPHL received urine samples from kidney transplant patients who were served free of charge by the government, potentially leading to a higher percentage of bacterial growth in patients undergoing long-term immunosuppressive therapy. Additionally, people with limited economic means often go for testing at NPHL after they have already taken antibiotics without any type of drug prescription and susceptibility testing, which could contribute to the development of ESBL by the time they reach the lab. Polluted river water and poultry feces act as environmental reservoirs where factors may limit the prevalence of ESBL *E. coli*. These environments may contain antibiotic residues from agricultural runoff or human waste, but overall exposure is typically lower than in clinical settings (Juncal *et al*., 2023).

In the study, the antibiotic susceptibility test was performed in *E. coli* (N = 617) isolated from water, urine and fecal sample for antibiotics categorized into A, B, C, E and O test/report group and 11 categories (CLSI, 2020; Magiorakos *et al*., 2010). Although the current study has limited scope in terms of detecting the mechanisms of resistance, in principle *E. coli* can develop resistance to antibiotics through several mechanisms.

The study showed almost all (96 %) of *E. coli* isolates were resistant towards erythromycin. Similar to the study *E. coli* was found to be highly resistant towards

erythromycin, macrolide in study conducted by Kazemnia *et al*. (2014) from Iran. *E. coli* can develop resistance to antibiotics through several mechanisms, including alterations in antibiotic targets (e.g., ribosomal mutations affecting erythromycin binding sites) and the efflux of antibiotics from bacterial cells (Li *et al*., 2019). Also, 96% of *E. coli* were resistant to ampicillin, test/group A, penicillin category which is in line with the systematic review done by Pormohammad *et al*. (2019). *E. coli* can develop resistance to ampicillin antibiotics through activation of efflux pump and production of enzymes that inactivate the antibiotic (beta-lactamases) (Nasrollahian *et al*., 2024).

In this study about one tenth of the *E. coli* showed resistance against imipenem, test group B, carbapenem category which is higher than reported by Nepal *et al*. (2017) from Om hospital, Kathmandu. The convergence of high antibiotic usage and misuse, insufficient regulatory oversight, dense populations, and healthcare infrastructure challenges create a perfect storm for the emergence and spread of carbapenemase-resistant *E. coli* in South Asian countries including Nepal (Luo *et al*., 2024; Rijal *et al*., 2021). Resistance to imipenem in *E. coli* can occur through various mechanisms, including the production of carbapenems, enzymes that hydrolyze carbapenem antibiotics and render them ineffective. Other mechanisms may involve alterations in the bacterial outer membrane permeability, preventing imipenem from entering the bacterial cell and exerting its antimicrobial activity (Mancuso *et al*., 2021).

In this study, 91 % of *E. coli* isolates showed resistance to amoxiclav antibiotics, beta lactam in test/report group B which is consistent with the findings of Badr *et al*. (2022) regarding *E. coli* isolates from animals and humans in Egypt, where resistance to amoxiclav ranged between 65 % and 95 %. Resistance to amoxiclav, a commonly prescribed antibiotic, can arise through various mechanisms such as beta-lactamase production, alteration of penicillin-binding proteins, and efflux pump mechanisms (Martínez-Casanova *et al*., 2021). In the same group B, 78 % of *E. coli* were resistance to cefepime, 50 % to ciprofloxacin and 32 % *E. coli* resistant to cefotaxime antibiotics which is in line with the summarized report showing 0-100 % resistant against cefepime in Bangladesh and 0-91 % *E. coli* resistant against cefotaxime in Southeast Asian countries (Pulingam *et al*., 2022). Cefepime is a fourth-generation cephalosporin antibiotic with broad-spectrum activity against Gram-negative bacteria. Resistance to cefepime in *E. coli* can arise through similar mechanisms as cefotaxime, primarily involving the production of ESBLs that hydrolyze and inactivate the antibiotic.

Additionally, alterations in outer membrane permeability or efflux pump mechanisms may contribute to decreased susceptibility to cefepime (Nasrollahian *et al*., 2024). Resistance to cefotaxime in *E. coli* often involves the production of extended-spectrum beta-lactamases (ESBLs), enzymes that hydrolyze and inactivate beta-lactam antibiotics (Martínez-Casanova *et al*., 2021). In current study 44 % *E. coli* were resistant towards amikacin which is similar to the study conducted in water and stool sample from India by Sahoo *et al*. (2012). Amikacin is an aminoglycoside antibiotic that inhibits bacterial protein synthesis. Resistance to amikacin in *E. coli* typically occurs through acquired mechanisms, such as the production of aminoglycoside-modifying enzymes (e.g., acetyltransferases, phosphotransferases) that chemically modify and inactivate the antibiotic, or through alterations in bacterial ribosomal targets, reducing the binding affinity of amikacin to its target site (Nasrollahian *et al*., 2024). In current study 49 % of the *E. coli* isolates were resistant to cotrimoxazole which is similar to the study conducted by Nji *et al*. (2021) & Phu *et al*. (2022) from LMICs and Vietnam.

In this study about one third of *E. coli* were resistant to ceftazidime antibiotics in group C, which was comparatively lesser resistant than chloramphenicol (42 %). This result is similar to the systematic review study conducted by Nji *et al*. (2021) & Phu *et al*. (2022) from LMICs and Vietnam. Chloramphenicol is a broad-spectrum antibiotic that inhibits bacterial protein synthesis. Resistance to chloramphenicol in *E. coli* can occur through acquired mechanisms, such as the production of chloramphenicol acetyltransferases (CATs) that modify and inactivate the antibiotic, or through mutations in bacterial ribosomal targets, reducing chloramphenicol binding affinity (Murray *et al*., 2024). Ceftazidime is a third-generation cephalosporin antibiotic with activity against Gram-negative bacteria. Resistance to ceftazidime in *E. coli* primarily occurs through the production of ESBLs, similar to cefotaxime and cefepime resistance mechanisms. Additionally, alterations in outer membrane permeability or efflux pump mechanisms may contribute to decreased susceptibility to ceftazidime (Mancuso *et al*., 2023).

In the study, within test /report group O, the majority of *E. coli* showed resistance against piperacillin, penicillin category. This result is similar to the study done by Kaushik *et al*. (2018) in poultry farms of India. Resistance to piperacillin, a broad-spectrum penicillin antibiotic, can occur through mechanisms such as beta-lactamase production, alterations in penicillin-binding proteins, efflux pump mechanisms, and

acquisition of resistance genes (Martínez-Casanova *et al*., 2021). Also, more than half of *E. coli* were resistant to nalidixic acid and more than three forth were resistant towards cefixime, cephems and Tetracycline, in test group O. The result coincides with systematic review conducted by Pormohammad *et al*. (2022) and by Phu *et al*. (2022). Resistance to nalidixic acid, a quinolone antibiotic, often arises due to mutations in the genes encoding DNA gyrase or topoisomerase IV, the targets of quinolones. These mutations prevent the binding of nalidixic acid to its target enzymes, reducing its effectiveness in inhibiting bacterial DNA replication and transcription (Nasrollahian *et al*., 2024). Resistance to cefixime, a third-generation cephalosporin antibiotic, primarily occurs through the production of extended-spectrum beta-lactamases (ESBLs) or AmpC beta-lactamases, enzymes that hydrolyze and inactivate the beta-lactam ring of cephalosporin antibiotics like ceftriaxone. Additionally, alterations in outer membrane permeability or efflux pump mechanisms may contribute to decreased susceptibility to ceftriaxone (Murray *et al*., 2024). Resistance to tetracycline, a broad-spectrum antibiotic, can occur through various mechanisms, including the production of tetracycline efflux pumps that actively remove the antibiotic from bacterial cells, reducing intracellular drug concentrations. Additionally, bacteria may acquire resistance genes encoding ribosomal protection proteins that prevent tetracycline from binding to the bacterial ribosome, thereby inhibiting protein synthesis (Gasparrini *et al*., 2020).

In this study nearly three-quarters of *E. coli* isolates were resistant to piperacillin tazobactam antibiotics. The study conducted by Shilpakar *et al*. (2021) in tertiary care hospital in Nepal showed 21 % of *E. coli* resistant to this antibiotic. In Nepal, the widespread adoption of excessive beta-lactam combination antibiotics is observed (Ghimire *et al*., 2023). About 11 % of World Organization for Animal Health (WOAH) member nations employ highest priority critically important antimicrobials (HPCIA) in poultry farms for prophylactic measures. The unregulated utilization of antibiotics without precise prescriptions can fuel the surge of antimicrobial resistance (AMR) (WOAH, 2024).

