ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

HEAVY METAL CONTAMINATION AND ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* IN THE AKAKI WASTEWATER TREATMENT PLANT AND AKAKI RIVER CATCHMENT: A ONE HEALTH FRAMEWORK

MSC THESIS

BY

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JUNE, 2026

BISHOFTU, ETHIOPIA

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A THESIS SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, PARASITOLOGY AND POULTRY HEALTH, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH

BY

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JUNE, 2026

BISHOFTU, ETHIOPIA

APPROVAL SHEET

Addis Ababa University

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As MSc One Health research advisors, we hereby certify that we have read and critically evaluated the thesis prepared under our supervision by Melaku Taye, entitled *"Heavy Metal Contamination and Antimicrobial Resistance of Escherichia coli in the Akaki Wastewater Treatment Plant and Akaki River Catchment: A One Health Framework."* We confirm that the work meets the academic standards required for the degree and recommend that it be submitted in partial fulfillment of the requirements for the Degree of Master of Science (MSc) in One Health.

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This is to certify that the thesis prepared by Melaku Taye entitled "**Heavy metal contami nation and Antimicrobial resistance of *E. coli* in the Akaki wastewater treatment plant and Akaki river catchment"** and submitted in partial fulfillment of the requireme nts for the degree of Master of Science in One Health complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Major advisor		

DEDICATION

I dedicate this work to the memory of my beloved father, who passed away during my early years of elementary education. Although he was not given the opportunity to witness my academic journey and achievements, his presence has always remained in my heart as a source of strength and inspiration. This accomplishment is a reflection of the foundation he began. I also dedicate this work with profound gratitude and love to my dear mother, whose sacrifices are beyond words. With unwavering dedication, she carried the burden of my education with limited resources, giving of herself completely to ensure my success. From my Doctor of Veterinary Medicine studies to my current Master's program in One Health, she has been my constant support, enduring every challenge so that I could continue forward. Her strength, resilience, and unconditional love have been the true pillars behind this achievement. This work stands as a tribute to both of them one who is no longer with me but lives through my journey, and one who gave everything so that I could reach this milestone.

STATEMENT OF AUTHOR

I, hereby declare that the thesis entitled "*Heavy Metal Contamination and Antimicrobial Resistance of E. coli in The Akaki Wastewater Treatment Plant and Akaki River Catchme nt: A One Health Framework"* is my original work and has not been submitted, either in whole or in part, for the award of any degree, diploma, scholarship or other academic qualification at any university or institution. All sources of information, including data, literature or ideas used or cited in this work have been properly identified with citations and references. All help in the preparation of this thesis has been appropriately acknowledged. This thesis was prepared for partial fulfillment of the requirement for the degree of Master of Science in One Health at the college of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia.

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BIBLIOGRAPHY

Melaku Taye Alemu was born in 1994 in South West Shoa Zone, Oromia regional state, Ethiopia. He obtain his Doctor of Veterinary Medicine (DVM) from Haramaya University Ethiopia. He has served in various professional and leadership positions in the livestock sector, including veterinary clinical practice and animal health management. Currently, he serves as an Animal Health Team Leader at the Oromia Agriculture Bureau. He is pursuing an MSc in One Health at Addis Ababa University under the NORHED II One Health Project, reflecting his commitment to advancing integrated approaches to human, animal, and environmental health.

ACKNOWLEDGEMENT

First and foremost, I give all glory and thanks to the Almighty God for His endless love, guidance, wisdom, strength, and blessings throughout my academic journey. I also express my heartfelt gratitude to the Blessed Virgin Mary and Saint Gabriel for their constant spiritual intercession and protection.

I sincerely thank my advisors, Dr. Takele Beyene (PhD), Dr. Lishan Asefa (MSc), and Professor Feleke Zewge (PhD)**,** for their invaluable guidance, constructive comments, encouragement, and continuous support throughout this research.

My sincere appreciation goes to the Livestock, Environment and One Health (LivE One Health) Project**,** the NORHED II One Health Program funded by NORAD**,** and the Africa Centre of Excellence for Water Management (ACEWM**)** for their financial, technical, and research support.

I am grateful to Mr. Abebe Olani and Mr. Abdi Ahmed of the Sebeta Animal Health Institute for their technical assistance. My special thanks also go to Mr. Ephrem Shimelis, Laboratory Technologist at the College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu Campus, for his invaluable assistance during the microbiological laboratory analyses, and to Ms. Zenebech of the CVMA Administrative Office for her kind guidance and administrative support from the beginning of my MSc study.

I also thank the Addis Ababa City Water and Sewerage Authority, the Akaki Wastewater Treatment Plant, the Aklilu Lemma Institute of Pathobiology, the ACEWM Core Water Laboratory, the CVMA Microbiology and Pharmacology Laboratories, the South West Shoa Zone Administration, the Oromia Agriculture Bureau, and all study participants for their cooperation and support.

Finally, I extend my deepest gratitude to my family for their unconditional love, prayers, patience, and encouragement, and to my friends and classmates for their friendship, moral support, and valuable cooperation throughout this study.

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LIST OF ABBREVIATIONS AND ACRONYMS

AACEWM	Addis Ababa Center of Excellence in Water Management
AMR	Antimicrobial Resistant
ARG	Antimicrobial Resistance Gene
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
CLSI	Clinical And Laboratory Standards Institute
EEPA	Ethiopian Environmental Protection Authority
EMB	Eosin Methylene Blue Agar
ESBL	Extended Spectrum β- Lactamase
HMs	Heavy Metals
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
MDR	Multi-drug Resistant
MARI	Multiple Antibiotic Resistance Index
mCIM	Modified Carbapenem Inactivation Method
QC	Quality Control
SOP	Standard Operating Procedure
UASB	Up flow Anaerobic Sludge Blanket Reactor
WWTP	Wastewater Treatment Plant

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ABSTRACT

Antimicrobial resistance (AMR) and heavy metal pollution in Wastewater treatment plants (WWTPs) and river catchments present significant global public health risks, potentially driving resistance via co-selection. This study assessed heavy metal contamination and AMR in *Escherichia coli within the Akaki WWTP* and river catchment, using a One Health framework, focusing on an extended spectrum beta-lactamase (ESBL) and carbapenemase production. A cross - sectional comparative study was conducted utilizing 232 samples from wastewater, river water, human stool, cattle feces, and milk. Six heavy metals (Cd, Pb, Cr, Cu, Mn, and Zn) were quantified by ICP-OES. *E. coli* was isolated using standard biochemical methods and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). Phenotypic resistance; ESBL and carbapenemase production were confirmed by disk diffusion, double-disk synergy tests (DDST) and modified carbapenem inactivation method (mCIM), respectively. Data were analyzed using logistic regression, chi-square, and one-way ANOVA ($p < 0.05$). Manganese (Mn) was the dominant contaminant, exceeding permissible across seasons. While, Zink (Zn) peaked during the rainy season. *E. coli* was isolated from 31 (13.4%) of samples, predominantly from water sources. High resistance was observed against Ampicillin (93.5%), Tetracycline (74.2%) , Sulphamethoxazole (64.5%), and Cefotaxime (58.0%). Multidrug resistance (MDR) rea ched 27 (87.1 %) and 14 (45.2%) were ESBL producers; no carbapenemase production was detected. Higher MDR and ESBL rates correlated with elevated Mn concentrations, indicating potential heavy metal–AMR co-selection.

The Akaki WWTP and river catchment are critical reservoirs for MDR and ESBL-producing *E. coli.* Evidence of Mn driven co-selection underscores the urgent need for integrated One Health surveillance, improved wastewater efficiency and strict antimicrob ial stewardship.

Keywords: *Antimicrobial resistance; Escherichia coli; Heavy metals; Manganese; Co-selection; One Health*

1. INTRODUCTION

1.1. Background of the Study

Antimicrobial resistance (AMR) is a major global public health threat that compromises the effective prevention and treatment of bacterial infections in humans and animals. Antimicrobial agents have played a critical role in reducing morbidity and mortality and improving animal health and productivity. However, the inappropriate and excessive use of these medicines has accelerated the emergence and spread of resistant microorganisms, reducing the effectiveness of available treatments. The World Health Organization (WHO) recognizes AMR as one of the leading global public health challenges of the 21st century. Recent estimates indicate that bacterial AMR was associated with 4.95 million deaths globally in 2019, including 1.27 million deaths directly attributable to antimicrobial-resistant infections(Murray *et al.*, 2022)

The environmental dimension of AMR is increasingly recognized within the one health framework that links human, animal and environmental health (Huijbers *et al*., 2020). Wastewater treatment plants (WWTPs) are recognized as environmental reservoirs and possible disseminators of antimicrobial-resistant bacteria (ARB) and of antimicrobial-resistance genes (ARGs) (Rizzo *et al*., 2019: Hazra *et al.*, 2022). Wastewater treatment plants receive a variety of mixed effluents from households, health care facilities and industries. These effluents contain antibiotics, heavy metals and other pollutants. Within these wastewater treatment plants the interactions between these pollutants and microorganisms can create a selective environment that allows certain microorganisms to survive longer than others. Also the transfer of resistance determinants between microorganisms can occur more easily in these environment (Plaza-Rodríguez *et al.*, 2021: Manaia, 2023). Selective heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr), zinc (Zn), copper (Cu), and manganese (Mn) are non-biodegradable pollutants that persist in the environment and tend to accumulate in soils and sediments. These metals exert long-term selective pressure on microbial populations and may contribute to the maintenance and dissemination of antimicrobial resistance. Due to the co-localization of metal

resistance genes and antimicrobial resistance genes (ARGs) on the same mobile genetic elements, such as plasmids and integrons, exposure to heavy metals can co-select for antibiotic resistance and facilitate cross-resistance mechanisms. Consequently, multidrug-resistant bacteria may persist even in the absence of direct antibiotic pressure (Edet *et al.*, 2023; Gillieatt and Coleman, 2024).

Among the antibiotic-resistant bacteria and their mechanisms of resistance extended spectrum β lactamases (ESBL) and carbapenemases produced by *Escherichia coli* are of utmost concern because they can degrade third generation cephalosporins and carbapenems, the latter of which are considered as the last-resort of antibiotics for treatment of serious infections (Algammal *et al*., 2023; Pulingam *et al.*, 2025). The occur rence of ESBL- and carbapenemase-producing *E. coli* in environmental settings is considered an important indicator of the dissemination of clinically relevant antimicrobial resistance genes beyond healthcare environments and their potential transmission among environmental, animal, and human populations (Berendonk *et al.*, 2015; Huijbers *et al*., 2020).

In Ethiopia AMR problems are increasingly becoming health and environmental issue. The use of antimicrobials in humans and in animals as well as lack of proper wastewater management are identified to be main causes of AMR problem. Akaki River, one of the most polluted water resources in Ethiopia, is facing complex mixture of pollution coming from industrial, hospital, domestic as well as agricultural sources and their partially or untreated effluents discharge into the river. The catchment area of the river is covering major parts of Addis Ababa and wastewater from all sources are discharge into the river without any treatment.(Worku *et al.*, 2022).

1.2. Problem Statements

The Akaki River catchment is one of the most environmentally impacted areas in Ethiopia, receiving municipal, hospital, industrial, and agricultural wastes from Addis Ababa and surrounding areas. The Akaki Wastewater Treatment Plant (WWTP), which serves as the major wastewater management facility in the catchment, receives mixed effluents containing antibiotics, heavy metals, resistant microorganisms, and other environmental pollutants. Although wastewater treatment processes are intended to reduce environmental contamination, WWTPs are increasingly recognized as important reservoirs and dissemination pathways of antimicrobial-resistant bacteria and resistance determinants because they concentrate contaminants from multiple sources. Consequently, treated and untreated effluents discharged into the Akaki River may contribute to the spread of antimicrobial resistance and environmental pollution within human, animal, and environmental interfaces.

Antimicrobial resistance (AMR), particularly among multidrug-resistant (MDR), extended-spectrum β-lactamase (ESBL)-producing, and carbapenemase-producing *Escherichia coli*, has become a major global public health concern affecting humans, animals, and the environment. Wastewater treatment plants (WWTPs) are increasingly recognized as important environmental reservoirs and dissemination pathways for antimicrobial-resistant bacteria because they receive municipal, hospital, and industrial wastewater containing antibiotics, resistant microorganisms, and other environmental contaminants (Edet *et al.*, 2023)

Heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), zinc (Zn), and manganese (Mn) are persistent environmental pollutants that may contribute to the maintenance and spread of antimicrobial resistance through co-selection mechanisms. Resistance genes to heavy metals and antibiotics may occur on the same mobile genetic elements, facilitating their persistence and dissemination in environmental settings even in the absence of direct antibiotic pressure (Gillieatt & Coleman, 2024)

Previous studies in Ethiopia have reported the occurrence of antimicrobial-resistant bacteria and heavy metal contamination in wastewater systems and surface waters (Worku *et al.*, 2022). However, these environmental hazards have largely been investigated separately, and little information exists regarding their potential interaction within wastewater-impacted ecosystems. In particular, there is limited evidence on the occurrence of pathogenic *E. coli*, their antimicrobial resistance profiles, ESBL and carbapenemase production, and their possible association with heavy metal contamination within the Akaki Wastewater Treatment Plant and Akaki River catchment.

The Akaki Wastewater Treatment Plant (WWTP) receives municipal, hospital and industrial wastewater for treatment and releases the treated wastewater into the Akaki River. The river is used for irrigation, animal watering and domestic use creating interfaces between the environment, human and animals. As such, the river can be a potential route for spread of environmental pollutants and antimicrobial resistant bacteria through the interconnected health systems of One Health domains. Previous studies identified water pollution and health risks to cattle in the Akaki River catchment area (Abosse *et al.*, 2025).

Despite the public health importance of this interface, a major knowledge gap remains regarding the distribution of pathogenic *E. coli*, the burden of antimicrobial resistance, and the potential association between heavy metal contamination and antimicrobial resistance within a One Health framework in Ethiopia. Therefore, this study was designed to assess heavy metal contamination, antimicrobial resistance patterns of pathogenic *E. coli*, including ESBL and carbapenemase production, and to explore their possible association in the Akaki WWTP and Akaki River catchment.

1.3. Objectives

1.3.1. General objective

To investigate heavy metal contamination and antimicrobial resistance of pathogenic *Escherichia coli* isolated from Akaki Wastewater Treatment Plant and Akaki River catchment, and their association within the One Health framework.

1.3.2. Specific objectives

- To determine the concentrations of selected heavy metals (Pb,Cd,Cr,Cu,Zn and Mn) in wastewater from five treatment stages of the Akaki wastewater treatment plant and in water from the Akaki river catchment
- To isolate and characterize pathogenic *Escherichia coli* from environmental, human and animal samples and determine their antimicrobial susceptibility, multidrug resistance (MDR) and prevalence of ESBL and carbapenemase production using phenotypic methods.
- To assess the distribution of antimicrobial-resistant *E.coli* across environmental, human and animal domains in the study area
- To evaluate the association between heavy metal contamination and antimicrobial resistance indicators (MDR, ESLB production, and resistance to selected antibiotics).

2. LITERATURE REVIEW

2.1. Antimicrobial Resistance as Global Health Threats

AMR is recognized as one of the most serious global public health threats affectin human s, animals and the environment (Pulingam *et al.,* 2022). The World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (WOAH) have adopted the One Health approach to coordinate global efforts aimed at controlling AMR across sectors (Organization, 2021: Aziz *et al.,* 2025). Contemporary reviews emphasize that while antibiotic misuse in clinical and agricultural settings remains central, environmental drivers especially chemical pollutants must be integrated into AMR frameworks to capture the full One Health picture. Globally, bacterial AMR was directly responsible for approximately 1.27 million deaths and associated with 4.95 million deaths in 2019 (Manaia, 2023).

Antimicrobial resistance is a growing global public health problem with far reaching impacts on human health, animal health, food security and the environment, and as such requires an urgent coordinated response (Founou *et al*., 2021). Instead of being viewed as a future public health threat, AMR is already a well-established global health challenge affecting health care systems, animal health, food systems and environmental sustainability worldwide. As has been previously stated, the projected number of deaths due to AMR by 2050 could surpass that of predicted malaria, HIV/AIDS and tuberculosis cases, becoming a major global public health problem (O'Neill, 2016).

A growing body of research has implicated agricultural production and the food system in the emergence and spread of antimicrobial resistance. As McKernan and colleagues noted, the use of antimicrobials often in an inappropriate or excessive manner in human and veterinary medicine as well as in agriculture, has the potential to select for and allow the persistence of resistant microorganisms (McKernan *et al.*, 2021). Antibiotic residues from animals and humans are released into the environment via a variety of routes including animal and human manure, wastewater from homes and hospitals, and industrial effluents,

and can maintain resistance determinants in environmental microorganisms and create selection pressure for the increase in resistant bacterial populations that can infect humans, animals and the environment and cause disease (Holmes *et al*., 2020).

