

Thesis Ref. No.: _____



**PHYSICOCHEMICAL, HEAVY METAL AND MICROBIOLOGICAL
EVALUATION OF KALITY WASTEWATER TREATMENT PLANT:
TREATMENT EFFICIENCY AND IMPLICATIONS FOR ANTIMICROBIAL
RESISTANCE WITHIN A ONE HEALTH NEXUS**

**COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF MICROBIOLOGY, PARASITOLOGY AND POULTRY HEALTH
MASTER OF SCIENCE PROGRAM IN ONE HEALTH**

MSC THESIS

BY

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JUNE, 2025

BISHOFTU, ETHIOPIA

**PHYSICOCHEMICAL, HEAVY METAL AND MICROBIOLOGICAL
EVALUATION OF KALITY WASTEWATER TREATMENT PLANT:
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Thesis submitted to College of Veterinary Medicine and Agriculture of Addis Ababa
University in partial fulfillment of the requirement for degree of Master of Science in
One Health

By

Tesfaye Fatalo

June, 2025

Bishoftu, Ethiopia

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As MSc in one health research advisors, we hereby certify that we have read and evaluated this thesis prepared under our guidance by Tesfaye Fatalo, entitled “*Physicochemical, Heavy Metal and Microbiological Evaluation of Kality Wastewater Treatment Plant: Treatment Efficiency and Implications for Antimicrobial Resistance Within a One Health Nexus,*” We recommend that it be submitted as fulfilling the MSc thesis requirement for the Degree of Master of Science in One Health.

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DEDICATION

I dedicate this work, first and foremost, to my beloved mother, Ayelech Chemiso, whose boundless love, unwavering sacrifices, and enduring wisdom laid the foundation for my life and achievements. Though she is no longer with me in this life, her strength continues to guide my steps and inspire my journey. To my dearest wife, Mekides Berhanu, I express my deepest gratitude. Your unwavering support, patient encouragement, and unconditional love have been my anchor through every challenge. Without you, this achievement would not have been possible. Finally, to my precious son, Besufekad Tesfaye, whose joyful love and grace fill my days with purpose and inspiration.

STATEMENT OF AUTHOR

I, **Tesfaye Fatalo**, solemnly declare that the thesis entitled “*Physicochemical, Heavy Metal and Microbiological Evaluation of Kaliti Wastewater Treatment Plant: Treatment Efficiency and Implications for Antimicrobial Resistance Within a One Health Nexus*” is my original work and has not been previously submitted in whole or in part for the award of any degree, diploma, scholarship, or other academic title at any university or institution. All sources of information, including data, literature, and intellectual contributions used or cited in this work, have been duly acknowledged with proper citations and references. Any assistance received in the preparation of this thesis has been appropriately credited. This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Science in One Health program at the College of Veterinary Medicine and Agriculture, Addis Ababa University, Ethiopia.

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ACKNOWLEDGEMENTS

First and foremost, I express my deepest gratitude to Almighty God for His divine guidance, strength and wisdom, which sustained me throughout the journey. My deepest appreciation goes to my advisors, Takele Beyene (Associate Professor) and Professor Feleke Zewge, whose constructive feedback, and encouragements were indispensable to this work. Also, I would like to thank Abebe Wirtu for his technical support.

This research was funded by NORAD through *NORHED II - One Health Project*, and I extend my sincere thanks to the Africa Center of Excellence for Water Management (ACEWM) project coordinators for their support. The laboratory analysis of this study was also partly supported by Addis Ababa University funded thematic research project “Improving Antimicrobial Stewardship Programs in Veterinary Medicine and the Agricultural Sector in Ethiopia through a One Health Approach (ASP-VMA).”