In the study, nearly three-quarters of *E. coli* isolated from water, urine and fecal samples were MDR which is similar to the systematic review conducted by Tweldemedhin *et al*. (2022) from Ethiopia. The detection of multidrug-resistant (MDR) *E. coli* in river water, human urine, and chicken fecal samples underscores the complex dynamics of antimicrobial resistance and its potential implications for public health (Walker *et al*.,

2022). The higher prevalence of multidrug-resistant (MDR) *E. coli* isolates in polluted river water and poultry feces compared to human urine can be attributed to intensive antibiotic usage in poultry farming and the presence of pollutants in the water sources. These pollutants include chemicals, antibiotic residues, and heavy metals, which create additional selective pressures for bacteria to develop multidrug resistance. Exposure to these pollutants in the environment can induce stress responses in bacteria, prompting them to develop resistance mechanisms as a survival strategy (Pormohammad *et al*., 2019). Moreover, the co-selection phenomenon, where the presence of one type of selective pressure (such as heavy metals) can inadvertently select for resistance to other unrelated agents (such as antibiotics), further exacerbates the development of multidrug resistance (Murray *et al*., 2024). The high-pressure environment provides a habitat conducive to the growth of antibiotic-resistant bacteria, facilitating the emergence and spread of various antibiotic resistance genes. This situation is exacerbated by the cleanliness, or lack thereof, of the surrounding environment, which is often mirrored in the quality of nearby flowing river water sources (Grenni, 2022). These water bodies are vital, as humans, plants, and animals rely directly on them for sustenance. Therefore, uncontrolled pollution in rivers is a clear indicator of deteriorating health conditions in both the physical and biological environment (Bengtsson-Palme *et al*., 2015). The detection of MDR *E. coli* in human urine signifies the possibility of colonization or infection with resistant strains, raising concerns about healthcare-associated infections or community-acquired illnesses. Inadequate sanitation, improper antibiotic use, and insufficient hygiene practices contribute to the proliferation and spread of MDR bacteria among human populations (Van Duin & Paterson, 2020). Furthermore, the identification of MDR *E. coli* in chicken fecal samples points to the reservoir of resistant bacteria in livestock, emphasizing the need for prudent antibiotic use in animal husbandry to mitigate the emergence and transmission of resistance (Racewicz *et al*., 2022).

The variation in multidrug resistance (MDR) rates of *E. coli* across different sample sources, such as urine, water, and feces, in low- and middle-income countries (LMICs) can be attributed to a myriad of interconnected factors. Firstly, the MDR rate in urine samples may be comparatively lower due to differences in antibiotic exposure as compared to its use in poultry farms and microbial interaction in pressurized areas as in polluted water sources. Conversely, the higher MDR rates observed in water and fecal samples could stem from environmental contamination (Tweldemedhin *et al*.,

2022). Polluted water sources in LMICs often contain a complex mixture of pollutants, including antibiotic residues and heavy metals, which can promote the selection and dissemination of MDR bacteria. Moreover, inadequate sanitation infrastructure and poor waste management practices may facilitate the spread of MDR *E. coli* through fecal contamination of water sources and environmental reservoirs (Guardabassi *et al*., 2020). Additionally, the prevalence of MDR *E. coli* in fecal samples from animals, such as poultry, could reflect the widespread use of antibiotics in agricultural practices (Poudel *et al*., 2024). In LMICs, where livestock farming may be less regulated, antibiotics are frequently administered to animals for growth promotion and disease prevention, contributing to the emergence of MDR strains (Abreu *et al*., 2023).

In this study about almost all of the ESBL *E. coli* were MDR which is similar to the study conducted by Muleme *et al*. (2023) from Uganda who reported 98 % MDR ESBL *E. coli*. Continuous exposure to low concentrations of antibiotics over extended periods, whether in the treatment of chronic infections or as growth promoters in veterinary practices, induces bacterial adaptation to these antibiotics (Serwecińska, 2020). ESBL-producing *E. coli* are recognized for their capability to generate diverse plasmid types, each capable of producing a range of enzymes that confer resistance to various classes of antibiotics (Gauba & Rahman, 2023). The presence of MDR ESBL *E. coli* in water, urine, and feces can be attributed to various interconnected factors. Firstly, the overuse and misuse of antibiotics in both human healthcare and agriculture contribute to the selection and proliferation of antibiotic-resistant bacteria, including ESBL *E. coli*. Antibiotics are commonly used to treat bacterial infections in humans and animals, and their widespread use exerts selective pressure on bacterial populations, driving the emergence of resistance mechanisms (Pormohammad *et al*., 2019). In healthcare settings, the frequent use of broad-spectrum antibiotics can promote the development of ESBL-producing strains of *E. coli*. These strains possess enzymes, beta-lactamases, that confer resistance to multiple classes of antibiotics, making them difficult to treat. Additionally, improper antibiotic prescribing practices, patient non-compliance, and inadequate infection control measures in healthcare facilities can further facilitate the spread of MDR ESBL *E. coli* (Tarrant *et al*., 2021). Additionally, the presence of ESBL-producing MDR *E. coli* in human urine samples underscores the threat posed by these bacteria to public health, particularly in the context of urinary tract infections where treatment options may be limited due to antibiotic resistance (Van Duin & Paterson, 2020).

The detection of multidrug-resistant MDR ESBL *E. coli* from polluted river water, poultry, and human urine samples indicates a concerning spread of antibiotic resistance across diverse environments and potentially among different reservoirs. This finding suggests that these environments serve as significant sources of MDR bacteria, highlighting the potential for transmission and the interconnectedness of human, animal, and environmental health in the dissemination of antimicrobial resistance (Ullah *et al*., 2023). Firstly, the presence of MDR ESBL *E. coli* in humans indicates the potential for difficult-to-treat infections, particularly urinary tract infections, which can lead to prolonged illness, treatment failures, and increased healthcare costs (Ramírez-Castillo *et al*., 2020). Moreover, the detection of MDR ESBL *E. coli* in animals, such as poultry, highlights the role of livestock as reservoirs for antibiotic-resistant bacteria, posing risks for food safety and potential transmission to humans through the food chain (Bezabih *et al*., 2020). Additionally, the presence of MDR ESBL *E. coli* in the environment, especially within a river basin, indicates widespread contamination and the potential for further dissemination through waterborne routes. This pose risks not only to human health through recreational water activities or consumption of contaminated water and food but also to the ecosystem, including aquatic organisms and biodiversity (Mahmud *et al*., 2020).

Bacterial resistance to Highly Important Critically Important Antibiotics (HPCIA) and Critically Important Antibiotics (CIA) presents a grave challenge to public health globally. HPCIA and CIA often serve as last-resort treatments, reserved for cases where other antibiotics have failed or when treating infections caused by bacteria resistant to multiple drug classes. When bacteria develop resistance to these vital antibiotics, it jeopardizes patient outcomes, prolongs illnesses, increases healthcare costs, and elevates the risk of mortality. Moreover, the spread of resistant bacteria undermines the effectiveness of entire antibiotic classes, exacerbating the global crisis of antimicrobial resistance (WHO, 2024).

The observation of significant association for beta lactam, penicillin, cephems and tetracyclines in *E. coli* in water sources is indicative of human impact creating selective pressure, favoring the survival and proliferation of resistant bacteria such as ESBL-producing *E. coli.* The presence of *E. coli* resistant to beta-lactam, penicillin, cephems, and tetracyclines in polluted water sources can lead to significant additional effects, including cross-resistance and co-resistance (Murrary *et al*., 2024). The observation of significant association for beta lactam, penicillin and cephems in ESBL *E. coli* isolated

from midstream, downstream, and tributaries indicates a concerning environmental and public health issue. It also implies that these antibiotics, while intended to control bacterial infections, are actually contributing to the selection pressure that fosters the survival and proliferation of ESBL *E. coli* in the environment (Muteeb *et al*., 2023).