2.2. Antimicrobial Resistance Mechanisms

AMR in bacteria can be inherent, acquired, or adaptive (B. Li *et al*., 2022).Microorganisms evolve or acquire resistance mechanisms to survive under toxic environmental conditions, often through horizontal gene transfer (HGT**)**, which enables the exchange of genetic material carrying resistance determinants between bacterial species (Abbas *et al.*, 2024). Acquired resistance arises either through spontaneous mutations in chromosomal genes or by obtaining mobile genetic elements such as plasmids, transposons, or integrin's from other bacteria via conjugation, transformation, or transduction (Gupta & Birdi, 2017).

Inherent (intrinsic) resistance results from natural structural or physiological characteristics that prevent antibiotic action and occurs independently of prior exposure. For instance, bacteria lacking cell walls (*Mycoplasma* spp.) are naturally resistant to β-lactam antibiotics (Zhao *et al.*, 2019). Adaptive resistance is a reversible physiological response that arises under specific environmental or stress conditions, such as exposure to sub-inhibitory antimicrobial concentrations or heavy metals (D'Aquila *et al*., 2023).

Acquired resistance can manifest as cross-resistance**,** multidrug resistance (MDR)**,** extensively drug resistance (XDR) or pan-drug resistance (PDR)**,** often mediated through target modification, enzymatic drug inactivation and efflux-pump overexpression (Belay *et al*., 2024). Among these mechanisms, production of β-lactamase enzymes remains one of the most important, as they hydrolyze the β-lactam ring of antibiotics, rendering them inactive (Gauba & Rahman, 2023). Extended spectrum β lactamases (ESBLs) and carbapenemases enzymes capable of hydrolyzing cephalosporins and carbapenems, respectively are now widespread in clinically and environmentally significant pathogens.

Genes encoding ESBLs and carbapenemases are commonly located on mobile genetic elements, facilitating their rapid dissemination across bacterial populations and ecosyste ms (Azab *et al*., 2021)

2.3. ESBL and carbapenemase mechanisms and environmental occurrence

Extended-spectrum β-lactamases (ESBLs) and carbapenemases are clinically important β-lactamase enzymes that hydrolyze broad-spectrum β-lactam antibiotics, including third-generation cephalosporins and carbapenems. Genes encoding these enzymes, such as *blaCTX-M, blaTEM, blaSHV, blaNDM, blaKPC,* and *blaOXA-48,* are frequently located on plasmids and other mobile genetic elements, facilitating rapid dissemination among bacterial populations (Findlay *et al*., 2022). Recent studies and surveillance reports show that increasing detection of ESBL and carbapenemase- producing Enterobacteriaceae in wastewater treatment plants, rivers and other aquatic environments worldwide (Hazra *et al*., 2022). Their occurrence in environmental systems highlights the importance of wastewater environments as reservoirs and transmission pathways for clinically important antimicrobial resistance determinants. The co- occurrence of heavy metals with these phe notypes has been reported in multiple field studies, suggesting possible co-selection or shared dissemination routes (Edet *et al*., 2023; Touahir *et al*., 2023).

2.4. Impacts of Antimicrobial Resistance

Antimicrobial resistance (AMR) has emerged as a major global concern due to the increasing prevalence of pathogens resistant to multiple drugs, often referred to as “Superbugs” (Algammal *et al*., 2023). The magnitude of this problem is alarming AMR was estimated to have caused 1.27 million deaths directly and contributed to 4.9 million deaths globally in 2019, surpassing the mortality burden of HIV/AIDS and malaria combined (Murray *et al.,* 2022). Resistant bacteria and residual antimicrobial compounds

are increasingly being detected not only in aquatic environments but also in human biological samples such as serum, urine, and breast milk, even among individuals not currently using antibiotics, indicating widespread environmental exposure (Weatherly & Gosse, 2017; Bever et al., 2018)

2.4.1. Impacts on human health

The rise of antimicrobial resistant infections significantly undermines medical care especi ally in healthcare settings where hospital acquired infections caused by resistant pathogen s are prevalent. Hospitals and long term care facilities are estimated to contribute up to one third of environmental antimicrobial resistance, mainly through the discharge of contaminated effluents (Hocquet *et al*., 2016). The reduction in the effectiveness of last resort antibiotics, such as carbapenems and colistin, presents a critical clinical challenge and limits therapeutic options for life-threatening infections (Hutchings *et al*., 2019)

2.4.2. Impacts on the Food Chain

The food chain serves as a key transmission route for antibiotic-resistant bacteria (ARB). Resistant spoilage and pathogenic microorganisms can enter foods at various points from farm to fork, and once contamination occurs at the farm level, these bacteria can persist on raw or undercooked food items (Teklemariam *et al*., 2023). Fresh produce, especially leafy greens and ready-to-eat foods, has been reported to harbor resistant pathogens and may act as vehicles of transmission to consumers (Losio *et al*., 2015). This pathway represents a direct link between agricultural antimicrobial use and public exposure to resistant strains.

2.4.3. Impacts on the environment

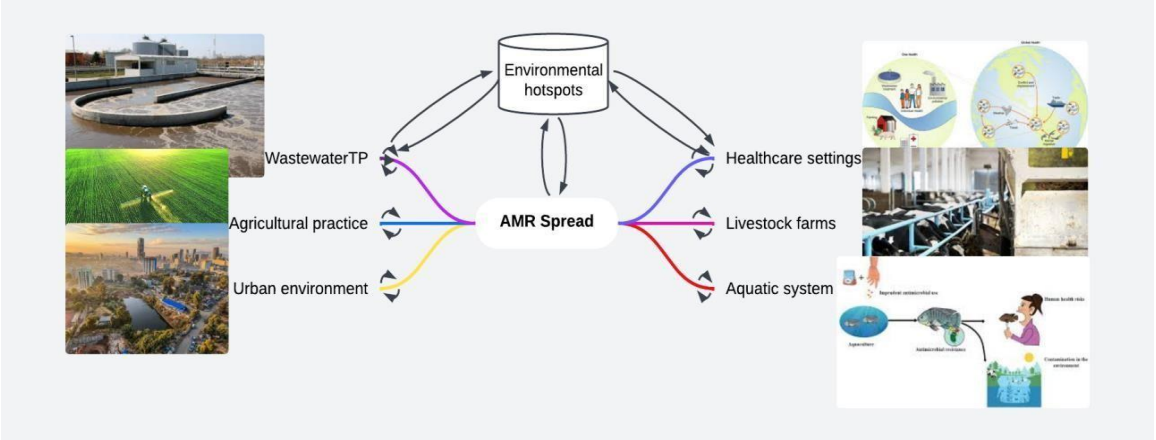
A large proportion of antimicrobials administered to humans and animals is excreted unchanged or as active metabolites via urine, feces, and manure, introducing antibiotic residues into the environment (Hussein *et al*., 2023). These residues exert selective pressure that promotes the persistence and proliferation of resistant microorganisms in soil and water systems. Antibiotics and resistance genes can disrupt natural microbial diversity essential for nutrient cycling, soil fertility, and ecosystem stability (Kraemer *et al*., 2019). Environmental contamination with antibiotics also alters microbial community structure and function, increasing the prevalence of pathogenic or opportunistic species *(Cycoń et al.,* 2019). Moreover, antibiotic pollution in aquatic environments has been associated with the proliferation of toxic cyanobacteria, contributing to eutrophication and potential public health hazards (Drury *et al*., 2013)

2.5. Environmental Hotspots for Antimicrobial Resistance

Antimicrobial resistant pathogens and their associated resistance genes are continually introduced into the environment through multiple critical hotspots, including hospitals, healthcare facilities, agricultural runoff, and wastewater treatment plants (Samreen *et al*., 2021). These environments act as reservoirs and dissemination points where resistance can evolve and spread among microbial communities. Inadequate management of healthcare and hospital waste plays a significant role in this process. When infectious waste materials are improperly handled or disposed of, pathogenic microorganisms may survive and subsequently infect humans, animals and environmental biota (Abosse *et al*., 2024). The pharmaceutical industry also represents a major contributor to environmental AMR. Discharges containing antibiotic residues, active pharmaceutical ingredients, and resistant microorganisms from manufacturing sites release selective agents into nearby ecosystems, facilitating the enrichment and persistence of resistant bacterial populations (Bjerke, 2025). Likewise, intensive agricultural practices are increasingly recognized as key drivers of

antimicrobial resistance. Soils and waters in agricultural areas are frequently exposed to stressors such as pesticides, fertilizers, and heavy metals, which together impose selective pressure on microbial communities (Rad *et al*., 2022).

The presence of heavy metals commonly used as feed additives, fertilizers, or fungicides has been linked to the co-selection of antibiotic-resistant strains in soil and water environments. *Escherichia coli*, in particular, has been reported to exhibit concurrent resistance to both heavy metals and antibiotics under such conditions (Vounba *et al*., 2019). Since the 1950s, antimicrobials have been widely used in livestock production for therapeutic, prophylactic, and growth promotion purposes. Although these practices contr ibute to productivity gains, the indiscriminate or non-therapeutic use of antibiotics in food animals has accelerated the emergence and dissemination of resistant bacteria that can be transmitted to humans through direct contact, food chain, and the environment (Rhouma *et al*., 2016). In Ethiopia, limited awareness and inappropriate perceptions regarding antimicrobial use (AMU) and resistance (AMR) among livestock producers have been identified as contributing factors, underscoring the need for enhanced education and responsible antibiotic stewardship within the agricultural sector (Tufa *et al*., 2023). The most common environmental hotspots that aggravate the dissemination of AMR pathogens are mentioned in Fig. 1



Source: (Kunhikannan *et al.*, 2021).

Figure 1: Environmental hotspots for emergence of AMR

2.5.1. Wastewater treatment plants

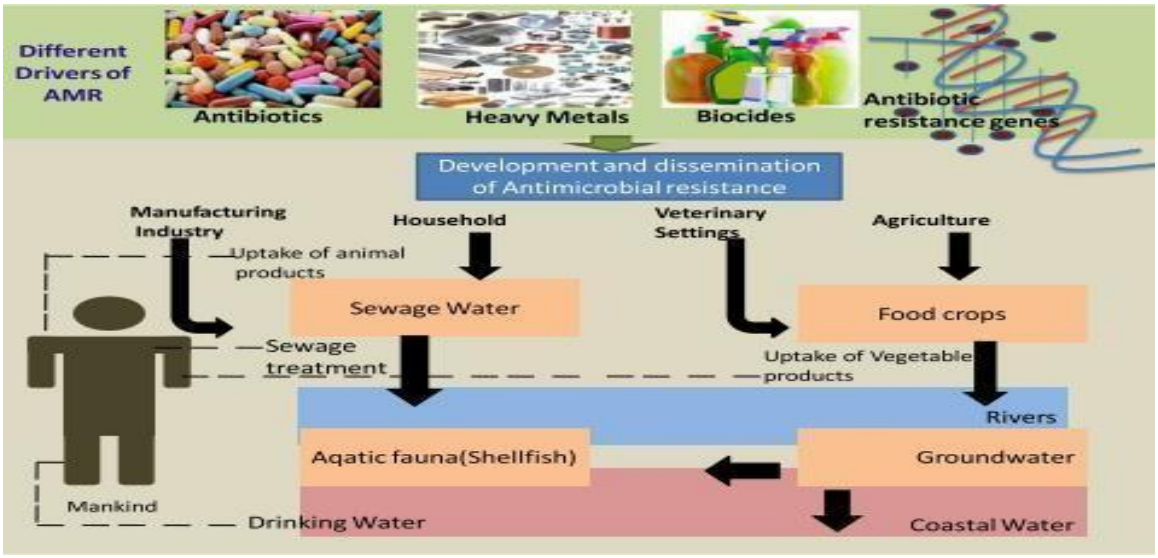
Wastewater treatment plants (WWTPs) are recognized as major environmental hotspots for the persistence and dissemination of antimicrobial resistance (AMR). They receive mixed influents from domestic, hospital, and industrial sources that contain antibiotics, heavy metals, biocides, microplastics, and resistant bacteria (Hazra *et al.*, 2022; Pulingam *et al.*, 2025). Within these systems, diverse microbial populations interact under continuous selective pressure, facilitating horizontal gene transfer (HGT) and the enrichment of antimicrobial resistance genes (ARGs).Although WWTPs are designed to remove organic matter and pathogenic microorganisms, conventional treatment technologies often fail to fully eliminate antibiotic residues, ARGs, or heavy metals. Consequently, treated effluents and sewage sludge can act as continuous sources of resistant bacteria and chemical contaminants when discharged into receiving water bodies or reused for irrigation (Triggiano *et al.*, 2020; Hazra *et al.*, 2022)

The combination of high microbial density, sub- inhibitory concentrations of antimicrobi als, and abundant mobile genetic elements provides ideal conditions for the survival and exchange of resistance determinants. The drivers of AMR in WWTPs are multifactorial. In addition to antibiotic misuse in human and veterinary medicine, environmental contaminants such as heavy metals, biocides and microplastics play critical roles in maintaining and propagating resistance (Chukwu *et al.*, 2023).

Heavy metals are persistent, non-biodegradable pollutants that accumulate in sewage sludge and sediments, exerting long-term selective pressure on microbial communities (Niu *et al.*, 2016). Metal resistance genes (MRGs) often co-occur with ARGs on plasmids, transposons, and integrons, allowing co- and cross-resistance and enabling simultaneous tolerance to both antibiotics and metals (Edet *et al*., 2023; Gillieatt & Coleman, 2024). Antibiotics excreted by humans and animals up to 85% of the administered dose in livestock reach WWTPs through sewage or runoff, where they maintain selective pressure on bacteria (Mutiyar & Mittal, 2014; Yap *et al*., 2023). Similarly, biocides, while effective for disinfection, may co-select for antibiotic resistance by inducing shared resistance

mechanisms such as efflux pumps and target-site modifications (Ruiz & Alvarez-Ordóñez, 2017; Shao *et al*., 2024). Microplastics, another emerging contaminant, provide surfaces for biofilm formation that enhance bacterial growth, gene exchange, and persistence Prata, 2018; Zhuge *et al.*, 2019; Nguyen *et al*., 2021)

In Addis Ababa, particularly in the Akaki River catchment, limited treatment efficiency and infrastructure challenges contribute to the continuous discharge of partially treated or untreated wastewater into the river system. This environment allows resistant bacteria, antibiotics, and heavy metals to co-exist and interact under sustained selective pressure (Adegoke *et al*., 2020; Zhao *et al.*, 2023). Different drivers of AMR along with environmental hotspots for AMR development and dissemination in wider ecosystem are elaborated in Figure 2.



Source: (Samreen *et al*., 2021)

Figure 2: Scheme of AMR drivers and their dissemination

Antimicrobial resistance (AMR) has become a major global public health concern, largely due to its ability to spread through mobile genetic elements such as plasmids, transposons, and integrons, which facilitate horizontal gene transfer among microorganisms (Sharma *et al.*, 2016).

Wastewater entering treatment facilities often contains a complex mixture of contaminants, including antibiotic residues, heavy metals, resistant microorganisms, resistance genes, and other contaminants of emerging concern such as microplastics (Hubeny *et al.*, 2021). Conventional wastewater treatment systems are not specifically designed to completely eliminate these pollutants, and therefore substantial amounts may persist throughout the treatment process and be released into receiving water bodies (Uluseker *et al.*, 2021). As a result, WWTPs may serve as reservoirs and transmission pathways for antimicrobial resistance determinants in the environment (Marutescu *et al*., 2023)

The coexistence of antimicrobial compounds, toxic metals, and other chemical stressors within wastewater creates favorable conditions for the selection and enrichment of resistant microbial populations (Krzeminski *et al.*, 2019).
In addition, the nutrient rich and microbially diverse environment within treatment systems promotes interactions among bacterial communities, increasing the likelihood of horizontal gene transfer and the spread of antimicrobial resistance genes (Kalli *et al.*, 2023). These processes may also alter microbial community structure and ecological balance in aquatic environments (Wang *et al.*, 2025).

Within the One Health framework, wastewater treatment plants represent a critical interface linking human, animal, and environmental health (Hassell *et al.*, 2019)Resistant bacteria and resistance genes originating from human and animal waste can circulate through sewage systems and subsequently spread to aquatic ecosystems through treated or untreated effluents (Berendonk *et al.*, 2015). In particular, β-lactamase-producing bacteria, including ESBL-producing organisms, have frequently been identified in influent and effluent wastewater and are considered important indicators of antimicrobial resistance dissemination in environmental settings (Huijbers *et al.*, 2020).

2.5.2. Drivers of antimicrobial resistance in wastewater treatment plants

The increasing burden of antimicrobial resistance is primarily driven by the extensive and often inappropriate use of antimicrobial agents in human medicine, veterinary practice, and agriculture. Wastewater treatment plants receive these antimicrobial residues along with resistant bacteria from multiple sources, creating environments where selective pressure can promote the persistence and amplification of resistance traits (Chukwu *et al*., 2023).