I sincerely thank the Addis Ababa City Water and Sewerage Authority and Kality WWTP for granting access to facilities and data, as well as the WWTP workers for their cooperation during fieldwork. My heartfelt thanks also go to the nearby residents of Kality WWTP who generously participated in this study. Furthermore, I would like to appreciate the contributions of all institutions-Aklilu Lemma Institute of Pathobiology, ACEWM-core water laboratory and CVMA-microbiology and pharmacology laboratory for providing essential technical and laboratory resources. I gratefully acknowledge the South Ethiopia Agricultural Research Institute, my employer, for their institutional support.

Finally, I would like to forward the immense gratitude to my “Families” for their unwavering support, love, patience, and encouragement throughout this journey. This achievement would not have been possible without the collective help of all mentioned, for which I am deeply thankful.

LISTS OF ABBREVIATIONS

AAWSA	Addis Ababa city Water and Sewerage Authority
ACEWM	Africa Center of Excellence for Water Management
AMR	Antimicrobial Resistance
ARGs	Antimicrobial Resistance Genes
AST	Antimicrobial Susceptibility Test
ATCC	American Type Colony Culture
BDL	Below Detection Level
BOD	Biological Oxygen Demand
CECs	Contaminants of Emerging Concerns
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standard Institute
COD	Chemical Oxygen Demand
CP	Carbapenemase Producer
DDST	Double Disk Synergy Test
EEPA	Ethiopia Environmental Protection Authority
ESBL	Extended Spectrum Beta Lactamase
GAP-AMR	Global Action Plan for Antimicrobial Resistance
HGT	Horizontal Gene Transfer
ICP-OES	Inductively Coupled Plasma-Optic Emission Spectrometry
IMVIC	Indole-Methyl Red-Voges Proskauer-Citrate
MARI	Multiple Antimicrobial Resistance Index
mCIM	Modified Carbapenem Inactivation Method
MDR	Multiple Drug Resistance
OR	Odds Ratio
PDR	Pan Drug Resistance
TDS	Total Dissolved Solids
USAB	Upflow Anaerobic Sludge Blanket
WWTP	Wastewater Treatment Plant
XDR	Extensively Drug Resistance

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ABSTRACT

Background: Antimicrobial resistance (AMR) poses a growing threat to global health and economic stability. Wastewater treatment plants (WWTPs) serve as critical hotspots for resistant pathogens due to contaminants of emerging concerns (CECs). Understanding the role of CECs in AMR within WWTPs and One Health domains is essential for effective mitigation. However, gaps remain in wastewater-based AMR epidemiology and its driving factors. This study aimed to evaluate CECs of Kality WWTP along antimicrobial resistance and efficiency of treatment within one health nexus.

Methods: A cross-sectional study was conducted at Kality WWTP and its surrounding areas. A total of 173 samples were aseptically collected from wastewater, humans, and cattle near and downstream to the WWTP. Physicochemical parameters, bacteria load, and heavy metals-including lead (Pb), chromium (Cr), copper (Cu), zinc (Zn), manganese (Mn), and cadmium (Cd) – were analyzed for using inductively coupled plasma optical emission spectrometry. To ascertain quantitative parameters, triplicate independent replication sets were utilized. *E. coli* was isolated, identified, and tested for extended spectrum beta lactamase (ESBL) and carbapenemase producing (CP) *E. coli* using the double disk synergy test and the modified carbapenem inactivation method, respectively. Data analysis employed logistic regression and one-way ANOVA, with statistical significance set at $p < 0.05$.

Results: Overall, 20.23% (35/173) of *E. coli* isolates were confirmed using biochemical and supplementary tests. The AMR study showed 97.1% of the isolates demonstrated multidrug resistances (MDR), with 20% of them were ESBL and 5.7% were CP *E. coli*. Isolates from the trickling filter exhibited co-resistance, both ESBL and CP *E. coli*, emphasizing rise of resistance mechanisms within the WWTP. The findings indicated a notable 1.47 log reduction in bacterial load, with an overall removal efficiency of 96.5%. Although effective removal rates were recorded for most physicochemical parameters and heavy metals, the concentrations of Cd (6.9%) and Mn (21.6%) remained high in the effluent.