The chi-square test, conducted at a significance level of $p = 0.05$, did not find any statistically significant associations between the *E. coli* isolates from water, human urine, poultry fecal samples; based upon resistance to the 17 antibiotics tested. The varied resistance patterns among *E. coli* isolates from water, human urine, and poultry fecal samples can be attributed to three major components: human, animal and environment. One of the major factors is antibiotic use practices in different sources. In human medicine, antibiotics are prescribed based on specific infections, with common antibiotics including amoxicillin, ciprofloxacin, and cephalosporins. Overuse and misuse, such as over-prescription and not completing the full course, contribute significantly to resistance development. Hospitals often use a broader range of powerful antibiotics, leading to different resistance patterns compared to community settings (World Bank, 2017). In veterinary medicine, antibiotics are used not only for treating infections but also as growth promoters in poultry, with common antibiotics being tetracyclines, sulfonamides, and macrolides. The regulations governing antibiotic use in animals vary significantly between regions, affecting the types and amounts of antibiotics used (Caneschi *et al*., 2023). Environmental sources contribute to resistance through agricultural runoff, where antibiotics used in agriculture enter water bodies, introducing a variety of antibiotics into the aquatic environment and creating a complex selective environment. Pharmaceuticals can also enter water sources through wastewater discharge, containing a mix of human antibiotics and their metabolites, while improper disposal of unused antibiotics into household waste or directly into water systems adds to environmental contamination (Grenni *et al*., 2022).

The similarity in resistance patterns of *E. coli* isolated from water, human urine and poultry feces suggests that *E. coli* from these diverse sources share common resistance traits. This could be due to various factors such as the spread of resistance genes across different environments through horizontal gene transfer, similar selective pressures due to the widespread use of antibiotics in human medicine and agriculture, or contamination pathways that connect these sources (Ramatla *et al*., 2023). The findings highlight the interconnectedness of the ecosystem when it comes to antibiotic resistance, emphasizing the need for a One Health approach to tackle this issue. This

approach involves integrating efforts across human health, animal health, and environmental health sectors to effectively monitor, control, and mitigate the spread of antibiotic-resistant organisms (WHO, 2023).

In this study, *bla CTX M-1* gene was detected in majority of ESBL *E. coli* isolates whereas *bla TEM* gene was detected in two-fifths, and *bla SHV* in one-sixth of ESBL-producing strains. However, *bla SHV* gene was not detected in the *E. coli* isolated from the human urine sample. This study also marks the detection of *bla CTX M-9* gene type in a water sample. Widespread prevalence of *bla CTX M-1* in *E. coli* exhibiting resistance to cefotaxime and ceftazidime, with a notable propensity for dissemination into the surrounding ecosystem has been reported by Bevan *et al*. (2017).

In this study, from urine sample *bla CTX M-1* and *TEM* genes were detected whereas *bla SHV* was not detected. This study coincides with a study conducted by Shah *et al*. (2020) at Sahid Ganga Lal hospital, Kathmandu. In clinical settings, the presence of various ESBL gene types in *E. coli* isolates highlights the importance of performing antimicrobial susceptibility testing to guide appropriate antibiotic therapy. It underscores the need for judicious antibiotic use, infection prevention measures, and surveillance strategies to monitor and mitigate the spread of multidrug-resistant bacteria (Harris, 2020).

Furthermore, the expression levels of *bla CTX M*, *bla TEM* and *bla SHV* beta-lactamases can vary among different variants, influencing their enzymatic activity and resistance phenotype (Hall *et al*., 2024). Variants with higher expression levels or greater stability may confer more robust resistance to beta-lactam antibiotics, allowing them to thrive in the face of selective pressures exerted by antibiotic usage, host immune responses, and other environmental factors (Zhang *et al*., 2022). Detection of various ESBL *E. coli* gene types indicate a complex antimicrobial resistance profile, conferring resistance to many beta-lactam antibiotics. The presence of multiple ESBL genes suggests diverse genetic mechanisms, increasing treatment failures and complicating patient management (Husna *et al*., 2023). Furthermore, the detection of multiple ESBL gene types in river water, human urine and chicken feces may indicate a high prevalence of antimicrobial resistance in the local microbial community, posing significant challenges for infection control and public health efforts, suggesting potential horizontal gene transfer between bacterial strains and facilitating the spread of antibiotic resistance genes within and between ecological niches (Guenther *et al*., 2011).

This study shows that there is a low to moderate significant correlation in the presence of ESBL *E. coli* across all three sample types (water, urine, and feces), with correlation coefficients ranging from 0.13 to 0.3. It suggests that there is a meaningful relationship between the presence of ESBL *E. coli* across these different sample types. Specifically, it implies that the factors influencing the presence of ESBL *E. coli* in one type of sample are likely related to the factors influencing its presence in the other types. The significant correlations suggest that there may be common sources contributing to the presence of ESBL *E. coli* in water, urine, and feces. This could be due to environmental contamination, human or animal waste, or other shared factors (Kawamura *et al*., 2017). The correlations imply that ESBL *E. coli* might be spreading through interconnected pathways. For example, contaminated water could be affecting both environmental samples and the human population, leading to the presence of ESBL *E. coli* in urine and feces (Salman *et al*., 2024). Significant correlations between these samples could indicate a public health concern, where contamination in one area (e.g., water) could be linked to the spread of ESBL *E. coli* in humans, evidenced by its presence in urine and feces. There are likely common or related sources and pathways contributing to its presence across these different sample types. This highlights the importance of addressing contamination sources and understanding the interconnected nature of environmental and public health factors to control the spread of ESBL *E. coli*.

This research underscores the necessity of regulating antibiotic usage in both poultry and clinical environments. The primary driver behind the escalating burden of antimicrobial resistance (AMR) organisms is the imprudent administration of antibiotics by the human population (Tang *et al*., 2023). The extensive discharge of waste and sewage into river systems has led to the degradation of natural water sources, transforming rivers into conduits for waste (Akhtar *et al*., 2021). The Bagmati river, receiving waste from hospitals, households, slaughterhouses, and poultry farms, acts as a major reservoir for antimicrobial-resistant (AMR) and ESBL-producing organisms Purifying water from upstream areas as it flows through the Kathmandu valley is essential. This proactive measure is critical in reducing the risk of ESBL *E. coli* transmission to both human and animal populations.

The isolation of ESBL producing *E. coli* in river water, human urine, and poultry fecal samples in Kathmandu, Nepal, highlights an alarming situation in line with the global concern in increasing antimicrobial resistance (AMR), over the years and their linkage between human, animal and environment for their transmission.

The current study is the comprehensive effort to identify the presence and possible linkage between human, animal and environment in their transmission of ESBL-producing *E. coli* within the river basin of the Kathmandu Valley. The findings from this study provide crucial baseline data that are required to further strengthen water safety plan/measures and public health policies & strategies in protecting the people as per the mandatory provision of the constitution of Nepal. .

ESBL-producing *E. coli* varied widely across different sources in Bangladesh: environmental samples ranged from 16% to 62%, human samples from 11% to 23%, and animal samples from 9% to 34% (Islam *et al*., 2023), which is similar to the current study findings, may be due to similar ecological and anthropogenic factors influencing the spread of AMR. The presence of ESBL-producing *E. coli* in water samples at high rates could point to inadequate wastewater treatment or the contamination of natural water bodies with human or animal waste, closely linked reservoirs, and possibly due to shared water sources, agricultural practices. The pervasive nature of these bacteria across diverse environments emphasizes the need for comprehensive one health approach based coordinated efforts to address AMR, considering the interconnectedness of environmental, human, and animal health.

In contrast, Madagascar reported significantly higher prevalence rates of ESBL-producing *E. coli*, with 100% in surface water, 30% in human samples, and 57% in chicken samples (Milenkov *et al*., 2024); Indonesia and Malawi also reported higher percentage: 40% ESBL *E. coli* in human samples, 67% in animal samples, and 66% in water samples in Indonesia; and 42% in human samples, 30% in animal samples, and 66% in river water samples in Malawi (Puspandari *et al*., 2021), may be due to significant contamination of water sources, differences in sanitation infrastructure, or higher levels of antibiotic use in both human medicine and animal husbandry, cultural practices, agricultural methods, and the regulatory framework surrounding antibiotic use vary significantly between the regions. This comparison clearly highlights the importance of considering local contexts when addressing AMR and the need for tailored interventions that account for regional differences in risk factors and transmission dynamics.