In addition to antibiotics, wastewater often contains contaminants of emerging concern, including disinfectants, pharmaceutical residues, and heavy metals, all of which may contribute to the development and maintenance of antimicrobial resistance. The effectiveness of wastewater treatment technologies, particularly the availability of advanced tertiary treatment methods, plays an important role in determining the extent to which these contaminants and resistant organisms are removed before environmental discharge (Hazra and Durso, 2022).

Heavy metals are of particular concern because they are non-biodegradable and can accumulate in wastewater, sewage sludge, and surrounding ecosystems over time. Their long-term persistence can exert continuous selective pressure on microbial communities, favoring the survival of metal-resistant bacteria (Niu *et al.*, 2016). Importantly, genes responsible for heavy metal resistance are often genetically linked with antimicrobial resistance genes on shared mobile genetic elements such as plasmids, transposons, and integrons (Gillieatt and Coleman, 2024).

This genetic linkage can promote co-selection, whereby exposure to heavy metals indirectly selects for antibiotic-resistant bacteria even in the absence of direct antimicrobial pressure. Heavy metal–antibiotic co-resistance may therefore amplify resistance gene dissemination across different ecological settings and contribute significantly to the spread of multidrug-resistant organisms (Edet *et al*., 2023).

2.6. Contribution of Heavy Metals to Microbial Resistance

Heavy metals are naturally occurring elements, but their accumulation in the environment due to industrialization, mining, agriculture, and urbanization has created toxic ecological conditions (Hubeny *et al.*, 2021; Goswami *et al.*, 2023). Unlike organic pollutants, heavy metals are non-biodegradable and persist for long periods in soil, water, and sediments, where they interact with microbial communities (Tufa *et al*., 2020).

Research has shown that exposure to heavy metals such as copper (Cu), zinc (Zn), cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As) can co-select for antibiotic resistance genes (ARGs) (Ramos *et al*., 2020; Jain *et al.*, 2021; Rana *et al.*, 2022).

2.7. Mechanisms of Heavy Metal Mediated AMR Co-Selection

Heavy metals such as lead (Pb), cadmium (Cd), copper (Cu), zinc (Zn), and chromium (Cr) are persistent environmental contaminants that can significantly influence the development and spread of antimicrobial resistance (AMR) among bacteria. Unlike antibiotics, metals are non-biodegradable and can persist in wastewater and sediments for long periods, exerting continuous selective pressure on microbial communities (Niu *et al*., 2016).

There are two major mechanisms through which heavy metals mediate AMR co-selection: Co-resistance occurs when genes conferring resistance to both antibiotics and metals are physically linked on the same mobile genetic elements (MGEs) such as plasmids, integrons, or transposons (Gillieatt and Coleman, 2024). When a bacterium is exposed to heavy metals, the selective pressure not only favors metal resistance but also indirectly selects for antibiotic resistance genes located on the same genetic structure. This mechan ism has been widely observed in *E. coli* and other Enterobacteriaceae isolated from wast ewater environments, where exposure to Cu, Zn, or Cd correlates withincreased abundan ce of β- lactamase (bla_{CTXM}), carbapenemase (bla_{NDM}), an d tetracycline resistance genes(Qiu *et al*., 2019;Chukwu *et al.*, 2023).The presence of th

ese genetic linkages enhances the persistence and dissemination of multidrug resistant (MDR) bacteria even in the absence of antibiotic use (W. Abbas *et al.,* 2024). Cross-resistance occurs when a single cellular mechanism provides protection against both antibiotics and heavy metals, most commonly through multidrug efflux pumps. These pumps actively expel a wide range of toxic compounds, including antibiotics (e.g., βlactams, quinolones) and metal ions, from bacterial cells include the CzcCBA efflux system conferring resistance to Zn, Cd, and Co and the CusCFBA system for Cu and Ag resistance, both of which are functionally related to antibiotic efflux pumps such as AcrABTolC. Activation of these efflux mechanisms increases bacterial tolerance to antibiotics, contributing to the maintenance of resistant populations under heavy metal exposure (Fallah *et al.,* 2021; Gillieatt and Coleman, 2024).

Recent studies have shown that co-regulation of MRGs and ARGs can occur under environmental stress, where exposure to metals upregulates genes related to both metal detoxification and antibiotic resistance (Vounba *et al*., 2019; Adegoke *et al*., 2020).

In wastewater treatment plants and river catchments like Akaki, the coexistence of antibiotics and heavy metals enhances horizontal gene transfer (HGT) through plasmid exchange, transformation, and conjugation, promoting the spread of ARGs across environmental, human, and animal microbial populations (Rizzo *et al.*, 2019; Hazra *et al.,* 2022) .

2.8. Pathways of Exposure to Heavy Metals and Associated AMR

The dissemination of AMR through heavy metal pollution is strongly linked to environm ental exposure pathways affecting humans, animals and ecosystems alike. Under the One Health framework, these exposure routes reflect interconnected dynamics between envir onmental contamination microbial evolution and public health.

2.8.1. Environmental exposure

Heavy metals (HMs) enter the environment through multiple anthropogenic sources, including Industrial effluents from mining, smelting, and manufacturing, urban runoff and storm water drainage, discharge from wastewater treatment plants, and agricultural inputs like fertilizers, pesticides, and animal manure. These pollutants accumulate in soil and sediment, transforming the microbial landscape and serving as hotspots for resistance gene selection (Ramos *et al.,* 2020; Bhowmik *et al*., 2023). The soil ecosystem, in particular, serves as a major reservoir for both heavy metal resistance genes (HMRGs) and antibiotic resistance genes (ARGs), as shown in contaminated urban and industrial zones *(Rabow et al.*, 2023). In wastewater, both HMs and antibiotics co-occur, creating synergistic selection pressures. Studies have shown that sub-inhibitory concentrations of heavy metals, such as Cu and Zn, can facilitate horizontal gene transfer (HGT) of plasmid-borne ARGs among microbial populations (Zhang *et al.,* 2018; Y. Li *et al.,* 2023;).

2.8.2. Animal exposure

Animals, particularly livestock, are frequently exposed to HMs through contaminated drinking water and feed (from irrigated fields or processed fodder), application of manure from treated or exposed animals and use of HM containing supplements (e.g., ZnO, $CuSO_4$) in animal husbandry. This leads to alterations in the gut microbiota, favoring the survival and multiplication of resistant strains. Resistant bacteria and ARGs are subsequently shed in feces and enter the environment, amplifying the One Health cycle (Rabow *et al.*, 2023; Talim *et al*., 2024).

2.8.3. Human exposure

Humans become exposed to heavy metals and associated AMR through consumption of contaminated food and water, inhalation of particulate matter in polluted air (particularly around mining or industrial zones), Contact with contaminated soil, especially among children and farmers (Goswami *et al*., 2023).

A systematic review by (Hubeny *et al.,* 2021) linked industrial wastewater containing both antibiotics and HMs to increased AMR burdens in downstream water bodies. In urban areas of Ghana and Vietnam, untreated effluents from car-washing bays and hospitals were found to harbor both high HM loads and resistant pathogens (Abagale *et al., 2013).*

2.9. The One Health implication of Heavy metals

Heavy metal contamination contributes to the persistence and dissemination of antimicrobial resistance (AMR), posing important challenges to human, animal and environmental health. Heavy metals are non-biodegradable pollutants that can accumulate in environmental compartments and exert long-term selective pressure on microbial communities (Hazra and Durso, 2022). The co-occurrence of heavy metal resistance genes (HMRGs) and antimicrobial resistance genes (ARGs) on shared mobile genetic elements, such as plasmids, transposons, and integrons, facilitates co-selection and may promote the maintenance and spread of antimicrobial resistance even in the absence of direct antibiotic exposure (Niu *et al.*, 2016; Gillieatt and Coleman, 2024). Consequently, environmental contamination with heavy metals may contribute to the emergence and persistence of multidrug-resistant bacteria across interconnected human, animal, and environmental systems. This highlights the importance of addressing heavy metal pollution within a One Health framework that recognizes the close relationship between environmental quality, animal health, and public health (Berendonk *et al.,* 2015; Huijbers *et al.,* 2020)

2.9.1. Impact on human health

Exposure to antimicrobial-resistant bacteria linked to heavy metal pollution has been increasingly documented in human populations. Humans are exposed to heavy metal AMR interactions through multiple pathways: Populations living near contaminated water bodies, industrial sites, or waste treatment facilities are at higher risk of encountering resistant pathogens. Studies have shown elevated levels of resistance genes in residents near heavy metal contaminated soils, such as the 35th Avenue Superfund Site in Birmingham, Alabama (Goswami *et al.,* 2023). Poor and marginalized communities, often located near industrial zones, disproportionately face exposure to environmental pollutants and associated AMR risks. These include respiratory illnesses, skin infections, and gastrointestinal diseases caused by resistant microbes (Tufa *et al*., 2020;Gozi *et al.*, 2021). Workers in mining, tannery, and industrial effluent zones face elevated risks through direct contact (Odumbe *et al.*, 2023).

Heavy metals may enter the human body through contaminated drinking water, food crops, fish, milk, and other animal products originating from polluted environments. Chronic exposure to heavy metals such as lead, cadmium, chromium, and manganese has been associated with adverse health effects including neurological disorders, kidney and liver damage, developmental abnormalities, reproductive problems, and increased cancer risk. In addition to their direct toxic effects, heavy metals may indirectly influence human health by promoting the persistence of antimicrobial-resistant microorganisms in the environment, thereby creating an additional public health concern within the One Health framework (Hubeny *et al*., 2021). Environmental AMR determinants infiltrate healthcare systems, where resistant infections such as those by *Pseudomonas aeruginosa* and *Enterococcus faecium* pose treatment challenges (Rana *et al.*, 2022). Globally, the synergy of metals and antibiotics is accelerating the rise of multi drug resistant pathogens, with direct consequences for morbidity, mortality and healthcare costs (Ranjbar and Alam, 2024).

2.9.2. Impact on animal health

Animals, particularly livestock, are not only exposed to heavy metals through feed and water but also serve as amplifiers and reservoirs of resistant microbes. Livestock gut microbiota alteration exposure to metals like Cu, Zn, and Pb promotes shifts in microbial populations, increasing the prevalence of AMR strains (Martins *et al.*, 2014; Vats *et al.*, 2022) These animals can shed resistant bacteria through feces, contaminating the environment and spreading AMR to other animals and humans. Aquatic and wild life species exposed to metal polluted environments exhibit elevated rates of resistance, disrupting ecosystem health and biodiversity (Edet *et al*., 2023; Fu *et al*., 2023)

2.9.3 *Impact on environmental health*

Heavy metal contamination alters the diversity, structure, and function of soil and aquatic microbiomes by reducing microbial diversity, favoring resistant taxa, and disrupting nutrient cycling, particularly under elevated concentrations of metals such as Pb, As, and Zn. (Goswami *et al*., 2023). Metagenomics predictions show that soils polluted with metals harbor significantly higher levels of antibiotic resistance genes (ARGs) compared to non contaminated soils, even when no antibiotics are present (Fu *et al.*, 2023). This per sistence poses risks to ecosystem services by affecting processes such as organic matter decomposition and nitrogen cycling, while environmental justice concerns arise because polluted areas disproportionately affect marginalized communities, thereby compounding both ecological and public health risks. (Goswami *et al*., 2023).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted in the Akaki River catchment, located in the southern part of Addis Ababa, Ethiopia, including the Akaki (Chefe) Wastewater Treatment Plant (WWTP) and selected surrounding peri-urban areas. The Akaki River originates from the Entoto highlands and flows through Addis Ababa before discharging into the Aba Samuel Reservoir, covering an approximate distance of 40 km. The catchment extends over about 403 km² and is characterized by rapid urbanization, population growth, and increasing industrial activities (Aliyu *et al.*, 2022; Worku *et al.*, 2022).

As one of the major drainage systems of Addis Ababa, the river receives wastewater and runoff from domestic, industrial, healthcare, and agricultural sources. The river is widely used by surrounding communities for irrigation, livestock watering, and other domestic activities, creating important interactions among environmental, human, and animal populations. Consequently, the catchment represents a suitable setting for investigating environmental contamination and antimicrobial resistance within a One Health framework.

Within the catchment, the Akaki (Chefe) WWTP serves as a major municipal wastewater treatment facility. The plant is located in the Akaki industrial zone at approximately 8°53′13″ N latitude and 38°47′1″ E longitude, at an elevation of about 2104 m above sea level. It operates in two phases with a total treatment capacity of approximately 25,000 m³ per day and occupies an area of about 40 hectares. The treatment process includes preliminary screening and grit removal, anaerobic treatment using an Upflow Anaerobic Sludge Blanket (UASB) reactor, secondary treatment through trickling filters, and final sedimentation units for sludge stabilization and solid–liquid separation (Cgcoc, 2022).

Because the WWTP receives municipal, hospital, and industrial wastewater and discharges treated effluent into the Akaki River, both the treatment plant and the receiving river system

represent important interfaces for the circulation of environmental contaminants, including heavy metals and antimicrobial-resistant bacteria(Hiruy *et al*., 2022). Therefore, the study area provides an appropriate setting to assess heavy metal contamination, antimicrobial-resistant *Escherichia coli*, and their potential association across environmental, human, and animal domains.

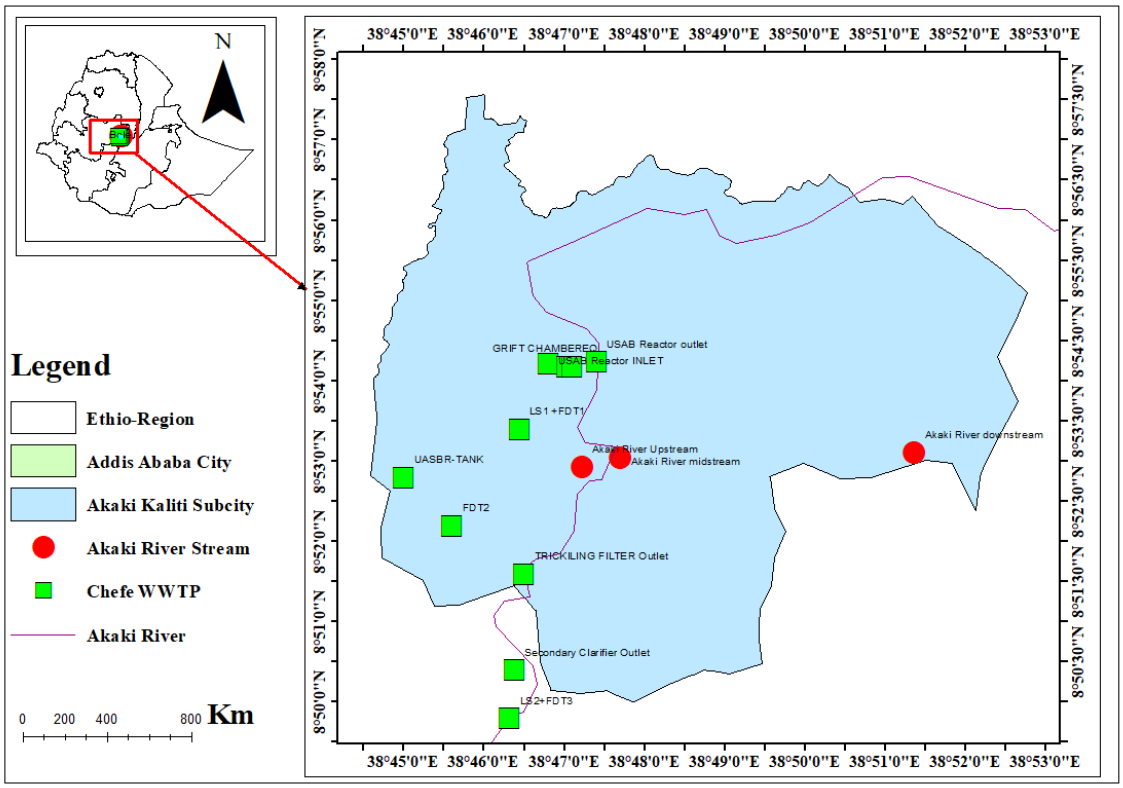


Figure 3: Sampling point, Akaki River Ethiopia, 2026

Source: ARC-GIS

3.2. Study Design

A comparative cross-sectional study design based on a One Health approach was employed to investigate heavy metal contamination and antimicrobial resistance in *Escherichia coli* from environmental (water and wastewater), human, and animal sources within the Akaki River catchment. The study was conducted from November 2025 to May 2026, encompassing both the dry season (Bega) and the short rainy season (Belg). Environmental sampling was performed during representative dry-season conditions and again during March 2026, when Belg rainfall occurred in the study area. Inclusion of both sampling periods enabled assessment of seasonal variation in heavy metal contamination and antimicrobial resistance patterns. This design facilitated the simultaneous comparison of heavy metal concentrations, antimicrobial susceptibility profiles, and the occurrence of ESBL-producing *E. coli* across human, animal, and environmental domains.