In the current study, *CTX-M* beta-lactamase gene could be detected from 90% of *E. coli* isolates, which is in line with global trends, where the *CTX-M* gene has become the most prevalent, surpassing the *TEM* and *SHV* genes. Over the years, the *CTX-M* gene has rapidly spread and become the dominant ESBL type worldwide, largely due

to its association with mobile genetic elements like plasmids, which facilitate horizontal gene transfer across different bacterial species. This widespread dissemination is likely driven by factors such as the overuse and misuse of antibiotics in both clinical and agricultural settings, which create selective pressure for bacteria carrying these resistance genes. Additionally, the persistence and global spread of *CTX-M* could be linked to international travel, trade, and the movement of people and animals, further emphasizing the need for coordinated global efforts to monitor and control the spread of AMR genes. (Yu *et al*., 2024)

Limitations of the study

- The proximity of the sample collection sites in human, animal and environment could not be well correlated due to collection of samples from the sites not directly linked to the single sources of water.
- The link between the clinical isolate, patient, their solid waste disposal systems in the river or environment could not be established for the direct correlation between human, poultry and environmental isolates.
- The link between the poultry feces and their disposal into nearby river sources could not be tracked to establish their direct contribution to antimicrobial resistance in environmental isolates.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

1. Thermotolerant *E. coli* was detected from upstream, mid-stream and downstream of Bagmati river, stone spouts, shallow wells, and tributaries, except in 2/7 upstream sites ,indicating fecal contamination in the flow of river within the Kathmandu valley, with considerably low level of contamination in upstream and higher in downstream and tributaries. In addition to *E. coli*, other Enterobacteriaceae (*Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*) were also detected in water samples.
2. ESBL *E. coli* were detected in the midstream, downstream, and tributaries of the Bagmati river, while not in the upstream, stone spout, and shallow well samples. Detection of ESBL *E. coli* in 40 % of the water sampling sites lying in mid & downstream clearly indicates that fecal pollution of the river water has increased in tandem with the human habitation.
3. *E. coli* was detected in poultry fecal samples from all 13 poultry farms. *E. coli* was detected in 8% (22/266) of the poultry feces collected from poultry farms located in different locations within the Kathmandu Valley. However, ESBL *E. coli* was not detected in poultry feces from 15% (2/13) of the poultry farms located in Sanglaphata & Suryabinayak. Compared to the other eleven farms, two farms had limited access to outsiders, regular hand and foot washing, routine cleaning, consistent vaccination and health check-ups by veterinary doctors, and use of clean utensils for feedings which indicates good biosecurity practices, which might have contributed to improve poultry health and lower antimicrobial resistance.
4. *E. coli* and ESBL *E. coli* were detected in urine samples from patients visiting NPHL where 29 % of the *E. coli* isolates were ESBL, necessitating review/changes in treatment strategies, heightened infection control practices, and close monitoring to manage and prevent the spread of these resistant bacteria.
5. Overall, ESBL *E. coli* isolates detection was higher in human urine samples (19 %; 18/96) and followed by poultry feces (5 %, 22/420) and water (5 %; 40/797)

suggesting antibiotic exposure in human community is relatively higher in compared to water and poultry sources.

6. Three beta lactamase gene types *bla CTX M-1 & bla CTX M-9, bla TEM* and *bla SHV* were detected in *ESBL E. coli* samples isolated from water and poultry feces; while only *bla CTX-M, bla TEM* was detected in *E. coli* isolated from urine samples, with indicating similar gene types in ESBL *E. coli* isolates from all 3 origins pertaining to similar sources of transmission.
7. The non ESBL *E. coli* and ESBL *E. coli* isolates from water, human urine and poultry feces showed low to moderate degree of correlation (r = 0.13 - 0.33). The correlation findings showed some degree of relationship between the presence or abundance of *E. coli* and ESBL *E. coli* in water, human urine and chicken feces, indicating gene transfer possibilities and interlinkage in between ESBL *E. coli* isolates within key components: environment, animal and human of One Health approaches.

5.2 Recommendations

1. Strengthen surveillance, monitoring and containment programs
   i. Institute and implement robust AMR surveillance system detecting AMR: in environment including drinking and wastewater system; animal health including poultry, livestock & animal feeds; in human health system including patients and infection prevention & control. This includes regular testing of water sources, poultry farms, animal feeds, and healthcare facilities to detect and track the spread AMR including ESBL *E. coli.*
   ii. Establish an AMR, AMU, & AMC database for different sectors, their analysis and dissemination to the concerned authorities for early containment interventions. This includes monthly/quarterly/annual AMR reports, publication in peer reviewed professional journals.
2. Enhance sanitation, hygiene and infection prevention & control practices
   i. Institute and effectively implement water safety plan in the water sources, for potable water in the sources, ultimately contributing to potable water supply in the system.
   ii. Institute and strictly adhere to wastewater treatment procedures before disposal of solid-liquid wastes in the river flow.
   iii. Strengthen biosecurity in poultry farms contributing to reduce antimicrobial resistant bacterial isolates and good quality chicken meats in supply chain

Institute strict adherence to infection prevention and control measures in human health facilities including hand hygiene and sanitation, use of PPE, to reduce further transmission of the resistant bacteria from health facility to community.

3. Promote rational use of antibiotics
    i. Enforce regulations to limit the use of antibiotics in livestock only for therapeutic purposes, discouraging their OTC sales.
    ii. Strengthen antibiotic stewardship through educating healthcare professionals on the need and understanding of the antimicrobial susceptibility trends in prescription, when to use empirical treatment, prescribing antibiotics only, when necessary, awareness in the public on the appropriate use of antibiotics and ensuring full course use of antibiotics when prescribed, and awareness to farmers in rational use of antibiotics in livestock & poultry farming.
4. Continuation of research activities for more knowledge generation
    i. Real time collection of samples from all three linked sources (human, animal and environment) to better correlate the antibiotic resistance transmission in the city, considering One Health approach.
    ii. Whole genome sequencing can be utilized to identify phenotypically unexpressed ESBL genes in *E. coli*. By sequencing the entire genome, researchers can identify the presence of resistance genes that are silent under certain conditions but have the potential to be activated, contributing to antibiotic resistance.

These recommendations aim to reduce the spread of AMR including ESBL *E. coli*, safeguarding both public health and environmental safety.

CHAPTER 6

6. SUMMARY

Antibiotic resistant bacteria can pose a significant threat to human population. The pathogenic bacteria can be transmitted to humans through several pathways: ingestion of contaminated food and water, consumption of infected chicken meat, direct contact with the infected person or introduced to the person's body through indwelling devices during the treatment procedures in the hospitals.

The current study aimed to investigate the antibiotic resistance and investigate the possible linkage in targeted bacteria isolated from human urine, chicken feces, water from Bagmati river (upstream-downstream) and its tributaries, water from spring spouts, shallow wells during Jan 2020 to Sept 2023.

During the study period, 264 water samples from 88 sites (samples in triplicates) collected from Bagmati river (up-stream-7, mid-stream-37, down-stream-16, tributaries-6), stone spouts-14, and shallow wells-8; poultry feces from 13 poultry farms, and 1,220 human urine samples from suspected UTI patients were analyzed following standard microbiological methods (APHA,1999)

In total, 1,295 bacterial isolates were isolated from water, urine and fecal samples, among which 48 % (617/1,295) of the isolates were *E. coli* and 6 % (80/1,295) were ESBL *E. coli*. Among 617 *E. coli* 13 % (80/617) were ESBL *E. coli*.

A total of 288 thermotolerant *E. coli* were isolated from water samples, of which 14 % (40/288) were ESBL *E. coli*. ESBL *E. coli* were not detected in upstream, stone spout, and shallow well samples. However, ESBL *E. coli* were isolated from 30 % (26/88) of the sites: 32% midstream (, 12/37), 50% downstream (, 8/16) and 100% of the tributaries (6/6). Apart from *E. coli*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter areogenes*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* were also isolated from the water samples.

In 1,220 urine samples processed at NPHL, significant growth was observed in 8 % (96/1,220) of samples, with 66 % (63/96) *E. coli* and 19 % (18/96) ESBL *E. coli*. Apart from *E. coli*, *Citrobacter freundii* (17%, 16/96), *Citrobacter koseri* (5 %, 5/96), *Enterobacter aerogenes* (3 %, 3/96), *Klebsiella oxytoca* (2 %, 2/96), *Klebsiella pneumoniae* (4%, 4/96), *Proteus mirabilis* (2 %, 2/96) and *Proteus vulgaris* (1 %, 1/96) were also isolated.

A total of 402 bacterial isolates were detected from 390 poultry fecal samples collected from 13 poultry farms, among which 66 % (266/402) were *E. coli* and 5 % (22/402) were ESBL *E. coli*. ESBL *E. coli* was detected in 85 % (11/13) of poultry farms. Apart from *E. coli*, *Citrobacter freundii* (17.9 %, 72/402), *Citrobacter koseri* (8.2 %, 33/402), *Proteus mirabilis* (2.5 %, 10/402), *Proteus vulgaris* (1 %, 5/402), *Salmonella* Typhi (1 %, 5/402), *Salmonella* Paratyphi (1%, 3/402), *Shigella dysenteriae* (2 %, 7/402) and *Acinetobacter* spp. (0.24 %, 1/402) were also detected.

For antibiotic susceptibility testing, a total of 17 different antibiotics were selected, grouped into five test/report groups (A, B, C, O, and U) and 11 antimicrobial categories. Antibiotic susceptibility test was performed using modified Kirby and Bauer disc diffusion method as according to CLSI guidelines (CLSI, 2020). Among the *E. coli* isolates, 92.48 % (568/617) were resistant to erythromycin, which belongs to the macrolide category. About 94 % (580/617) of *E. coli* isolates were resistant to ampicillin, test/report group A, penicillin category.

In test/report group B, the highest resistance was observed in *E. coli*, with 89 % (549/617) showing resistance to amoxicillin/clavulanic acid, Beta lactam category, followed by 83 % (511/617) resistance to cefepime in cephems category, 74 % (474/617) to piperacillin tazobactam, beta lactam category, and 54 % (333/617) resistance to cotrimoxazole, folic acid inhibitors. *E. coli* showed the lowest resistance rate of 16 % (97/617) to imipenem in the carbapenem category, followed by 62 % (384/617) resistance to cefotaxime in the cephems category, and 44 % (271/617) resistance to amikacin in the aminoglycosides category.

In test/report group C, 63 % (389/617) of *E. coli* isolates were resistant to ceftazidime in the cephems category, which is less than the 69 % resistance to chloramphenicol in the quinolones category.

In test/report group O, 91 % (561/617) of *E. coli* were resistant to piperacillin in the penicillin category, followed by 88 % (544/617) to tetracycline, 87 % (536/617) to cefixime, and 60 % (371/617) to nalidixic acid.

Among the *E. coli* isolates, 53 % (325/617) were resistant to nitrofurantoin in the furans category, according to test/report group U.

All the ESBL-E coli isolates from Water, and 94% of the isolates from urine and chicken feces were MDR, while 94 %, 67 % and 81 % of the non-ESBL isolates from Water , Urine and Chicken feces were MDR respectively.

The multiplex PCR for the detection of genes contributing to resistance in ESBL E coli revealed that *bla CTX M-1* in 90 % (72/80), *bla TEM* in 40 % (32/80) and *bla SHV* in 15 % (12/80) of the isolates. *bla SHV* gene type was not detected in isolates from human urine samples. *bla CTX M-9* gene was detected in one bacterial isolate from one water sample.

The correlation coefficient showed low to moderate correlation between urine and water (r = 0.3), water and feces (r = 0.17) and urine and feces (r = 0.13) samples. The study unveils low but significant correlation in the antibiotic resistance profiles of ESBL *E. coli* across river water, human urine, and chicken fecal samples, indicating a the possible cross-linkage and a similarity map revealing less distance between ESBL *E. coli* isolated from water and human urine in comparison to isolates from water and poultry feces.

Implementation of comprehensive one health approach-based surveillance & evidence-based response systems spanning across various environments, animal health, and human health sectors needs to be further strengthened to effectively monitor and combat antimicrobial resistance.

It is recommended that real-time sample collection be implemented using a One Health approach, where data from human, animal, and environmental sources are gathered and analyzed simultaneously. This coordinated effort would enable dynamic surveillance and rapid detection of possible emerging threats, as evidence for policy/practice interventions.

Adopting antibiotic stewardship programs alongside strengthened infection control, robust surveillance & response, public awareness campaigns, and better access to quality assured diagnostics is crucial for combating antimicrobial resistance and protecting public health.

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APPENDICES

APPENDIX I

LIST OF MATERIALS AND EQUIPMENTS USED IN STUDY

I List of equipment used in the study

- Autoclave
- Electrophoresis set (Electrophoresis gel doc, power supply, gel bed)
- Gas burner
- Glass wares (beaker, conical flask, petri discs, test tubes, glass slides)
- High speed centrifuge
- Hot air oven
- Incubator
- Inoculating wire and loop
- Measuring cylinder
- Microfuge tubes
- Microscope
- Refrigerator
- Transilluminator
- Water bath
- Weighing machine

II. List of culture media used in the study (HI Media, India)

i. Brilliant green lactose bile broth (Ref: M121-500G, Lot: 0000411396)

Ingredients	**gm/liter**
Tryptone	10.000
Lactose monohydrate	10.000
Dehydrated bile	20.000
Brilliant green	0.0133
Final pH (at 25°C) 7.2±0.2	

Preparation: As directed by the manufacturer, 40 grams of the medium was suspended in 1000mL of distilled water and then heated to boiling to dissolve completely. Then the medium was distributed in 15 mL test-tubes with inverted Durahm's tube and sterilized in the autoclave for 121°C for15 minutes at (15 lbs pressure). The sterilized medium was then cooled to room temperature.

ii. Eosin Methylene Blue Agar (Ref: M317-500G, Lot: 0000390741)

Ingredients	gm/liter
Peptone	10.000
Dipotassium hydrogen phosphate	2.000
Lactose	10.000
Eosine	0.400
Methylene blue	0.065
Agar	15.000
Final pH (at 25°C) 7.1±0.2	

Preparation: As directed by the manufacturer, 37.46 grams of the medium was dissolved in 1000mL distilled water. Then the medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. The sterilized medium was then poured in sterilized Petri-plate and then allowed to cool.

iii. Luria Bertani broth

Ingredients	gm/liter
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium chloride	10
Agar	15
Final pH (at 25°C) 7.4±0.2	

Preparation: As directed by the manufacturer, 40 grams of the medium was dissolved in 1000 mL distilled water and then heated to boiling to dissolve completely. Then the medium was distributed in 5mL test-tubes and sterilized in the autoclave for 121°C for15 minutes at (15 lbs pressure). The sterilized medium was then cooled to room temperature.

iv. Mac-conkey broth purple (Ref: M083-500G, Lot: 0000416352)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Final pH (at 25°C) 7.4±0.2	

Preparation: As directed by the manufacturer, 40.01 grams of the medium was suspended in 1000mL of distilled water and then heated to boiling to dissolve completely. Then the medium was distributed in 15 mL test-tubes with inverted Durahm's tube and sterilized in the autoclave for 121°C for15 minutes at (15 lbs pressure). The sterilized medium was then cooled to room temperature.

v. Mueller Hinton Agar (MHA) (Ref: M173-500G, Lot: 0000405494, 0000447589)

Ingredients	**gm/liter**
Beef infusion form	300.0
Casein acid Hyrolysate	17.50
Starch	1.50
Agar	17.0
Final pH (at 25°C)7.4±0.2	

Preparation: As directed by the manufacturer, 38 grams of the medium was suspended in 1000mL distilled water. Then the medium was then sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. The sterilized medium was then poured in sterilized Petri-plate and then allowed to cool.

vi. Nutrient Agar (NA) (Ref: MV070-500G, Lot: 0000417145)

Ingredients	**gm/liter**
Peptone	10.0
Sodium chloride	5.0
Beef extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25°C) 7.4±0.2	

Preparation: As directed by the manufacturer, 37 grams of the medium was suspended in 1000mL of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 15 minutes. The sterilized medium was then poured in sterilized Petri-plate and then allowed to cool.

vii. Nutrient Broth (Ref: M002-500G, Lot: 0000446475)

Ingredients	**gm/liter**
Peptone	10.0
Sodium chloride	5.0
Beef extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25°C) 7.4±0.2	

Preparation: As directed by the manufacturer, 13 grams of the medium was dissolved in 1000mL distilled water and then heated to boiling to dissolve completely. Then the medium was distributed in 5mL test-tubes and sterilized in the autoclave for 121°C for15 minutes at (15 lbs pressure). The sterilized medium was then cooled to room temperature.

viii. Tryptone broth (Tryptone water) (Ref: M463-500G, Lot: 000420332)

Ingredients	gm/Litre
Casein enzymic hydrolysate	10.0
Sodium chloride	5.0
Final pH (at 25°C) 7.5±0.2	

Preparation: As directed by the manufacturer, 15 grams of the medium was dissolved in 1000mL distilled water and then heated to boiling to dissolve completely. Then the medium was distributed in 5mLtest-tubes and sterilized in the autoclave for 121°C for15 minutes at (15 lbs pressure). The sterilized medium was then cooled to room temperature.