Sampling was carried out from multiple sources including river water (upstream, midstream and downstream), wastewater treatment plant stages (inlet to outlet), human stool, cattle fecal and milk samples. A structured questionnaire survey was also conducted among households to assess potential risk factors, antibiotic usage practices and awareness related to antimicrobial resistance. Water samples collected for heavy metal analysis were processed in triplicate independent replicates to ensure analytical precision and reproducibility. Heavy metal determination was performed at the ACEWM Core Water Laboratory, Addis Ababa University, using standardized analytical procedures.

Microbiological samples were transported under cold-chain conditions to the Microbiology Laboratory at Addis Ababa University, College of Veterinary Medicine and Agriculture. Laboratory analysis included isolation and confirmation of *E. coli*, followed by antimicrobial susceptibility testing (AST) using the Kirby–Bauer disk diffusion method. Phenotypic detection of ESBL and carbapenemase production was conducted using standard methods. This integrated study design provides a comprehensive framework to evaluate the distribution of heavy metals and antimicrobial-resistant *E. coli* (CLSI, 2024; EUCAST, 2024).

3.3. Study Domains and Sampling Points

The study was conducted across three interconnected domains environmental, human, and animal following a One Health approach to assess the distribution of heavy metals and antimicrobial-resistant *Escherichia coli* within the Akaki River catchment and WWTP.

3.3.1. Environmental domain

Environmental sampling included both wastewater and surface water sources. Wastewater samples were collected from different operational units of the Akaki Wastewater Treatment Plant specifically at key treatment stages, including the UASB reactor inlet, UASB reactor outlet, trickling filter outlet, secondary clarifier outlet, and final effluent discharge point. These samples were used to determine the concentration of selected heavy metals, including lead (Pb), cadmium (Cd), chromium (Cr), zinc (Zn), copper (Cu), and manganese (Mn).

In addition, surface water samples were collected from the Little Akaki River at strategic locations relative to the wastewater discharge point, including upstream (adjacent, <1000 m), midstream and downstream (distal, >1000 m) sites representing areas influenced by wastewater discharge. Sampling points were designed based on the right thumb rule that suggests complete mixing of contaminants of emerging concerns from effluent discharge with nearby water bodies occurs at a distance of at least 10 times the width of surface water (<20m) and the international guideline of water quality sampling for microbiological analysis (O'Hagan *et al.*, 2021)

Environmental samples were processed for *E. coli* isolation, antimicrobial resistance profiling, and phenotypic detection of extended-spectrum β-lactamase (ESBL) and carbapenemase production.

3.3.2. Human domain

The human study population consisted of individuals with varying levels of exposure to wastewater effluent, including WWTP workers and residents living in proximity to the treatment plant. Participants were categorized into two groups based on their distance from the WWTP, namely adjacent residents (<1000 m) and distant residents (>1000 m). This classification was based on previous studies assessing environmental exposure gradients (Rodríguez-Molina *et al*., 2022).

A semi-structured questionnaire was administered to collect information on socio-demographic characteristics, antibiotic usage practices, and awareness of antimicrobial resistance, thereby minimizing potential confounding factors. Stool samples were collected from eligible and consenting participants following ethical approval and under appropriate supervision. These samples were analyzed for the isolation of *E. coli*, antimicrobial susceptibility testing, and phenotypic detection of ESBL and carbapenemase-producing isolates.

3.3.3. Animal domain

The animal component of the study focused on cattle reared in areas surrounding the Akaki River and wastewater irrigation zones. Fecal and milk samples were collected to assess the presence of *E. coli* and to investigate antimicrobial resistance patterns among isolates.

These sampling sites were selected to capture potential transmission pathways between environmental contamination and livestock, thereby strengthening the One Health perspective of the study.

Sampling were follow purposive and convenience approaches, with inclusion/exclusion criteria and a brief questionnaire employed to mitigate confounding variables. Sampling points and volumes was adhere to ISO 19458:2007 (O'Hagan *et al.*, 2021). Standards and the established thesis sampling scheme.

3.3.4. Sampling approach and standards

A purposive and convenience sampling approach was employed because the study specifi cally targeted populations and sampling sites with potential exposure to wastewater conta mination guided by predefined inclusion and exclusion criteria. This approach ensured representation of key exposure groups across the study domains.

All sampling procedures including sample collection, handling and transportation were carried out in accordance with international standards for microbiological water analysis (ISO 19458:2007) and established laboratory protocols. Appropriate sample volumes and aseptic techniques were maintained to ensure data reliability and validity.

3.4. Sample Size Determination

Environmental sampling included both wastewater and river water collected during two different seasons (dry and rainy) to capture temporal variations. Wastewater samples were collected from the Akaki Wastewater Treatment Plant (WWTP) at key treatment stages, including the UASB inlet, UASB outlet, trickling filter outlet, secondary clarifier outlet, and final effluent discharge point were collected to determine the concentration of selected heavy metals, antimicrobial resistance profiles, and patterns of indicator pathogens, particularly ESBL and carbapenemase producing *E. coli*. Each treatment stage was sampled during both dry and rainy seasons, resulting in a total of 10 wastewater samples (5 stages × 2 seasons).

In addition, three surface water samples from the Akaki River (one adjacent point <1000 m or Upstream, Mid-stream and one distal point >1000 m or downstream from the effluent discharge) were purposively collected to assess *E. coli* occurrence, antimicrobial resistance profiles, and ESBL and carbapenemase production.

The sample size for human component was calculated using the formula for comparing two proportions. The calculation was based on previously reported prevalence estimates of

E. coli carriage among populations residing near and distant from wastewater treatment facilities (Rodríguez-Molina *et al.*, 2021). Prevalence values of 29% and 7% were adopted for exposed and non-exposed groups, respectively. A significance level of 5% and statistical power of 80% were used in the calculation (Efron, 1998) and further explained by Bolarinwa (2020) as shown below:

$$N = \frac{(Z\alpha/2 + Z\beta)^2 \, (P1\,(1 - P1) + P2\,(1 - P2))}{(P_1 - P_2)^2}$$

Where:

N = required sample size per subgroup population

Zα/2 = desired significance level at 5% (1.96)

Zβ = power of the test set at 80% (0.84)

P_1 = previous prevalence of *E. coli* carriage in nearby residents (0.29)

P_2 = previous prevalence in distant residents (0.07)

Stool samples were obtained from 100 volunteers who were selected intentionally. The cattle that lived, grazed, and drank in the territories defined above were regarded as the target animal population. The sample size of cattle was determined using the same formula for two sub-populations (adjacent and distant), without prior proportion report, with an effect size of 20% (Kim, 2016). For the pilot study, the animal domain, cattle were selected from areas located near and far from the river and wastewater sources. A total of 84 fecal samples were collected from cattle in both adjacent and distant groups. In addition, 32 milk samples were collected from lactating cattle. Milk sampling was included to strengthen the One Health perspective by assessing potential transmission pathways of antimicrobial-resistant *E. coli* through dairy products.

Inclusion and exclusion criteria for the study

To minimize potential confounding factors and improve comparability between exposure groups, separate inclusion and exclusion criteria were established for human and animal participants. Eligibility was assessed using structured questionnaires and field observations before sample collection.

Inclusion and exclusion criteria for human participants

For the human domain, eligible participants included adults aged between 18 and 67 years who had resided in the study area for at least six months and were willing to participate in the study by providing informed consent. The study targeted wastewater treatment plant workers and community residents living in areas adjacent to and distant from the wastewater treatment plant. To minimize potential confounding factors that could influence the occurrence of antimicrobial-resistant *Escherichia coli*, individuals with a history of employment in slaughterhouses or healthcare facilities, recent hospitalization, international travel, chronic medical conditions, or direct contact with patients during the previous months were excluded from the study. In addition, individuals younger than 18 years and older than 67 years were not included in the study according to Annex 2.

Inclusion and exclusion criteria for animal participants

For the animal domain, cattle raised within the study area and regularly exposed to the environmental conditions of the Akaki River catchment were included in the study. Fecal samples were collected from cattle located in areas adjacent to and distant from the river and wastewater sources, while milk samples were collected from lactating cattle to assess potential transmission pathways of antimicrobial-resistant *E. coli* through dairy products. To reduce potential sources of bias, cattle that had recently been purchased from outside the study area or those with poor body condition were excluded based on Annex 3 from the study. Slaughterhouse and animal farms can be a risk factor for *E. coli* transmission (Allende *et al*., 2025).

3.5. Sample Collection and Transport

Water samples (waste water and river water) were collected aseptically using 1-L sterile polyethylene bottles, stored in cool boxes (4°C), and transported to the laboratory within 6 hours. Human stools and animal fecal and milk samples were collected in sterile containers following aseptic procedures, labeled, and transported under a cold chain to the CVMA microbiology laboratory. All samples were processed within 24 hours of collection. Chain of custody forms and biosafety protocols (PPE use, disinfection, waste disposal) was strictly followed.

3.6. Laboratory Analysis

3.6.1. Heavy metal analysis

Heavy metal analysis was conducted on wastewater and river water samples collected from the Akaki Wastewater Treatment Plant (WWTP) and the Little Akaki River during two distinct sampling periods, representing the dry and rainy seasons. This repeated sampling approach was employed to capture seasonal variations in heavy metal concentrations and to improve the representativeness of environmental conditions.

Wastewater samples were collected during peak operational periods of the treatment plant using grab sampling techniques at predetermined locations, including key treatment stages. Similarly, surface water samples were collected from designated sites along the Akaki River. All samples were collected, preserved, and transported under appropriate conditions to maintain sample integrity prior to analysis(APHA, 2017).

Upon arrival at the laboratory, water samples were prepared using microwave-assisted acid digestion. Briefly, concentrated nitric acid (HNO_3) was added to the samples to facilitate digestion and to maintain metal ions in solution by preventing precipitation and minimizing adsorption losses to container surfaces (Bahiru, 2020). The digestion process was carried

out using a microwave digestion system under controlled temperature and pressure conditions.

Following digestion, the concentrations of selected heavy metals lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), zinc (Zn), and manganese (Mn) were determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) a widely accepted analytical technique for trace metal determination in environmental samples (EPA, 2007). The analysis was carried out at the ACEWM Core Water Laboratory, Addis Ababa University, using standardized analytical procedures, instrument calibration and quality-control measures to ensure the reliability of the results.

Quality assurance and quality control measures were strictly implemented throughout the analysis. These included the use of reagent blanks, duplicate samples and certified referen ce materials to validate analytical performance. Acceptable recovery rates were maintained within the range of 80-120%, ensuring the reliability of the results (APHA, 2017). The concentrations obtained were subsequently compared with the Permissible limits for wastewater and surface water established according to EEPA guidelines are presented in Table 1.

All sample preparation and analytical procedures were conducted in a clean laboratory environment using acid-washed glassware and appropriate personal protective equipment (PPE). The overall methodology was designed to ensure precise quantification of heavy metals and to allow comparison of contamination levels across sampling sites and between seasons.

Table 1: Permissible limits of heavy metals in wastewater and surface water

Heavy Metal	Symbol	Permissible Limit (mg/L)	Reference
Cadmium	Cd	0.01	EEPA (2003)
Lead	Pb	0.10	EEPA (2003)
Chromium	Cr	0.10	EEPA (2003)
Copper	Cu	1.00	EEPA (2003)
Zinc	Zn	5.00	EEPA (2003)
Manganese	Mn	0.20	EEPA (2003)

EEPA = Ethiopian Environmental Protection Authority

3.6.2. Microbiological analysis

Escherichia coli isolation and confirmation from one health domain

Microbiological analysis focused on the isolation and confirmation of *Escherichia coli* from environmental (wastewater and river water), human (stool), and animal (cattle fecal and milk) samples collected from the study area.

All samples were transported to the laboratory under cold chain conditions and processed within 6–12 hours of collection to maintain bacterial viability (APHA, 2017). Upon arrival, samples were handled aseptically following standard microbiological procedures. For water and wastewater samples, appropriate volumes were serially diluted using sterile physiological saline solution. For human stool, cattle fecal, and milk samples, pre-enrichment was carried out in buffered peptone water and incubated at 37°C for 18–24 hours to enhance bacterial recovery (ISO 16649-2, 2001). Following pre-enrichment, samples were inoculated onto MacConkey agar using standard streaking techniques and incubated at 37°C for 18–24 hours.

Colonies exhibiting typical lactose fermenting characteristics (pink colonies) on MacCon key agar were considered presumptive *E. coli* and were sub-cultured onto Eosin Methylene Blue (EMB) agar for further differentiation. After incubation, colonies showing a characteristic metallic green sheen on EMB agar were selected as presumptive *E. coli* isolates. To further assess potential pathogenic strains, selected isolates were streaked onto Sorbitol MacConkey agar (SMAC). Non-sorbitol fermenting colonies (colorless colonies) were considered indicative of possible pathogenic *E. coli* strains (O157:H7), while sorbitol-fermenting colonies were differentiated accordingly.

Presumptive isolates were then purified by sub-culturing on nutrient agar to obtain pure colonies. These isolates were subjected to a series of standard biochemical tests for confir mation, including IMViC tests (Indole production, Methyl Red, Voges–Proskauer, and Citrate utilization). Additional confirmatory tests, including Triple Sugar Iron (TSI) agar reactions (acid/alkaline reaction, gas production, and hydrogen sulfide production) and urease test, were performed to further validate the identification. Furthermore, selecte d isolates were confirmed using Matrix- Assisted Laser Desorption/Ionization Time of- Fl ight Mass Spectrometry (MALDI-TOF) providing species-level identification based on protein profiling. A reference strain, *E. coli* ATCC 25922, was used as a quality control organism throughout the isolation and identification procedures to ensure reliability and accuracy of results. Confirmed isolates were preserved in Brain Heart Infusion broth (OXOIDCM1135B, UK) with 20µl of 25% glycerol and stored for further analysis as mentioned in Annex 7.

3.6.3. Antibiotic susceptibility testing of E.coli isolates and AMR profiling

AMR profiling was conducted to assess the resistance patterns of *E. coli* isolates recovered from the Akaki Wastewater Treatment Plant (WWTP), the Akaki River and associated human and animal domains. This component is critical to understanding the environmental dimension of AMR within the One Health framework.

Antimicrobial susceptibility testing of the confirmed *E. coli* isolates was performed using the Kirby–Bauer disk diffusion method following standard guidelines provided in Annex 7 of this report. A bacterial suspension was prepared from isolated colonies grown on bacteria-identification agar and made up to a sterile saline solution. The solution was then standardized to a 0.5 McFarland turbidity using a turbidmeter and made up to a concentra tion of approximately 1.5×10^8 CFU/mL and evenly spread on to using a sterile bottle-top spreader over the surface of Mueller–Hinton agar (MHA) plates.

Among the tested bacterial isolates, the 13 antimicrobial agents were tested for susceptibility. These antimicrobial agents were grouped into several categories of antimicrobics including β-lactams (cefotaxime (CTX, 30 µg) - Disk diffusion method, ceftazidime (CAZ, 30 µg), amoxicillin–clavulanic acid (AMC, 20/10 µg), ampicillin (AMP, 10 µg)), carbapenems (meropenem (MEM, 10 µg)), sulfonamides (sulfamethoxaz ole (RL, 15 µg)) and co-trimoxazole (COT, 25 µg)), tetracyclines (tetracycline (TET, 30 µg)), aminoglycosides (kanamycin (K, 10 µg)), other (amikacin (AK, 30 µg)), quinolones (nalidixic acid (NA, 30 µg)) and ciprofloxacin (CIP, 5 µg)) and phenicols (chloramphenicol (C, 30 µg)).

The plates were then placed in the incubator at 37°C for 18-24 hours under aerobic conditions. The inhibition zones were measured to the nearest millimeter using a straight edge and results interpreted as mentioned in Table 2 according to (CLSI, 2024). *E. coli* ATCC 25922 was used as the quality control organism for all tested antimicrobials.

Multidrug resistance (MDR) was defined as resistance to at least three categories of antibiotics or resistance to at least one agent in three or more categories (Sweeney *et al.*, 2018). The Multiple Antibiotic Resistance Index (MARI) was calculated by dividing the number of antibiotics to which an isolate was found to be resistant by the total number of antibiotics tested (Ejikeugwu *et al*., 2022). Isolates found to be intermediate or resistant were considered non-susceptible to these antibiotics (Egwu *et al*., 2024; Vogt *et al*., 2022).