III. Biochemical Test Medium

i. Christensen Urea Agar (Ref: M111-500G, Lot: 0000381612)

Ingredients	gm/liter
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0
Final pH (at 25°C) 7.4 ±0.2	

Preparation: As directed by the manufacturer, 24 grams of the medium was suspended in 950 mL distilled water and sterilized by autoclaving at 121°C for 15 minutes. After cooling to about 45°C, 50 mL or 40 % urea was added and mixed well. Then 5 mL was dispensed in test tube and set at slant position.

ii. Hugh and Leifson's Medium (Ref: M395-500G, Lot: 0000399147)

Ingredients	gm/liter
Tryptone	2.0
Sodium Chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0
Final pH (at 25°C) 6.8±0.2	6.8±0.2

Preparation: As directed by the manufacturer, 9.4 grams of the medium was rehydrated in 1000 mL cold distilled water and then heated to boiling to dissolve completely. Then the medium was distributed in 100 mL amounts and sterilized in the autoclave for 121°C for15 minutes at (15 lbs pressure). To 100 mL sterile medium aseptically added 10 mL of sterile Dextrose and mixed thoroughly and dispensed in 5 mL quantities into sterile culture tubes.

iii. MR-VP Media (Ref: MV070-500G, Lot: 0000417145)

Ingredients	gm/liter
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25°C) 6.9±0.2	

Preparation: As directed by the manufacturer,17 grams of the medium was dissolved in 1000 mL distilled water. 3 mL of medium was distributed in each test tube after dissolving and autoclaved at 121°C for 15 minutes.

iv. Sulphide Indole Motility(SIM) Medium (Ref: M181-500G, Lot: 0000360926)

Ingredients	gm/liter
Beef Extract	3.0
Peptone	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH (at 25°C)7.3±0.2	

Preparation: As directed by the manufacturer,36 grams of the medium was suspended in 1000 mL distilled water and heated to dissolve completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized.

v. Simmon Citrate Agar (Ref: M099-500G; Lot: 0000440807)

Ingredients	gm/liter
Magnesium Sulfate	0.2
Mono- ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25°C) 6.8±0.2	

Preparation: As directed by the manufacturer, 24.2 grams of the medium was dissolved in 1000 mL distilled water and heated to dissolve completely. 3 mL medium was distributed in test tubes and sterilized by autoclaving at 121°c for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

vi. Triple Sugar Iron (TSI) (Ref: M021-500G, Lot: 0000416144)

Ingredients	**gm/liter**
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25°C) 6.8±0.2	

Preparation: As directed by the manufacturer, 65 grams of the medium was dissolved inch mL of distilled water and sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C. the medium was allowed to set in sloped form with a butt about 1 inch of thickness.

IV. Composition and preparation of different reagents

i. Catalase Reagent (3% H_2O_2)

Hydrogen peroxide : 3 mL

Distilled Water : 97 mL

Preparation: To 97 mL of D/W, 3 mL of hydrogen peroxide was added and mixed well.

ii. Oxidase

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD) 1 gm

Distilled Water 100 mL

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 Ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

iii. Preparation of 0.5 McFarland Standard

0.6 mL of 1% (w/v) solution of barium chloride solution was added to 99.4 mL of 1% (v/v) solution of concentrated sulfuric acid. The mixture was shaken well. This stock

solution was stored in a well-sealed container in the dark. A small amount of solution was taken a time in a clean test tube to compare the turbidity of the inoculums.

iv. 70% Ethanol (UN No. 1170; Lot: 202 00105)

Dissolve 70mL of 100% (Absolute ethanol) to 30mL of d/w.

APPENDIX II

COORDINATES OF SAMPLING SITES

Sites	Sample No.	Latitude (N)			Longitude (E)		
		º	'	"	º	'	"
UPSTREAM	1	27	49	21.34	85	22	38.75
	2	27	47	35.06	85	23	28.54
	3	27	46	53.19	85	23	12.16
	4	27	47	27.22	85	25	38.06
	5	27	46	49.77	85	25	34.27
	6	27	46	15.62	85	25	36.29
	7	27	45	48.83	85	25	24.86
MIDSTREAM	8	27	45	49.59	85	25	20.96
	9	27	45	41.77	85	25	19.73
	10	27	45	36.7	85	25	17.99
	11	27	45	31.48	85	25	16.6
	12	27	45	26.92	85	25	13.71
	13	27	45	20.29	85	25	14.04
	14	27	45	17.23	85	25	16.39
	15	27	45	12.4	85	25	18.01
	16	27	45	7.97	85	25	19.82
	17	27	45	2.6	85	25	22.65
	18	27	44	59.49	85	25	26.26
	19	27	44	51.38	85	25	17.52
	20	27	44	41.84	85	25	6.25
	21	27	44	28.95	85	24	46.08
	22	27	44	10.2	85	24	25.21
	23	27	44	4.8	85	24	1.69
	24	27	44	7.26	85	23	48.58
	25	27	44	15.99	85	23	33.45
	26	27	44	7.11	85	23	10.6
	27	27	43	46.78	85	23	4.02
	28	27	43	23.36	85	22	58.17
	29	27	43	11.15	85	22	56.77
	30	27	43	4.3	85	22	52.9
	31	27	42	59.82	85	22	45.19
	32	27	42	58.7	85	22	30.35
	33	27	42	42.23	85	22	21.21
	34	27	42	39.34	85	22	1.21
	35	27	42	39.33	85	21	45.14
	36	27	42	33.69	85	21	19.74
	37	27	42	46.41	85	20	59
	38	27	42	17.7	85	20	58.79
	39	27	42	1.45	85	20	55.26

	40	27	41	57.16	85	20	49.24
	41	27	41	43.03	85	20	59.38
	42	27	41	16.23	85	20	51.43
	43	27	41	9.78	85	20	36.9
	44	27	40	47.35	85	20	12.38
DOWNSTREAM	45	27	40	44.09	85	19	54.27
	46	27	40	55.31	85	19	44.49
	47	27	41	11.48	85	19	23.12
	48	27	41	18.66	85	19	2.81
	49	27	41	30.08	85	18	39.75
	50	27	41	33.06	85	18	16.29
	51	27	41	22.8	85	18	5.45
	52	27	41	7.31	85	18	0.98
	53	27	40	48.41	85	17	57.59
	54	27	40	31.26	85	17	44.33
	55	27	40	8.18	85	17	41.55
	56	27	39	53.26	85	17	47.56
	57	27	39	48.43	85	17	46.97
	58	27	39	38.52	85	17	47.33
	59	27	39	28.4	85	17	36.67
	60	27	39	25.34	85	17	36.55
TRIBUTARY	61	27	40	42.12	85	20	2.36
	62	27	41	12.91	85	19	28.05
	63	27	41	27	85	18	52.44
	64	27	41	30.44	85	18	8.33
	65	27	40	58.79	85	17	55.6
	66	27	39	47.24	85	17	47.99
STONE SPOUT	1	27	41	8.33	85	20	32.11
	2	27	40	41.63	85	19	51.64
	3	27	40	39.7	85	19	46.63
	4	27	40	48.5	85	19	40
	5	27	40	39.59	85	19	36.42
	6	27	40	47.63	85	19	34.44
	7	27	40	36.77	85	19	51.53
	8	27	40	45.17	85	19	32.45
	9	27	42	2.5	85	19	50.69
	10	27	42	11.55	85	20	5.72
	11	27	42	1.73	85	19	10.66
	12	27	41	37.77	85	18	11.91
	13	27	41	55.84	85	18	12.19
	14	27	42	8.97	85	18	11.42
SHALLOW WELL	1	27	40	47.57	85	20	4.48
	2	27	40	42.24	85	19	52.92
	3	27	41	13.56	85	19	34.67

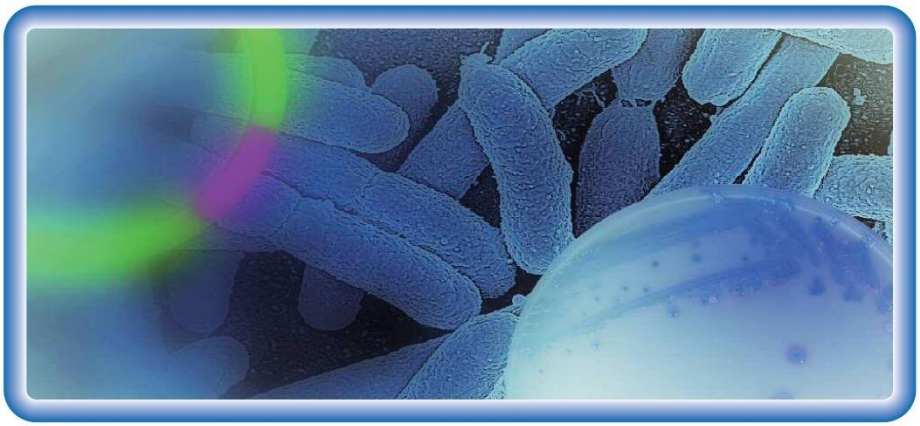
	4	27	41	14.97	85	19	30.14
	5	27	41	11.95	85	19	40.02
	6	27	41	18.04	85	19	24.58
	7	27	41	35.58	85	18	9.59
	8	27	41	34.15	85	18	5.81
POULTRY FARM	1	27	46	31.63	85	18	55.97
	2	27	46	24.55	85	18	49.73
	3	27	46	24.1	85	18	57.68
	4	27	46	10.71	85	18	58.78
	5	27	43	12.15	85	24	30.35
	6	27	43	17.41	85	24	25.88
	7	27	43	17.62	85	24	20.7
	8	27	39	55.78	85	24	32.63
	9	27	36	27.4	85	21	45.89
	10	27	36	26.73	85	21	45.75
	11	27	36	26.71	85	21	45.73
	12	27	36	25.07	85	21	52.63
	13	27	40	27.72	85	16	56.89

APPENDIX III

A BROUCHER OF ESBL GENE DETECTION KIT

Cica Geneus® ESBL Genotype Detection KIT

Kanto Reagents



Extended Spectrum β-Lactamase：ESBL is an enzyme hydolyzes wide β-lactam antibiotics such as penicillin, cephem, monobactam and so on.
Recently, an increase of ESBL producing bacteria has become a big problem and requires to infection control.
Cica Geneus® ESBL Genotype Detection KIT can detect 6 representative ESBL genotypes by two kinds of multiplex PCR. This kit was collaborativery deveroped by Prof. Dr. Sugai and Assistant Prof. Dr. Kayama at Hiroshima University.

Characteristics

- This kit can detect 6 ESBL genotype (TEM group, SHV group, CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, and CTX-M-9 group)
- Required time is approximately 3 hours.
- Suitable for use in common thermal cycler.

Composition (30 rxns)

Reagent		Volume
A	AptaTaq DNA Master(5×Conc.)*1	240 μL×1
B	PCR supplement	240 μL×1
C	Primer mix α	120 μL×1
D	Primer mix β	120 μL×1
E	Positive control	240 μL×1
F	Loading buffer(6×Conc., Orange-G)	240 μL×1

*1 AptaTaq DNA Master(5×Conc.) is the product of Roche Diagnostics K.K.

Work Flow

Isolate ESBL producing bacteria*2
↓
DNA Extraction*3
↓
Multiplex PCR
↓
Agarose gel electrophoresis
↓
Detection

*2 CHROMagar™ ESBL (72101) is recommended.
*3 Cica Geneus® DNA extraction reagent is sold separately.

Kanto Chemical

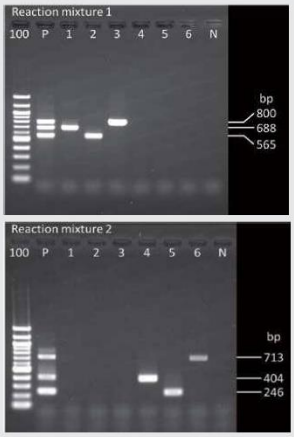


Fig.1 Example of the electrophoretic pattern

100 : 100 bp DNA Ladder, PC: Positive control (Reagent E),
1 : CTX-M-1 positive bacteria, 2 : CTX-M-9 positive bacteria,
3 : TEM positive bacteria, 4 : CTX-M-2 positive bacteria
5 : CTX-M-8 positive bacteria, 6 : SHV positive bacteria,
N : Negative control (TE Buffer)

Table1 Target genes and its amplicon size

	Target gene	Amplicon size(bp)
Reaction mixture.1	*bla* TEM	800
	bla CTX-M-1 group	688
	bla CTX-M-9 group	565
Reaction mixture.2	*bla* SHV	713
	bla CTX-M-2 group	404
	bla CTX-M-8 group	246

① Please refer to an attached document.
② From an electrophoretic pattern of two multiplex PCR (Figure. 1), you can estimate ESBL genotype (Table.1) in comparison with the band of the positive control.

- This kit can not identify bacterial species.
- Some strains may possess some types of ESBL genes.
- This kit can detect representative ESBL genes, but some ESBL genes can not be detected. (Genes not in 6 ESBL genotype group and some subtypes)
- Sometimes genotype test results may differ from drug sensitivity test results.
- This kit is for research and experimental use.

Product Information

Product No.	Product Name	Package size	Stored at
08112-96	Cica Geneus® ESBL Genotype Detection KIT	30 rxns	-20 ℃~-25 ℃
08178-96	Cica Geneus® DNA Extraction Reagent	120 rxns	2 ℃~8 ℃

Multiprex PCR kit series

Product No.	Product Name	Package size	Stored at
08180-96	Cica Geneus® Staph POT KIT	120 rxns	-20 ℃~-25 ℃
08180-97		30 rxns	-20 ℃~-25 ℃
08187-96	Cica Geneus® Pseudo POT KIT	50 rxns	-20 ℃~-25 ℃
08062-96	Cica Geneus® Acineto POT KIT	30 rxns	-20 ℃~-25 ℃
08362-97	Cica Geneus® E.coli POT KIT	30 rxns	-20 ℃~-25 ℃
-	Cica Geneus® Improved Carbapenemase Genotype Detection KIT (tentative)	30 rxns	-20 ℃~-25 ℃

Related reagent

Product No.	Product Name	Package size
72101	CHROMagar™ ESBL (prepared)	10 sheets
46509-79	10×TAE buffer	1 L
14575-43	Ethidium bromide solution	10 mL
01098-23	Agarose KANTO LE	100 g
01016-96	AptaTaq DNA Master(5×Conc.)	500 μL

Please acsess Cica-web to catch the detail of our reagent information !

- This product is developed with Hiroshima University.

KANTO CHEMICAL CO., INC.
REAGENT DIVISION
East Muromachi Mitsui BLDG, 2-1, Nihonbashi Muromachi 2-chome,
Chuo-ku, Tokyo, 103-0022, JAPAN
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2018 Jan.

APPENDIX IV

MANUSCRIPT I

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.