Table 2: Breakpoints of disk diffusion methods used to determine antimicrobial susceptibility of *E coli* Kirby-Bauer disk diffusion

Class of antibiotics	**Antibiotics**	**Code**	**Disk potency (µg)**	**Zone Diameter (m m)**		
				S	**I**	**R**
Aminoglycosides	Amikacin	AK	30	≥20	15–19	≤14
	Kanamycin	K	10	≥18	14–17	≤13
Cephalosporin's	Ceftazidime	CAZ	30	≥21	18–20	≤17
	Cefotaxime	CTX	30	≥26	23–25	≤22
Penicillin's	Amoxicillin	AMP	10	≥18	14–17	≤13
	Ampicillin	AMC	10	≥18	14–17	≤13
Carbapenem	Meropenem	MEM	10	≥23	20–22	≤19
Quinolones	Ciprofloxacin	CIP	5	≥26	16–25	≤15
	Nalidixic Acid	NA	30	≥19	14–18	≤13
Sulfonamide	Sulfamethoxazole	RL	15	≥17	13–16	≤12
	Co-trimoxazole	COT	1.25/23.75	≥16	11–15	≤10
Tetracycline	Tetracycline	TE	30	≥15	12–14	≤11
Phenicol	Chloramphenicol	C	30	≥18	13–17	≤12

Source: *CLSI M100-Ed34, Clinical and Laboratory Standards Institute, 2024.*

S = Susceptible; I = Intermediate; R = Resistant. All zone diameters in nearest whole number, mm = millimeter, µg = microgram (disk concentration).

3.6.4. Phenotypic detection of ESBL and carbapenemase

Phenotypic Detection of ESBL and Carbapenemase confirmation of extended-spectrum β- lactamases (ESBL) production was performed using the Double Disc Synergy Test (Wayne, 2020). Inoculum preparation and patching of the bacterial suspension onto Mue ller–Hinton agar using a cotton swab was performed as outlined in Annex 9. Amoxicillin–clavulanic acid (20/10 μg), cefotaxime (30 μg) and ceftazidime (30 μg) discs were placed on the agar surface at the recommended distance apart from each other. After incubation at 37°C for 18–24 hours, clear extensions of the inhibition zones between the amoxicillin–clavulanic acid disc and the two cephalosporin discs, of the test isolates indicated them to be ESBL producers. As quality control strain *Escherichia coli* ATCC 25922 was used.(John-Onwe *et al*., 2022).

Detection of Carbapenemase production was performed by the Modified Carbapenem Inactivation Method (mCIM). The test was performed according to the protocol described in Annex 10 (Van der Zwaluw *et al*., 2015). A meropenem disc (10 μg) was placed into a tube containing tryptone soya broth (sterile, OXOID-CM0129B, UK) which had been inoculated with the test organism. The tube was then incubated for 4 hours at 37°C after which time the disc was removed from the broth and placed on the surface of a Mueller–Hinton agar plate that had been previously inoculated with a meropenem-susceptible *E. coli* ATCC 25922 (used as an indicator organism). After incubation overnight at 37°C at 100% relative humidity the inhibition zone was measured using an electronic caliper. Inhibition zones of 6–15 mm or zones of 16–18 mm containing visible growth were considered to be indicative of carbapenemase production. Inhibition zones of ≥19 mm indicated that the isolate did not produce carbapenemases. The results were interpreted according to the (CLSI, 2024).

3.7. Data Management and Analysis

All data generated from field sampling, laboratory analysis and microbiological assays were systematically recorded, checked for completeness and entered in to Microsoft excel. The cleaned data were then exported to STATA software version 17 for statistical analysis and also to Statistical Package for the Social Sciences (SPSS, version 26) or R software. Heavy metal concentrations were summarized using descriptive statistics including mean, standard deviation, and range to describe the central tendency and determined as frequencies and proportions for categorical variables across sampling sites and waste treatment stages. One way analysis of variance ANOVA and Turkey's post hoc test for pairwise comparisons were employed to evaluate the existence of statistical mean differences in heavy metal concentrations between groups and within groups between WWTP and river sampling sites. The logistic regression model with OR was used to evaluate the strength of association between different variables. For non-normally distributed data, equivalent nonparametric tests such as the Kruskal Wallis test was applied. The Pearson chi-square test ($\chi 2$) or Fisher's exact tests was used to assess the associations between categorical variables, such as sampling source (environmental, human, animal) and the presence of ESBL or carbapenemase-producing *E. coli*.

3.8. Ethical Considerations

The study was conducted to comply with the ethical guidelines relevant to human and animal studies. The clearance for animal study was obtained from the Institutional Animal Research Ethics Committee of the College of Veterinary Medicine and Agriculture Addis Ababa University (Certificate Ref. No. **VM/ERC/08/113/18/2026**). Similarly, clearance for human study was obtained from the Institutional Research Ethics Review Committee of the Aklilu Lemma Institute of Pathobiology, Addis Ababa University (Certificate Ref. No. **ALIHR- IRERC- 005/2018/2026**). Written informed consent was obtained from the residents of the WWTP participating in the study based on Annex 1. All procedures were conducted according to human and animal research ethics. All research procedures was adheres to the ethical principles outlined in the Declaration of Helsinki for human research and in the OIE Terrestrial Animal Health Code for animal welfare (Shrestha & Dunn, 2019). All human subjects, including wastewater treatment plant workers and nearby residents, who were voluntarily recruited for the study were thoroughly explained about the objectives, potential benefits, and possible risks before samples were collected from them based on annex 1. Participants were also clearly informed about their rights to withdraw from the study at any stage without any reason or penalty and that their information would be kept confidential and in anonymous form and would not be shared with any third party except for the purpose of the study. Sampling of animals was done using humane methods of animal handling to minimize stress and discomfort to animals. Non-invasive samples such as fecal specimens were collected by experienced personnel under the close supervision of veterinarian. The biological samples and data obtained from the study were used for research purpose only. Sample collection, transportation, processing, storage and disposal were carried out following the biosafety guidelines of Addis Ababa University and relevant laws of country. Copies of approval certificates are attached in Annex 11 and Annex 12.

4. RESULTS

4.1. Detection of Heavy Metals

Concentrations of the heavy metals studied have been found to vary between sampling points and seasons (Table 3). Cadmium has not been detected at any of the locations studied during either the dry or the rainy seasons. The results for Manganese have been found to be the highest of the heavy metals studied with all river samples exceeding levels found in the wastewater treatment plants. For most of the detectable metals an increase in concentrations were found at all locations during the rainy season compared with the dry season. In addition chromium, copper and zinc which were found at very low levels during the dry season were found at several of the locations during the rainy season. Lead was found at low concentrations during both seasons but was found at higher levels during the rainy season.

Prior to comparing heavy metal concentrations among sampling sources, a tests of normality and homogeneity of variance were performed. The results for the dry seasons are presented in Table 4. The normality and homogeneity tests showed that the majority of the data sets violated the assumptions for parametric testing (Shapiro-Wilk test, $p < 0.05$). Therefore, the non-parametric Kruskal-Wallis test was used to compare the heavy metals from the different sampling sources during the dry season ($p < 0.05$) significant differences were found at trickling filter, clarifier outlet, effluent, UASB inlet, UASB outlet and all river sampling locations. Similarly, the results of the normality, homogeneity and Kruskal-Wallis tests for the rainy season are presented in Table 5. The Shipro- Wilk test again indicated significant differences from normality ($p < 0.05$) for all sampling sources. Accordingly, nan-parametric statistical analysis was employed. As shown in Table 5, significant differences in heavy metal concentrations were detected among most sampling sources during the rainy season ($p< 0.05$). However, no statistically significant differences was observed at the clarifier outlet ($\chi^2 = 10.381$, $p = 0.1095$). Overall, heavy metal concentrations tended to be higher during the rainy season than during the dry season,

possibly due to increased runoff and contaminant mobilization associated with rainfall events.

Table 3: Heavy metals concentration (ppm) in different sampling points during dry and rainy season

Sample sites	Season	Cd	Pb	Cr	Cu	Mn	Zn
River upstream	Dry	BDL	0.04±0.03[a]	BDL	BDL	0.41±0.01[a]	BDL
	Rainy	BDL	0.07±0.13[a]	0.00±0.03[a]	0.05±0.04[a]	1.43±0.01[a]	0.18±0.01[a]
River midstream	Dry	BDL	0.02±0.03[a]	BDL	BDL	1.14±0.01[a]	BDL
	Rainy	BDL	0.07±0.13[a]	0.03±0.03[a]	0.05±0.04[a]	1.45±0.01[a]	0.20±0.01[a]
River downstream	Dry	BDL	0.02±0.03[a]	BDL	BDL	1.16±0.01[a]	BDL
	Rainy	BDL	0.08±0.14[a]	0.04±0.02[a]	0.11±0.05[a]	1.45±0.01[a]	0.19±0.01[a]
UASB inlet	Dry	BDL	0.02±0.02[b]	BDL	0.07±0.02[a]	0.16±0.00[c]	BDL
	Rainy	BDL	0.03±0.05[b]	0.01±0.01[b]	0.07±0.01[a]	0.45±0.01[b]	0.19±0.01[a]
UASB outlet	Dry	BDL	BDL	BDL	BDL	0.19±0.01[bc]	BDL
	Rainy	BDL	0.09±0.10[a]	BDL	0.08±0.04[a]	0.47±0.01[b]	0.15±0.01[b]
Trickling filter	Dry	BDL	0.003±0.006[b]	BDL	BDL	0.14±0.01[c]	BDL
	Rainy	BDL	0.20±0.13[a]	0.04±0.02[a]	0.04±0.03[b]	0.67±0.01[b]	0.10±0.01[c]
Clarifier outlet	Dry	BDL	0.04±0.03[b]	BDL	BDL	0.23±0.00[bc]	BDL
	Rainy	BDL	0.14±0.09[ab]	0.03±0.03[a]	0.03±0.03[b]	0.46±0.00[b]	0.13±0.00[bc]
Effluent	Dry	BDL	0.02±0.03[b]	BDL	BDL	0.19±0.00[bc]	BDL
	Rainy	BDL	0.29±0.08[a]	0.01±0.01[ab]	0.02±0.03[b]	0.50±0.01[b]	0.10±0.01[c]

BDL = Below Detection Limit. Values are mean ± SD (ppm). Mn values exceed WHO provisional guideline (0.08 mg/L). Superscript letters within the same column indicate significant differences between seasonal values at the same site (Kruskal-Wallis, $p < 0.05$). † ETHEPA effluent discharge limit for Mn = 5,000 µg/L (5.0 mg/L). ‡ WHO 2021 provisional guideline value for Mn in drinking water = 0.08 mg/L (revised from former 0.4 mg/L). NR = Not reported. UASB = Up flow-Anaerobic sludge blanket reactor, mg/L = Milligram per liter, ppm =Part per million

Table 4: Kruskal-Wallis H test results- heavy metal concentrations across sampling sites during the dry season.

Sampling Source	Barlett p-value	Shapiro p-value	X^2	P- value
TF	0.389	0.00004	14.415	0.0132*
Clarifier outlet	0.398	0.00021	14.850	0.0110*
Effluent	<0.001	0.00037	14.500	0.0127*
UASB inlet	NR	0.00011	14.875	0.0109*
UASB outlet	NR	0.00011	16.875	0.0047*
River Upstream	0.066	0.00003	14.776	0.0114*
River Midstream	0.066	0.00001	14.776	0.0114*
River Downstream	0.066	0.00001	14.776	0.0114*

*H = Kruskal-Wallis test statistic. * = significant at p < 0.05. NR = Bartlett's test not reported for UASB sites. TF = Trickling filter, UASB = Up flow Anaerobic sludge blanket reactor,* X^2 *=Chi-square*

Table 5: Kruskal- Wallis H test results- heavy metal concentrations across sampling sites during the rainy season.

Sampling Source	Barlett p-value	Shapiro p-value	X^2	p- value
TF	0.001	0.000112	19.05	0.0040*
Clarifier outlet	0.026	0.000268	10.381	0.1095ns
Effluent	0.008	0.001077	17.381	0.0069*
UASB inlet	0.029	0.000155	17.733	0.0069*
UASB outlet	0.003	0.005087	17.098	0.0089*
River Upstream	0.004	0.000001	13.929	0.0304*
River Midstream	0.008	0.000001	13.919	0.0306*
River Downstream	0.003	0.000002	14.562	0.0240*

*H = Kruskal-Wallis test statistic; * = significant at p < 0.05; ns=not significant; NR = Bartlett's test not reported for UASB sites; TF = Trickling filter, UASB = Up flow Anaerobic sludge blanket reactor,* X^2 *= Chi-square*

4.2. Isolation and confirmation of *Escherichia coli* isolates

A total of 232 samples from environmental (wastewater and surface water), human (stool) and animal (faeces and milk) sources were processed for isolation and confirmation of pathogenic *E. coli* (Table 6). Out of 232 samples processed, 31 (13.4%) were confirmed based on cultural, biochemical tests and MALDI-TOF identification.

Detections of pathogenic *E. coli* were generally found to vary depending upon the source from which the samples were collected. For the river water samples collected from all three sections (upstream, midstream and downstream) during the two seasons, all samples from all sections during both the dry and the wet season tested positive for pathogenic *E. coli*, giving an overall occurrence of 100% for all the river water samples collected. In wastewater samples collected during the two seasons, there were higher occurrences during the wet season (80.0%) than in the dry season (40.0%). For animal derived samples, there were higher occurrences in cattle feces collected from adjacent areas (11.9%) than from very distant areas (7.1%). In addition, there were higher occurrences in milk samples collected from adjacent areas (25.0%) than from very distant areas (6.3%). Human participants who resided near the WWTP had a higher occurrence of pathogenic *E. coli* (8.0%) than did individuals from very distant areas (4.0%).

The presence of pathogenic *E. coli* was found to be associated with the sampling source (Pearson $\chi^2 = 70.2$, $P < 0.001$). The results of the multivariate Firth's penalized logistic regression analysis are presented Table 7.Using Firth's penalized logistic regression, it was found that the sampling source and the distance from the wastewater treatment plant were significant for the occurrence of *E. coli*. Comparing with human samples, the odds for the occurrence of *E. coli* from wastewater samples (OR = 21.00, 95 % CI: 4.72–93.40, $P < 0.001$) and from river water samples (OR = 21.49, 95 % CI: 7.64–4673.87, $P < 0.001$) were significantly higher than those from other sources. The odds for the occurrence of *E. coli* from cattle feces (OR = 1.62, 95 % CI: 0.55–4.71, $P > 0.05$) and from milk samples (OR = 2.91, 95 % CI: 0.85–9.91, $P > 0.05$) were higher than those from human samples; however, but not significant. In contrast, samples obtained from adjacent areas to the point source (OR = 0.051, 95 % CI: 0.01–0.18, $P < 0.001$) and from very distant areas (OR = 0.023, 95 % CI: 0.01–0.09, $P < 0.001$) had significantly lower odds for the occurrence of *E. coli* than those from point-source environmental samples. Firth's penalized regression model was applied for the analysis of the river water group in which all samples tested positive for *E. coli* and complete separation occurred in this group.

Table 6: Distribution of *Escherichia coli* across sampling sources

Sampling sources	Positive	Total (n)	%
WWTP (dry season) Reference	2	5	40
WWTP (rainy season)	4	5	80
River water (US, MS, DS ; Dry, Rainy)	6	6	100
Adjacent Human	4	50	8
Distant Human	2	50	4
Adjacent Cattle	5	42	11.9
Distant Cattle	3	42	7.1
Milk Adjacent	4	16	25
Milk Distant	1	16	6.3

Key: Pearson χ^2 *(8)* $\approx$ *70.23, P= 0.000 or* 4.43×10^{-12}, *WWTP= Wastewater treatment plant,* US=Upstream, MS= Midstream, DS= Downstream

Table 7: Multivariate logistic regression- Firth's method for *E. coli* occurrence across variables

Variables	Level	OR (Firth)	95% CI	P value
Sampling sources	Human	Ref	–	_
	Cattle	1.62	0.55 -4.71	0.375
	Milk	2.91	0.85 -9.91	0.1
	Water	21.49	7.64-4673.87	0.001
	WWTP	21.00	4.72-93.40	0.001
Distance from WWTP	Point source (Environmental)	Ref		
	Adjacent	0.051	0.01-0.18	0.001
	Distant	0.023	0.01-0.09	0.001

***Key**: OR- odds ratio (Firth's penalized), CI- confidence interval, Ref- Reference category, WWTP wastewater treatment plant.*

4.3. Antimicrobial Susceptibility Test

Resistance data of 31 pathogenic *E. coli* isolates were determined by using antimicrobial susceptibility tests and the results are presented in Table 8. High percentages of resistance to Ampicillin (93.5%), Tetracycline (74.2%), Sulphamethoxazole (64.5%), and Cefotaxi me (61.3%) were determined among the tested *E. coli* isolates. In addition, the percentages of resistance to ceftazidime (45.2%) and co- trimoxazole (48.4%) were found to be consi derable among the study *E. coli* isolates. These results indicated that there is a high level

of resistance to the most of the β-lactam antibiotics and other commonly used antimicrobial agents among *E. coli* isolates in the study area.

Isolates remained susceptible to ciprofloxacin (93.5%), chloramphenicol (93.5%), meropenem (90.3%) and aminoglycosides (such as amikacin, 87.1% and kanamycin, 80.6%) with the exception of a few isolates showing intermediate meropenem susceptibility. In contrast, high numbers of isolates were resistant to third generation cephalosporins (ciprofloxacin 75.6%, cefotaxime 96.6% and ceftazidime 96.6%) as mentioned in the (Table 8), which could indicate the presence of pathogenic *E. coli* isolates producing Extended-Spectrum β-lactamases (ESBL). The results obtained in this study show a considerable number of resistant isolates from environmental, human and animal sources, which could pose public health risks, and which are most likely to be transmitted through the One Health interconnection between humans, animals and the environment.

Table 8 : Antimicrobial susceptibility profile of *Escherichia coli* (N = 31)

Antimicrobial agents	**Antimicrobial classes**	**R (%)**	**I (%)**	**S (%)**
Tetracycline	Tetracycline	23 (74.2%)	2 (6.5%)	6 (19.4%)
Kanamycin	Aminoglycoside	0 (0%)	6 (19.4%)	25 (80.6%)
Ampicillin	Penicillin	29 (93.5%)	2(6.5%)	0 (0%)
Co-trimoxazole	Sulfonamide	15 (48.4%)	1 (3.2%)	15 (48.4%)
Sulphamethoxazole	Sulfonamide	20 (64.5%)	0 (0%)	11 (35.5%)
Nalidixic acid	Quinolone	4(12.9%)	11 (35.5%)	16 (51.6%)
Ciprofloxacin	Fluoroquinolone	0 (0%)	2 (6.5%)	29 (93.5%)
AMC	Penicillin	2 (6.5%)	10 (32.3%)	19(61.3%)
Amikacin	Aminoglycoside	1 (3.2%)	3 (9.7%)	27 (87.1%)

Chloramphenicol	Phenicol	1(3.2%)	1 (3.2%)	29 (93.5%)
Cefotaxime	Cephalosporin	18 (58.1%)	5 (16.1%)	8 (25.8%)
Ceftazidime	Cephalosporin	14 (45.2%)	7 (22.6%)	10 (32.3%)
Meropenem	Carbapenem	0 (0%)	3 (9.7%)	28 (90.3%)

***Key**: N= Total E.coli isolates, AMC= Amoxicillin clavulanate, R- resistant, I= Intermedi ate S= Susceptible*

4.4. Antimicrobial Resistance Profile of *E. coli*

Diversity in the level of antimicrobial resistance among pathogenic *E. coli* from wastewater, surface water, stool, fecal and milk samples was observed (Table 9). High resistance rates were recorded for ampicillin and tetracycline followed by sulphamethoxa zole, co-trimoxazole, cefotaxime and ceftazidime. Among tested antibiotics, cefotaxime ($\chi^2 = 17.45$, $P = 0.042$) showed significant variation while resistance to sulphamethoxazole was on border line ($P = 0.052$). However, no significant variation was observed among tested antibiotics for other samples types ($P > 0.05$). The isolates were highly susceptible to ciprofloxacin, kanamycin, amikacin, chloramphenicol and meropenem.

Table 9: Antimicrobial resistance profile of *E. coli* isolates across sampling sources

Antimicrobials	WWTP	AH	DH	AC	DC	AM	DM	US LAR	MS LAR	DS LAR	Overall	*χ2, P value*
Tetracycline	100(6/6)	50(2/4)	50(1/2)	100(5/5)	66.6(2/3)	25(1/4)	0(0/1)	100(2/2)	100(2/2)	50(1/2)	64.5(20/31)	χ^2 =14.42 0.108
Kanamycin	0(0/6)	0(0/4)	0(0/2)	0(0/5)	0(0/3)	0(0/4)	0(0/1)	0(0/2)	0(0/2)	0(0/2)	0 (0/31)	NA NA
Ampicillin	100(6/6)	75(3/4)	50(1/2)	100(5/5)	100(3/3)	100(4/4)	100(1/1)	100(2/2)	100(2/2)	100(2/2)	93.5(29/31)	10.29, 0.328
Co-trimoxazole	0(0/6)	75(3/4)	50(1/2)	80(4/5)	66.6(2/3)	100(2/4)	100(1/1)	50(1/2)	50(1/2)	0(0/2)	48.4(15/31)	12.11, 0.207
Sulphamethoxazole	16.6(1/6)	100(4/4)	100(2/2)	100(5/5)	66.6(2/3)	75(3/4)	100(1/1)	50(1/2)	50(1/2)	0(0/2)	64.5(20/31)	16.80, 0.052
Nalidixic acid	33.3(2/6)	25(1/4)	50(1/2)	0(0/5)	0(0/3)	0(0/4)	0(0/1)	0(0/2)	0(0/2)	0(0/2)	12.9(4/31)	8 .01, 0.533
Ciprofloxacin	0(0/6)	0(0/4)	0(0/2)	0(0/5)	0(0/3)	0(0/4)	0(0/1)	0(0/2)	0(0/2)	0(0/2)	0(0/31)	NA NA
AMC	0(0/6)	0(0/4)	0(0/2)	0(0/5)	0(0/3)	0(0/4)	0(0/1)	50(1/2)	50(1/2)	0(0/2)	6.4(2/31)	14.43, 0.108

Amikacin	0(0/6)	0(0/4)	0(0/2)	20(1/5)	0(0/3)	0(0/4)	0(0/1)	0(0/2)	0(0/2)	0(0/2)	3.2(1/31)	5.37, 0.801
Chloramphenicol	0(0/6)	0(0/4)	0(0/2)	0(0/5)	0(0/3)	0(0/4)	0(0/1)	50(1/2)	0(0/2)	0(0/2)	3.2(1/31)	14.98, 0.091
Cefotaxime	83.3(5/6)	100(4/4)	50(1/2)	20(1/5)	33.3(1/3)	100(4/4)	100(1/1)	100(1/2)	0(0/2)	0(0/2)	58(18/31)	17.45, 0.042
Ceftazidime	33.3(2/6)	100(4/4)	50(1/2)	40(2/5)	33.3(1/3)	75(3/4)	100(1/1)	50(1/2)	50(1/2)	50(1/2)	54.8(17/31)	6.98, 0.640
Meropenem	0(0/6)	0(0/4)	(0/2)	0(0/5)	0(0/3)	0(0/4)	0(0/1)	0(0/2)	0(0/2)	0(0/2)	0(0/31)	NA NA

***Key**: χ^2 = Pearson chi-square test; P < 0.05 indicates statistically significant association between sampling source and antimicrobial resistance pattern; WWTP-Wastewater treatment plant, AH (Adjacent human), DH (Distal human), AC (Adjacent cattle), DC (Distal cattle), AM (Adjacent milk), DM (Distal milk), USLAR (Upstream little Akaki river), MSLAR (Midstream little Akaki river), DSLAR (Downstream little Akaki river), AMC-Amoxicillin clavulanic acid , NA (Not applicable)*

4.5. Antimicrobial Resistance Patterns of *E. coli* Isolates

Among 31 confirmed pathogenic *Escherichia coli* isolates tested for antimicrobial susceptibility, 87.1% (27/31) of the tested isolates were found to be multidrug resistant (MDR) defined by resistance to drugs belonging to three or more different classes. The detailed Antimicrobial resistance patterns and Multiple Antibiotic Resistance Index (MARI) values of the MDR *E. coli* isolates are presented in Table 10. The common resistance patterns among tested isolates included Ampicillin (AMP) in combination with Sulphamethoxazole (RL), Co-trimoxazole (COT), Tetracycline (TE), Cefotaxime (CTX), and Ceftazidime (CAZ). Among the tested isolates, the resistance pattern of AMP–COT–RL was observed in 14.8% (4/27) of MDR isolates followed by AMP–RL–CTX (11.1% or 3/27), AMP–TE–RL–CAZ (11.1% or 3/27), and AMP–TE–RL–CTX (11.1% or 3/27). The Multiple Antibiotic Resistance Index (MARI) of tested MDR isolates ranged from 0.23 to 0.38 and were found to be greater than 0.31 for majority of the tested isolates. The resistance to tested drugs was found to be maximum for drugs belonging to the classes of β-lactams, sulfonamides, and tetracycline. None of the tested isolates showed complete resistance to meropenem; however, some of the tested isolates showed intermediate level of susceptibility to meropenem.

Table 10: Antimicrobial resistance pattern of MDR Escherichia coli isolates (N=27)

AMR patterns	Sampling sources	MARI	Isolate n (%)
AMP, COT, RL	Human, Cattle/Milk	0.23	4 (14.8)
AMP, RL, CTX	Milk	0.23	3 (11.1)
AMP, TE, RL	Cattle feces	0.23	2 (7.4)
AMP, TE, CAZ	River water	0.23	1 (3.7)
AMP, CAZ, CTX	Milk	0.23	1 (3.7)
AMP, TE, NA, CTX	WWTP	0.31	1 (3.7)
AMP, TE, RL, CAZ	Human, Cattle feces	0.31	3 (11.1)
AMP, TE, RL, CTX	WWTP, River water	0.31	3 (11.1)
AMP, COT, RL, CAZ	Cattle feces,	0.31	2 (7.4)
AMP, COT, RL, CTX	Milk	0.31	2 (7.4)
AMP, TE, COT, RL	Cattle feces	0.31	2 (7.4)
AMP, TE, COT, AK, RL	Cattle feces	0.38	1 (3.7)
AMP, TE, RL, CAZ, CTX	Human, Cattle feces, Milk	0.38	2 (7.4)

***Key**: AMP- Ampicillin, TE30- Tetracycline, RL- Sulphamethoxazole, CTX- Cefotaxime, COT-Cotrimoxazole, CAZ – Ceftazidime, NA - Nalidixic acid; AK- Amikacin; MARI - Multiple Antibiotic Resistance Index.*

$$\text{MARI} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics tested}}$$

A MARI value greater than 0.20 was considered indicative of exposure to environments where antibiotics are frequently used.

4.6. Phenotypic Detection of ESBL and Carbapenemase-Producing *E. coli*

The double-disc synergy test (DDST) was carried out for phenotypic detection of Extended-spectrum β-lactamases (ESBL) in 31 confirmed pathogenic *Escherichia coli* isolates. Moreover, all non-susceptible isolates to meropenem (intermediate were further screened for production of different carbapenemases by employing the modified carbapenem inactivation method (mCIM). Out of 31 isolated *E. coli* strains, 14 (45.2%) of them were ESBL producers. 17 (54.8%) were ESBL negative (Figure 4). ESBL producing isolates were found in all sampling domains. The distribution of the ESBL producing isolates within the sampling domains were as follows: wastewater treatment plant samples 3/6 (50.0%), river water samples 1/6 (16.7%), human stool samples 5/6 (83.3%), milk samples 2/5 (40.0%), cattle fecal samples 3/8 (37.5%). The highest percentage of ESBL producing E. coli strains were found from human stool samples followed by wastewater and animal samples. This shows that *E. coli* resistant to β-lactam antibiotics are spreading in the environment and between humans and animals and therefore require serious attention.

All three meropenem non-susceptible isolates tested negative for production of carbapenemases by mCIM; therefore, no phenotypically detectable carbapenemase-producing *E. coli* was identified among study isolates. None of the tested isolates co-produced ESBL enzymes and carbapenemases.

The study established that there is high prevalence of ESBL producing pathogenic *E. coli* in the study area and implicated wastewater systems and environment interfaces as critical reservoirs and pathways for spread of clinically relevant antimicrobial resistant bacteria within a One Health framework.

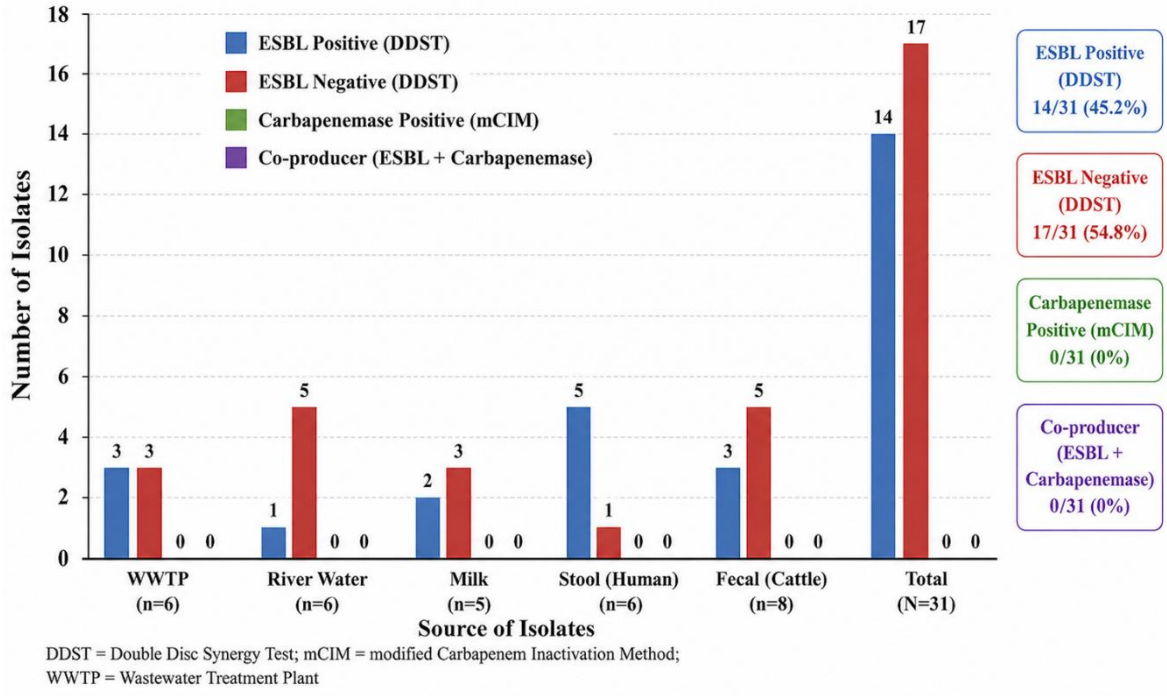


Figure 4: Phenotypic detection of ESBL and carbapenemase production by *E. coli*

4.7. Heavy Metal–AMR Co-selection Analysis

An exploratory descriptive comparison of heavy metal occurrence and antimicrobial resistance indicators across environmental sampling sites is presented in Table 11. The analysis compared the distribution of detected heavy metals (Pb, Cr, Cu, Zn, and Mn) with the occurrence of multidrug-resistant (MDR) and ESBL-producing *Escherichia coli* isolated from wastewater treatment stages and river water samples collected during both dry and rainy seasons.

Heavy metal analysis revealed persistent environmental contamination, with manganese (Mn) consistently detected at all sampling locations during both seasons, whereas lead (Pb), chromium (Cr), copper (Cu), and zinc (Zn) were detected more frequently during the rainy season. Antimicrobial susceptibility testing showed a high prevalence of MDR (87.1%) and ESBL-producing *E. coli* (45.2%). Descriptively, sites with relatively greater heavy metal

occurrence, particularly downstream river locations and selected wastewater treatment units, also tended to show the presence of MDR and ESBL-positive isolates.

However, no isolate-level statistical correlation or molecular analysis was performed. Therefore, the observed relationship represents an exploratory and descriptive comparison only and should be considered indicative of a possible association rather than evidence of a causal relationship between heavy metal contamination and antimicrobial resistance.

Table 11: Exploratory descriptive comparison of heavy metal occurrence and AMR

Sampling site	Heavy metal occurrence	MDR detected	ESBL detected
River upstream	Mn detected	Yes	Yes
River midstream	Mn, Pb, Cr, Cu, Zn detected	Yes	Yes
River downstream	Mn, Pb, Cr, Cu, Zn detected	Yes	Yes
UASB inlet	Mn, Pb, Cu detected	Yes	Yes
UASB outlet	Mn, Pb, Cu, Zn detected	Yes	Yes
Trickling filter	Mn, Pb, Cr, Cu, Zn detected	Yes	Yes
Clarifier outlet	Mn, Pb, Cr, Cu, Zn detected	Yes	Yes
Final effluent	Mn, Pb, Cr, Cu, Zn detected	Yes	Yes

Key: Mn = Manganese; Pb = Lead; Cr = Chromium; Cu = Copper; Zn = Zinc

5. DISCUSSION

Wastewater treatment plants have crucial roles to protect environmental health and human health. However, they can also act as a reservoir and a pathway for dissemination of antimicrobial-resistant microorganisms and chemical pollutants into the environment when they are not removed completely during treatment processes (Uluseker *et al.*, 2021). Persist ent and bio- recalcitrant heavy metals exert long-term selective pressure on microbial populations even in the absence of exposure to antibiotics can coexist and co- selected with resistance determinants to other classes of antimicrobials (Gillieatt and Coleman, 2024). The current study utilized a One Health approach to investigate levels of heavy metal contamination and antimicrobial-resistant *E. coli* in wastewater and river environments of the Akaki WWTP catchment.