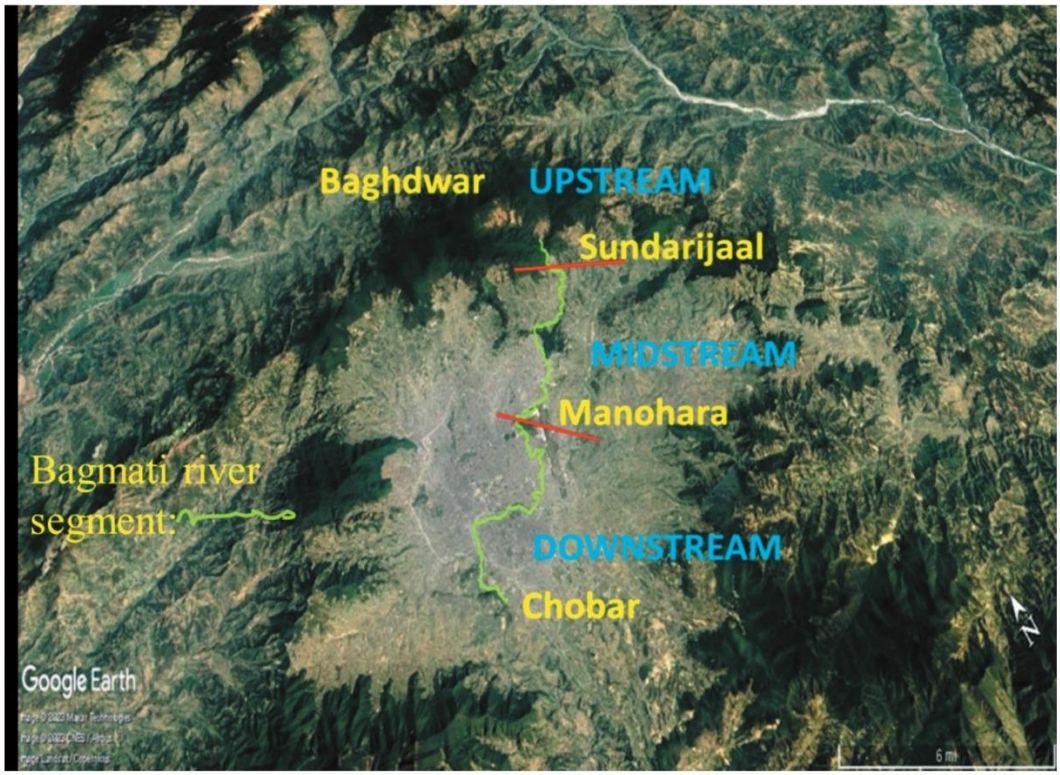

Figure 1: Map area of the Bagmati river basin showing upstream, midstream and downstream river segments.

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).

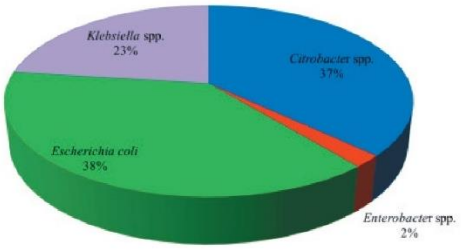

Figure 2: Distribution percentage of coliforms in different water samples

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

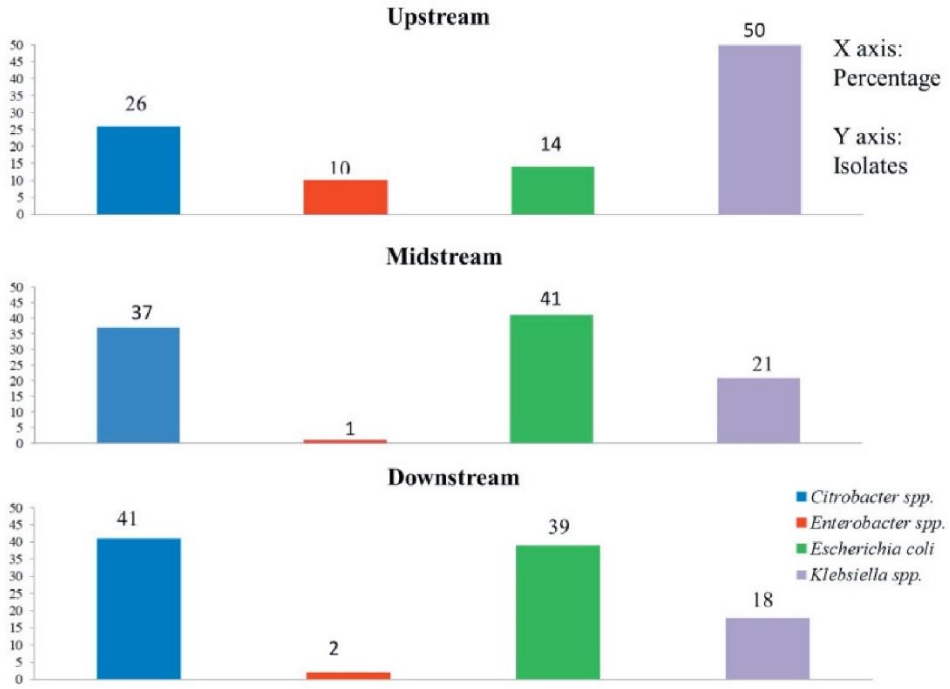

Figure 3: Distribution pattern of bacterial isolates in water streams

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).

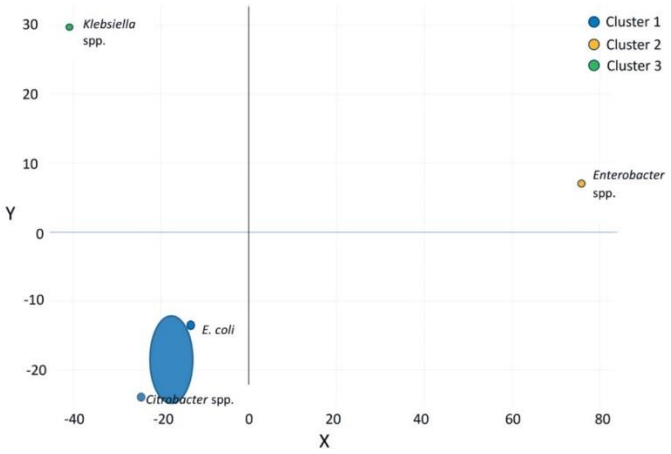

Figure 5: Similarity map of coliforms based on similarity index

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.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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APPENDIX V

MANUSCRIPT II

Original Article

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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).

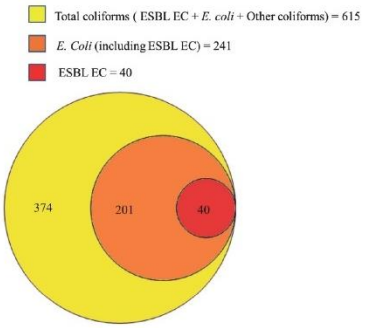

Figure 1. Prevalence of ESBL EC and E. coli within isolated total coliforms.

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).

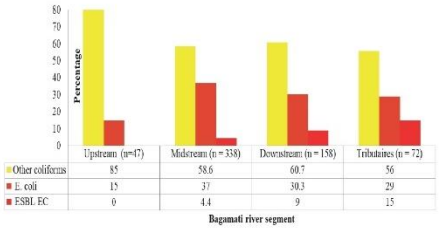

Figure 2. Prevalence of isolates in different segments of Bagmati river.

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).

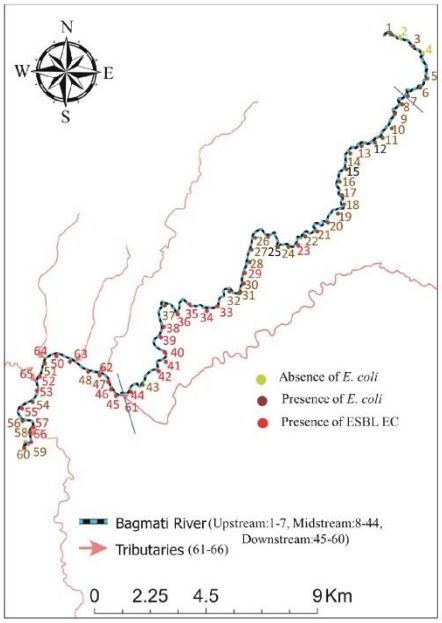

Figure 3. Map showing the sites with the presence of E. coli and ESBL EC.

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
Ceflxime	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).

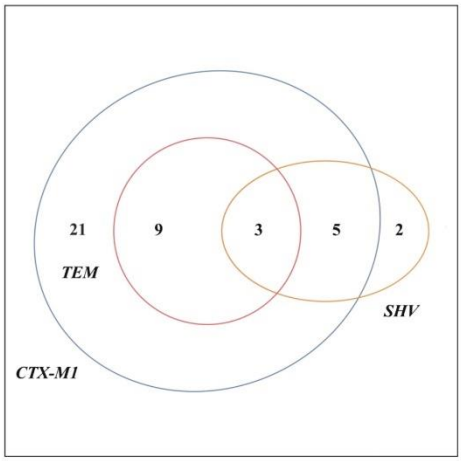

Figure 4. Euler diagram showing the combination of ESBL gene in ESBL EC.

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.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORAL PRESENTATION CERTIFICATE

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APPENDIX VII

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APPENDIX VIII

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to

Bindu Ghimire

For Poster Presentation on title of

Co-existence of extended spectrum beta lactamases bla CTX-M-1, bla CTX-M-9, bla TEM and bla SHV in Escherichia coli from Bagmati river

In the 10th National Summit of Health and Population Scientists in Nepal
10-12 April 2024, Kathmandu, Nepal.
Organized by Nepal Health Research Council (NHRC)

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