Here the major findings of this study are presented and discussed in four interconnected themes, i.e. distribution of environmental chemicals including heavy metals and their environmental implication; antimicrobial susceptible profile and its determinants; occurrence of MDR and ESBL-producing *E. coli* isolates and correlation between environmental chemical pollution and antimicrobial resistant bacteria.

The results of heavy metal distribution in wastewater treatment plant and river catchment showed significant spatial and seasonal variations. Among the investigated heavy metals, Manganese (Mn) was the most consistently detected and occurred at higher concentrations than the other metals throughout the study period. Manganese is naturally occurring element in the Earth's crust and can also be introduced into environment from industrial sources such as mining, refining, and from urban runoff. Within the context of wastewater treatment, manganese can occur in both dissolved and particulate form. Under anaerobic conditions found in many wastewater treatment processes such as UASB reactors, insoluble manganese compounds can be reduced to more soluble forms and thus increase the mobility of metal and its persistence in the environment (Abd-El-Kader *et al.*, 2020). The Presence of high concentrations of Mn in treated wastewater indicates that the metal is not being completely

removed from the wastewater and thus more efficient removal strategies for dissolved metals are needed (Abd-El-Kader *et al.,* 2020; Hu *et al.,* 2018).

In contrast, some of the highest metal concentrations were found during the rainy season and are thought to have been picked up in surface runoff from urban, industrial and agricultural sources. Heavy metals in wastewater-impacted surface waters have been found to display similar seasonal patterns of distribution and mobility (Shuralla *et al.*, 2024). The persistence of Mn throughout the treatment process and across river sampling locations suggests that the existing treatment system may have limited capacity to remove dissolved manganese effectively, particularly under anaerobic treatment conditions. The other heavy metals (Pb, Cr, Cu, and Zn) were detected only on few occasions, mainly during the rainy season and apparently originate from different diffuse environmental sources that release these metals in an episodic manner. The increase of Pb concentrations from upstream to the downstream sites of the Akaki River likely reflects the contribution of wastewater effluent and urban runoff (Tesfaye *et al*., 2019)

On the other hand, Cd was not detected in all samples studied during both study periods. This result indicated that there might be a very low level of Cd contamination in the study area. Compared with earlier studies on heavy metal pollution in the Akaki catchment (Girma *et al.*, 2023). The present study generally showed lower levels of all heavy metals. This could be due to several reasons including the difference in time of sampling, nature of wastewater and industrial pollution load etc. However, persistence of Mn in all wastewater and river samples studied and occurrence of other metals in a seasonal manner could pose ecological risk through bio-accumulation and potential long-term ecological effects (EEPA, 2003).

The findings of this study demonstrate that the effluent discharged from wastewater treatment plants into the Akaki River through its tributaries during dry and rainy seasons contributes to the occurrence and distribution of heavy metals in the study area. Manganese (Mn) was detected in all the stages of wastewater treatment plants and sampling points along the Akaki River during the two seasons. This implies that there are environmental sources of contamination of Mn and that the existing systems for removing polluting substances from

wastewater are not effective. Problems related to water quality and health risk to animals and human beings through water have been reported in the area (Abosse et al., 2025).

The recovery of pathogenic *E. coli* from environmental, human and animal sources highlights the interconnected nature of antimicrobial resistance transmission within the study area and supports the relevance of the One Health approach for understanding environmental dissemination pathways. The findings reveal the extent of fecal contamination of samples and the spread of antimicrobial resistant bacteria through various environmental interfaces and animal–human contact in the study area. *E. coli* can be used as an ideal indicator organism for the assessment of the environmental dissemination of AMR in wastewater-impacted ecosystems (Berendonk *et al*., 2015; Uluseker *et al.*, 2021). The widespread resistance to commonly used antimicrobials such as ampicillin, tetracycline and sulfonamides likely reflects long-term selective pressure arising from their extensive use in both human and animal health sectors. Similarly, resistance rates to third generation cephalosporins (such as ceftriaxone, cefotaxime and ceftazidime) were ranging from 82% to 94% (Emurotu *et al*., 2024; Kotlarska *et al.*, 2015). Wastewater receiving environments harbor the largest amount of antibiotic resistant bacteria including *E. coli* due to the input of resistant bacteria and resistance genes from human, healthcare, agricultural and livestock sources and continue to select resistant bacteria in these environments (Uluseker *et al.*, 2021). Furthermore, susceptibility test revealed that ciprofloxacin (60%), amikacin (69%) and meropenem (81%) showed high susceptibility percentages as these are less used and therefore less expected to be present in the environment (Bessa *et al*., 2014).

The MARI values for MDR *E. coli* isolates in this study (0.23-0.38) reflects exceeding 0.20 suggests that the study environment is subjected to considerable antimicrobial selective pressure. Similar findings have been reported in wastewater-associated environments where continuous inputs of antimicrobial residues, resistant bacteria and resistance genes contribute to the maintenance and dissemination of resistance(La Rosa *et al*., 2025; Papajová *et al*., 2022). According to (Khan *et al*. (2015), MARI values above 0.20 are indicative of bacterial populations originating from environments with frequent antimicrobial exposure.

The study found the prevalence of *E. coli* strains from environmental samples which were *E. coli* strains from human samples tested for production of Extended-spectrum β-lactamases (ESBL) to be lower than previously found by (Ahmad Zahra *et al.*, 2025) who found that 63.64% of *E. coli* isolates from various samples throughout Pakistan produced ESBL and were clinically relevant β-lactamase producers. None of the tested *E. coli* strains produced any tested for production of carbapenemases however, a small number of environmental isolates had intermediate Meropenem values indicating the possible presence of the organisms which are classified as Carbapenem-resistant Enterobacteriaceae (CRE) in the environment. Continued surveillance for CRE in the environment and animals is required as reports of CRE from around the globe are increasing (Rizi *et al*., 2023)The prevalence of ESBL-producing *E. coli* strains in this study found higher percentages of *E. coli* strains from human samples that produced ESBL compared to percentages found in environmental and animal samples. The relatively higher occurrence of ESBL-producing isolates among human samples may reflect greater exposure to antimicrobial agents through clinical treatment, self-medication practice and community antibiotic use. Human-associated environments are recognized reservoirs of ESBL-producing Enterobacteriaceae, which can subsequently disseminate into wastewater systems and surrounding environmental compartments through fecal contamination (Huijbers *et al.*, 2020). The findings of the presence of resistant *E. coli* strains in all three compartments in this study support the interconnection of resistance in environmental, animals and human health in the One Health context.

An exploratory comparison further suggested that sampling locations with relatively higher heavy metal contamination, particularly manganese (Mn), tended to exhibit higher frequencies of multidrug-resistant (MDR) and ESBL-producing *E. coli*. Similar co-occurrence patterns have been reported in wastewater-impacted environments where heavy metals and antimicrobial-resistant bacteria persist within the same ecological niches (Gillieatt and Coleman, 2024; Krzeminski *et al.,* 2019). Previous studies have shown that heavy metal resistance genes and antimicrobial resistance genes may be co-located on shared mobile genetic elements, such as plasmids, transposons and integrons, allowing heavy metal exposure to indirectly maintain antimicrobial resistance through co-selection mechanisms (Hazra *et al.,* 2022; Niu *et al*., 2016). However, because no isolate-level statistical

correlation analysis or molecular characterization of resistance determinants was performed, the observed relationship should be considered exploratory and indicative only rather than evidence of a causal association between heavy metal contamination and antimicrobial resistance

Collectively, the findings suggest that wastewater and river ecosystems within the Akaki catchment may function as environmental reservoirs where chemical pollutants and antimicrobial-resistant bacteria coexist. Such environments may facilitate the persistence and dissemination of resistance across human, animal and environmental interfaces, emphasizing the importance of integrated surveillance and wastewater management under a One Health framework.

Study limitations

This study has some limitations that should be considered when interpreting the findings. Molecular characterization of antimicrobial resistance genes was not performed due to resource limitations; therefore, resistance mechanisms were assessed only using phenotypic methods. The study also relied on culture-based bacterial detection and a relatively small number of confirmed *E. coli* isolates (n = 31), which may have limited the detection of some associations. In addition, the purposive sampling approach focused on high-risk areas surrounding the WWTP and may limit the generalizability of the findings. Furthermore, the assessment of heavy metal–AMR relationships was exploratory and did not include isolate-level molecular characterization or statistical correlation analyses. Despite these limitations, the study provides important baseline evidence on heavy metal contamination and antimicrobial-resistant *E. coli* within a One Health framework and supports the need for future studies incorporating larger sample sizes, molecular approaches, and longitudinal designs.

6. CONCLUSIONS AND RECOMMENDATIONS

Heavy metal concentrations varied by location and season. Manganese (Mn) was consistently detected across sites and seasons of the study, while Lead (Pb), Chromium (Cr), Copper (Cu) and Zinc (Zn) were mainly detected during the rainy season. Cadmium (Cd) remains below the detection limit throughout the study period, suggesting seasonal influences on contaminant distribution and the continued impact of wastewater discharge on environmental quality. Multi-drug resistant (MDR) and extended spectrum β-lactamase (ESBL) - (producing E. coli were detected in environmental, human and animal samples. These findings indicate the circulation of clinically important resistant bacteria across interconnected ecosystems and underscore the need for integrated One Health strategy to mitigate antimicrobial resistance. The observed coexisting of heavy metals contamination and resistant *E. col* suggests a potential ecological link. However, in the absence of molecular characterization and statistical correlation analysis, this relationship remains preliminary and requires confirmation through larger-scale studies incorporating molecular and analytical approaches.

Based on the results and implications of this study, the following recommendations were developed

- Establish integrated one health surveillance systems to routinely monitor antimicrobial resistance, heavy metal contamination, and resistant bacteria in environmental, human, animal, and food production systems.
- Improve wastewater treatment and strengthen routine monitoring of effluent quality to ensure effective removal of chemical contaminants, including heavy metals and antimicrobial-resistant bacteria, before discharge into the environment.
- Promote responsible antimicrobial use and stewardship in both human and animal health sectors to reduce the emergence and spread of antimicrobial resistance.
- Enhance public awareness of antimicrobial resistance, environmental contamination pathway, and associated public health risks.

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8. APPENDICIES

Annex 1: Participant informed consent form

PARTICIPANT INFORMED CONSENT FORM

Name/ID of study Participant: _____ Age: ____Sex _____

Study investigator name: **Melaku Taye** Study location(s): Akaki Wastewater Treatment Plant and Akaki River Catchment.

I agree to be studied with regard to the title "Heavy Metal Contamination and Antimicrobial Resistance of *E .coli* in the Akaki Wastewater Treatment Plant and Akaki River Catchment:" Akaki river and Akaki wastewater treatment plant, Akaki Kality sub city, Addis Ababa, Ethiopia and provide information as a resident of the Akaki river catchment studying Heavy Metal Contamination and Antimicrobial Resistance of *E. coli* in the study area and/or provide stool samples of his/her cattle for fecal sample analysis.

For the study on the above research topic, the researcher asked information from residents who live in Akaki river catchment and wastewater treatment plant area on the following issues of animals such as watering and grazing habits, were the animals ever sick, treated and from where were they purchased in the past months. In addition to the above information, the researcher also asked for the stool samples of the cattle along with their fecal samples. There are no serious risks associated with the research procedures. The researcher will provide no direct benefits to participants. Participants have the right to be informed about their participation. All results from the study will be kept confidential. Sufficient time was given to participants to consider whether or not to participate in the study. It is on the above noted circumstances that I am willing to give my informed consent to participate in and to cooperate with all aspects of the study.

Participant Name: _____Signature: _____Date: _____

Investigator Name: _____Signature: _____ Date: _____

Annex 2: Semi-Structured Questionnaire for Human Participants

Section I: Demographic Information

1. What is your age category?

A. Young (<18 years)

B. Adult (18–67 years)

C. Older adult (>67 years)

2. How far are you from the wastewater treatment plant (WWTP)?

A. Greater than 1000 m

B. Less than 1000 m

C. WWTP worker

Section II: Occupational and Travel History

3. Slaughterhouse work or visit.

A. Yes, worked there

B. Yes, visited

C. No

4. International travel within last 6 months?

A. Yes

B. No

Section III: Health and Medical Information

5. Has he taken any antibiotics within the last 6 months?

A. Yes

B. No

6. Has patient with suspected or confirmed infectious disease been hospitalized in the last 6 months or have you had close contact with such patient?

A. Yes, hospitalized

B. Yes, contact with patients

C. No

7. Any chronic medical condition (e.g. repeated cases of urinary tract infections)?

A. Yes

B. No

Annex 3: Semi-Structured Questionnaire for Cattle

1. Age category of the animal:

A. Calf (<6 months)

B. Young stock (7–12 months)

C. Heifer (1–2 years)

D. Adult (2–5 years)

E. Older cattle (>5 years)

2. Has the animal received any veterinary treatment or use of antibiotics in the last six (6) months?

A. Yes

B. No

3. What is the body condition of the animal?

A. Excellent

B. Good

C. Fair

D. Poor

4. What is the main source of drinking water for the animals?

A. Pond

B. Well

C. Little Akaki River

5. Approximate distance from the animal holding area to the nearest WWTP.

A. Greater than 1000 m

B. Less than 1000 m

6. Was the animal purchased from another area during the past six months?

A. Yes

B. No

7. Is the animal provided with pasture / forage which is irrigated with water from the river?

A. Yes

B. No

8. Is there any poultry, dairy, beef or pig farms in the vicinity?

A. Yes

B. No

Annex 4: Heavy Metals Analysis

Materials and Reagents

- Microwave digester, auto sampler, ICP-OES, digestion vessel, concentrated nitric acid, deionized water

Procedures

- ml of concentrated nitric acid (65% HNO_3) was added to 5 ml of wastewater sample in a vessel.
- Samples were digested in the microwave digester at 180°C for 15 minutes.
- Cooled and diluted with 35 ml of 5% HNO_3.
- The digestate was transferred to a 50 ml tube for metals analysis.
- ICP-OES was calibrated with multi-element standards and blanks used. The auto sampler recognized and quantified the metals.

Annex 5: *E. coli* isolation and confirmation

Procedure

- Samples were aseptically collected and pre-enriched in buffered peptone water.
- A loopful of enrichment broth was streaked onto MacConkey agar and incubated at 37°C for 24 hours.
- Typical lactose-fermenting colonies were sub-cultured onto eosin methylene blue (EMB) agar and incubated overnight.
- Colonies exhibiting a characteristic metallic green sheen on EMB agar were selected and purified on nutrient agar.
- Presumptive isolates were subjected to biochemical characterization using IMViC tests.
- Additional confirmation was performed using Triple Sugar Iron (TSI) agar and urease tests.
- Confirmed *E. coli* isolates were preserved in Brain Heart Infusion (BHI) medium until further laboratory analysis.

Annex 6: Antimicrobial Susceptibility Testing

Purpose:

To determine the antimicrobial resistance (AMR) profiles of *Escherichia coli* isolates.

Materials and Reagents:

- Mueller–Hinton agar (MHA) plates (Oxoid, UK)
- Sterile cotton swabs
- Sterile saline solution
- 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL)
- Reference strain: *E. coli* ATCC 25922 (Quality control strain)

Procedures:

- 3-5 confirmed colonies from overnight incubated fresh nutrient agar were suspended.
- Turbidity was adjusted to 0.5 McFarland standard.
- The bacterial suspension was streaked evenly on MHA plates using a sterile swab.
- The plates were allowed to dry for 3–5 minutes.
- Antimicrobial disks were aseptically applied on the dried plates with a dispenser.
- Plates were incubated aerobically at 37°C for 18–24 hours.
- The zone of inhibition diameters (mm) were recorded after overnight incubation using a digital caliper or ruler.
- Results were interpreted based on Clinical and Laboratory Standards Institute (CLSI, 2022) guidelines as Sensitive (S), Intermediate (I), and Resistant (R).
- All measurements were recorded for subsequent AMR analysis.
- *E. coli* ATCC 25922 was used as a quality control strain during the procedure.

Annex 7*:* Phenotypic Detection of ESBL-Producing *E. coli*

Procedure

- Fresh bacterial colonies were suspended in sterile saline and adjusted to a 0.5 McFarland turbidity standard.

- The suspension was uniformly inoculated onto Mueller–Hinton agar plates.

- Amoxicillin–clavulanic acid, cefotaxime, and ceftazidime discs were placed on the agar surface at the recommended distances.

- Plates were incubated aerobically at 37°C for 18–24 hours.

- Enhancement of the inhibition zone between the amoxicillin–clavulanic acid disc and either cephalosporin disc was interpreted as evidence of ESBL production.

- *E. coli* ATCC 25922 was included as the quality control strain. .

Annex 8: Phenotypic Detection of Carbapenemase-Producing E. coli

Procedure:

- 3-5 pure colonies of *E. coli* isolate inoculated into 2 ml of Tryptone Soya Broth agar (OXOID - CM0129B, UK).
- Meropenem disk was added to it and incubated for 4 hours at 37°C.
- *E. coli* ATCC 25922 suspension in sterile saline adjusted to 0.5 McFarland.
- Suspension evenly streaked on MHA plate and allowed to dry.
- Meropenem disk from TSB culture placed on MHA plate.
- After overnight incubation, zone of inhibition recorded and interpreted.
- Isolates with less than 15 mm of inhibition diameter are considered carbapenemase producers and greater than 19 mm .considered non-carbapenemase

Annex 9: MALDI-TOF MS Procedure for Confirmation of Escherichia coli Isolates

- Presumptive *E. coli* isolates obtained after culture and biochemical identification were selected for confirmation
- A fresh bacterial colony (18–24 h culture) was transferred onto a MALDI target plate.
- The sample spot was allowed to air dry at room temperature.
- Approximately 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution was added to the dried sample.
- The target plate was inserted into the MALDI-TOF MS instrument for analysis.
- Generated protein spectra were compared with the reference database available in the instrument software.
- Identification results were interpreted according to the manufacturer's recommended criteria.
- Isolates identified as *Escherichia coli* with acceptable confidence scores were considered confirmed.

- Confirmed isolates were subsequently included in antimicrobial susceptibility testing and resistance analysis.
- Quality control procedures were performed according to the laboratory's standard operating procedures.

Annex 10: Ethical statement for animal-based research activities

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
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Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/08/113/18/2026

Name of Applicant: **Melaku Taye (DVM, MSc Student)**

Address: Department of Microbiology, Parasitology and Poultry Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Heavy metal contamination and antimicrobial resistance of E. coli in the Akaki wastewater treatment plant (WWTP) and Akaki river catchment ESBL, Carbapenemase and co-selection analysis within a One Health framework*

Date of application: **December, 2025**

Nature of the project: **Field investigation**
Target animal species: **Cattle**
Number of animals involved: **84**
Study area: **Akaki, Ethiopia**

Minutes No. and date of review: **VM/ERC/08/18/026, 27/03/2026**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of Student Melaku Taye.

Professor Getachew Terefe (DVM, PhD)
Chairman

Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine & Agriculture

Signature

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Please quote Our Ref. No. When replying

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Fax 251-11-4339933 Tel. +251 114338450 P.o.x. Box}34 Bishoftu, Ethiopia

Annex 11: Ethical principles for human based research activities

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Addis Ababa University
Aklilu Lemma Institute of Health Research

Ethical Clearance Certificate

Ref. No.: ALIHR IRERC-005/2018/2026

Date: June 9, 2026

Title: [Heavy Metal Contamination and Antimicrobial Resistance of *E. Coli* in the Akaki Wastewater Treatment Plant (WWTP) and Akaki River Catchment: A One Health Assessment]

Principal Investigator: Melaku Taye,

Recommendation by the ALIHR -IRERC

Dear: Melaku,

The ALIHR-IRERC has reviewed your above mentioned research proposal and noted its merit. The IRERC would like to remind you, as PI, to submit progress reports of the work every 6 months and the final report upon completion of the study. Furthermore, you are expected to notify the ALIHR-IRERC ahead of time for any amendment or modification in the protocol or premature suspension or termination of the study.

STATUS: Approved

National Ethics Review Board Clearance: not required

IRERC Chairperson: Nigatu Kebede, Prof.
Signature: _____

IRERC Secretary: Abebe Animut, PhD
Signature: _____

Approved by
Name: Prof. Wakgari Deressa, Director, ALIHR
Signature: _____
Date: _____

CC:
- IRERC Office
- CPR Office
- ALIHR-IRERC

Tel: +251-11-8550186
Email: alihr@aau.edu.et
www.aau.edu.et

Annex 12: Some photos credited during research activities

Water sample from Akaki River

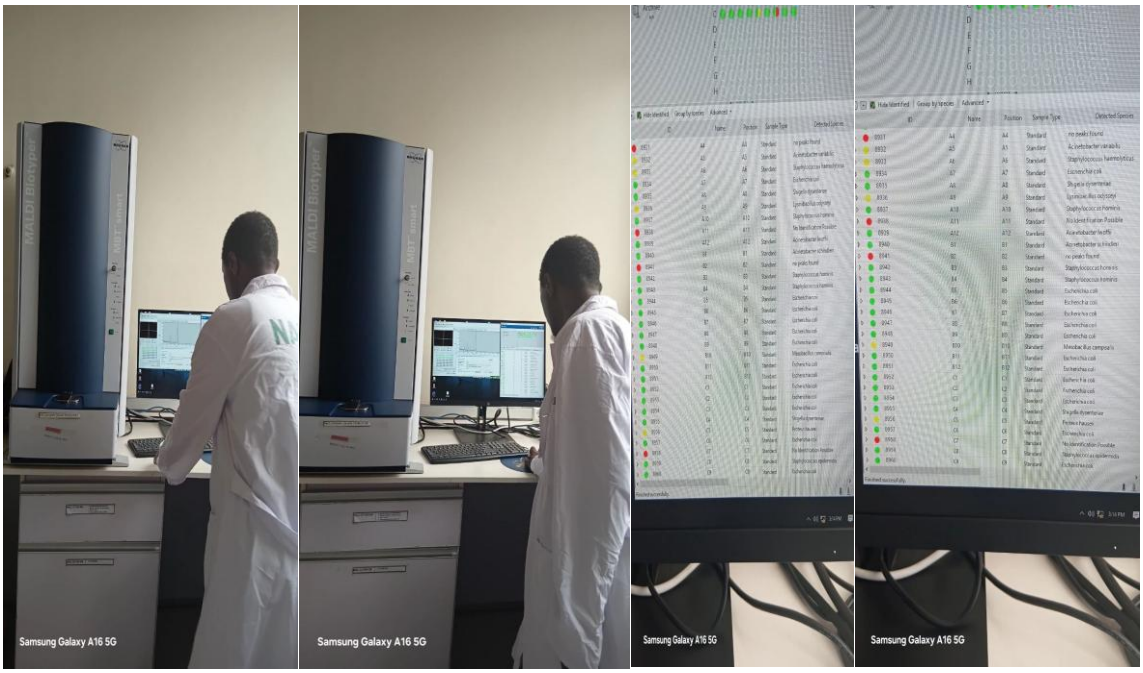
Some of sampling points of WWTP


TSI Tests SMAC

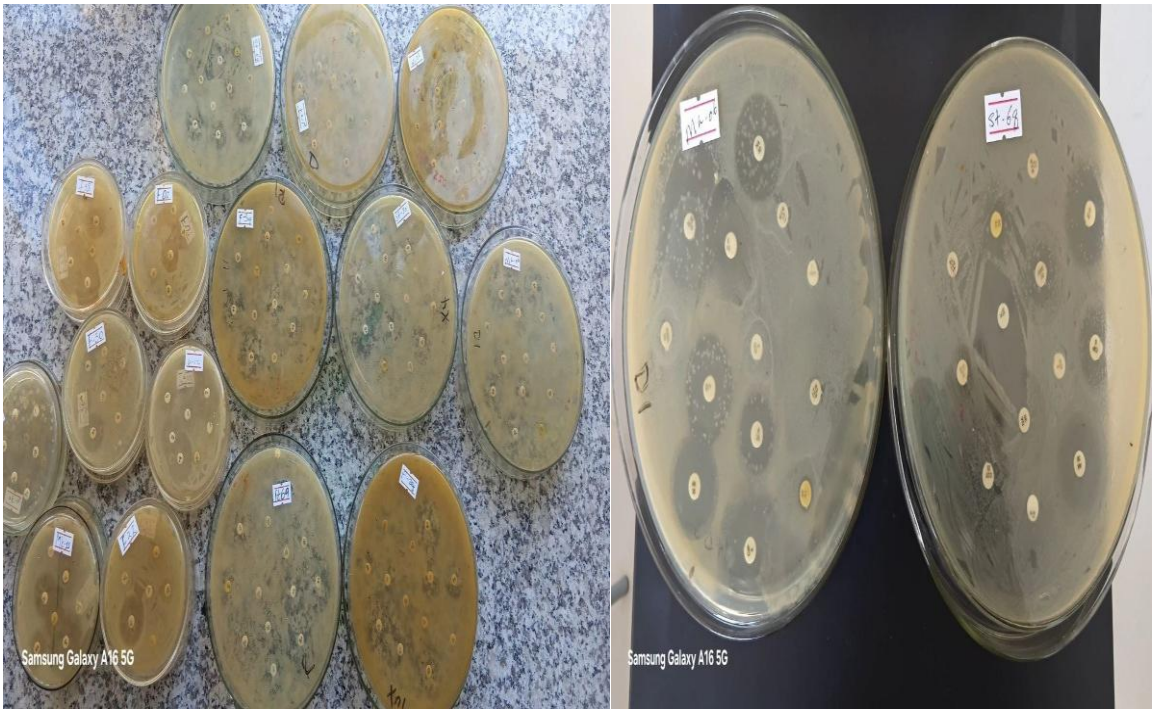
Biochemical Tests



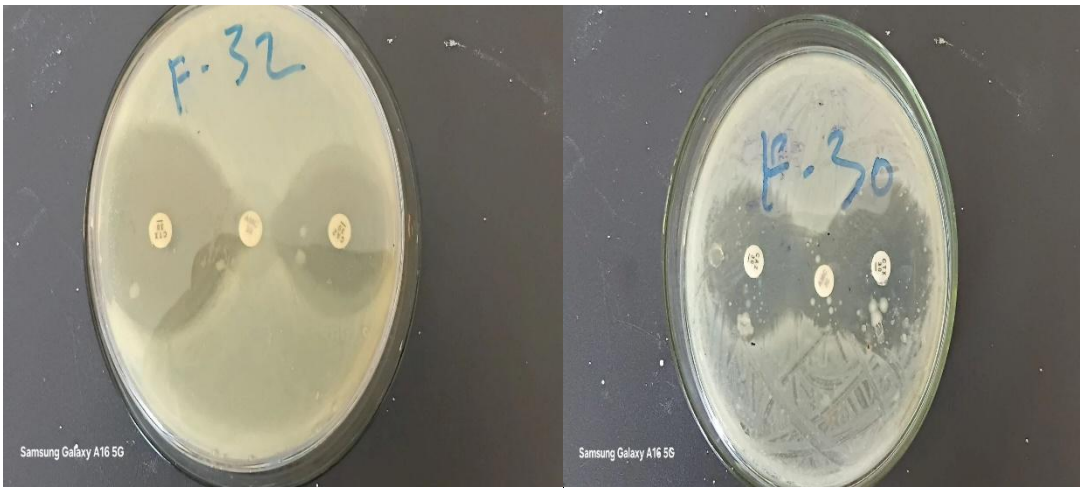
Sample preparation *Microwave digestion* *ICP-OES* Heavy metal analysis



MULDI-TOF Analysis at Sebeta AHI



Antimicrobial Susceptibility Test (AST)



Double Disk Synergy Test (DDST)

Annex 13: Plagiarism Check Report

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HEAVY METAL CONTAMINATION AND ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* IN THE AKAKI WASTEWATER TREATMENT PLANT AND AKAKI RIVER CATCHMENT: A ONE HEALTH FRAMEWORK

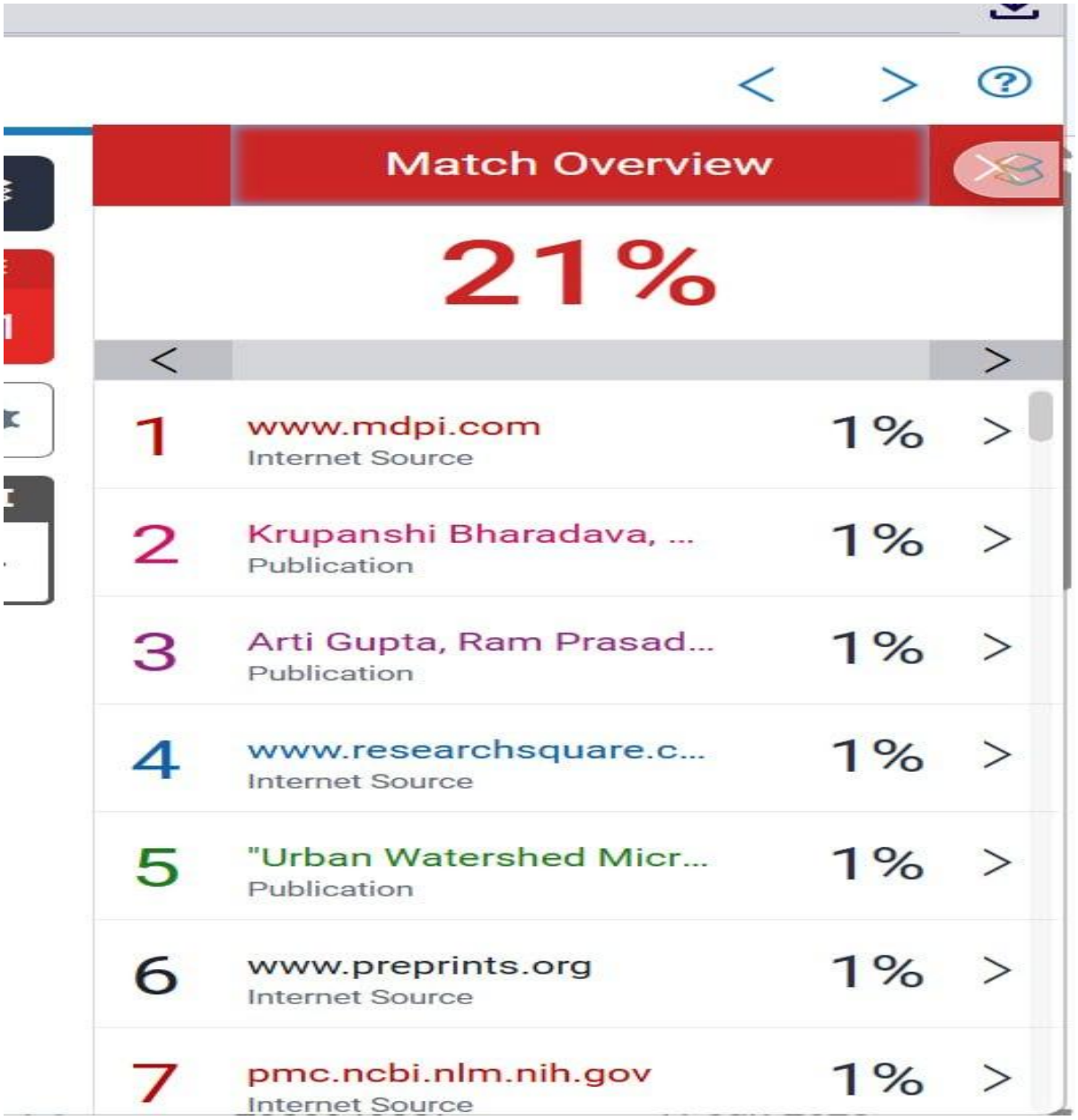
MSC THESIS

BY

MELAKE TAYE

ABSTRACT

Antimicrobial resistance (AMR) and heavy metal pollution in Wastewater treatment plants (WWTPs) and river catchments present significant global public health risks, potentially driving resistance via co-selection. This study assessed heavy metal contamination and AMR in *Escherichia coli within the Akaki WWTP and river catchment*, using a One Health framework, focusing on an extended spectrum beta-lactamase (ESBL) and carbapenemase production. A cross - sectional comparative study was conducted utilizing 232 samples from wastewater, river water, human stool, cattle feces, and milk. Six heavy metals (Cd, Pb, Cr, Cu, Mn, and Zn) were quantified by ICP-OES. *E. coli* was isolated using standard biochemical methods and MALDI-TOF. Phenotypic resistance, ESBL and carbapenemase production were confirmed by disk diffusion, double-disk synergy tests (DDST) and mCIM, respectively. Data were analyzed using logistic regression, chi-square, and one-way ANOVA ($p < 0.05$). Manganese (Mn) was the dominant contaminant, exceeding permissible across seasons. While, Zink (Zn) peaked during the rainy season. *E. coli* was isolated from 31 (13.4%) of samples, predominantly from water sources. High resistance was observed against Ampicillin (93.5%), Tetracycline (74.2%), Sulphamethoxazole (64. 5%), and Cefotaxime (58.0%). Multidrug resistance (MDR) reached 27 (87.1 %) and 14 (45.2%) were ESBL producers; no carbapenemase production was detected. Higher MDR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APPROVAL SHEET

This Research proposal entitled **Heavy Metal Contamination and Antimicrobial Resistance *of Escherichia coli* in the Akaki Wastewater Treatment Plant and Akaki River Catchment: A One Health Framework** has been submitted by **Melaku Taye** for presentation with my approval as college advisor.

Advisors Name: Dr.Takele Beyene (Assoc.Professor, PHD)

Signature: _____ Date of Submission: _____

Advisors Name: Dr. Lishan Asefa (DVM, MSC,)

Signature: _____ Date of Submission: _____

Professor Feleke Zewuge (PHD) Signature: _____Date of Submission_____