Conclusions: These findings underscore the critical role of WWTP as hotspots for MDR pathogens and highlight the need for improved wastewater treatment and sanitation practices to safeguard the environment and public health within a one health nexus.

Keywords: *Antimicrobial resistance; Bacterial load; Carbapenemase and ESBL producing E. coli; Heavy metals; One health; Removal efficiency; WWTP*

1. INTRODUCTION

1.1. Background

The discovery of penicillin in the 1940s marked a groundbreaking advancement in treating infections in humans and animals. At the time, no one anticipated that bacteria could develop resistance to antibiotics (Vercelli *et al.*, 2022). Currently, antimicrobial resistance (AMR) has now emerged as a chronic public health problem with 700,000 deaths worldwide, if no action is taken to reduce its burden, the forecasted number will reach 10 million deaths per year globally by 2050 resulting in a cumulative economic loss of more than \$100 trillion (Tang *et al.*, 2023). Antimicrobial resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) are identified as contaminants of emerging concerns (CECs) for the healthcare system and pose significant risks to the public and environmental health (Rizzo *et al.*, 2019).

Currently, the emergence and spread of AMR pathogens carrying genes encoding extended spectrum beta lactamase (ESBL), carbapenemase or metallo-beta-lactamase are considered urgent health problems worldwide particularly in areas where environmental hotspots play a critical role in pathogens transmission (Islam *et al.*, 2023; Zhang *et al.*, 2018). The ESBL expressing genes are well known AMR genes responsible for the emergence of multidrug resistance (MDR) strains of pathogens (Aslam *et al.*, 2018).

To understand the dynamics of AMR from a One-Health perspective, monitoring and surveillance programs of human and animal health as well as the environment play a significant role (Djordjevic *et al.*, 2024). *Escherichia coli* is one of the most important members of the family Enterobacteriaceae which has been widely demonstrated with the emergence of β -lactamase and an outstanding indicator species to investigate the transmission of AMR through fecal contamination of water (Mohsin *et al.*, 2017). It is used as a potential indicator for monitoring and surveillance of the occurrence and level of antimicrobial resistance in wildlife (Anjum *et al.*, 2021). This is due to its high survival rate even at low level in environment (van Elsas *et al.*, 2011). Furthermore, it

monitored from both healthy and diseased targets, and survive in different environments (Montalbano Di Filippo *et al.*, 2022).

Among the anthropogenic factors aggravating AMR spread, wastewater treatment plants, healthcare settings, agricultural practice are now widely considered as a hotspot area for the proliferation and dissemination of AMR along its ARGs. Among those environmental pathways, wastewater treatment plant (WWTP) is widely considered as a hotspot area for the proliferation of ARB along its resistant genes due to CECs (Adesoji *et al.*, 2020).

The conventional municipal wastewater treatment plants are designed to remove nutrients and organic materials efficiently and poorly effective in the removal of emerging contaminants (Anthony *et al.*, 2020; Rizzo *et al.*, 2020). The incomplete removal of contaminants through WWTPs makes the effluent a source of pollutants for receiving ecosystems and exerts significant risks on aquatic ecosystem (Schmidt *et al.*, 2020). The effluent wastewater could contain multidrug resistance that can cause infection and transmit resistance genetic markers in the environment (von Wintersdorff *et al.*, 2016).

It significantly alters the microbial community structure and diversity of water ecosystems (Lu *et al.*, 2022). Chlorination based disinfection technique don't effectively remove ARB and ARGs (Zheng *et al.*, 2017). AMR should be monitored and managed under the one-health concept, considering the interconnection between humans, animals and the environment (Mudenda *et al.*, 2023). However, this approach often focuses on human and animal health and rarely on the environment (Gholizadeh *et al.*, 2023).

1.2. Statements of Problem

The emergence of extended spectrum beta lactamase (ESBL) and carbapenemase producing (CP) *E. coli* is a significant public health concern worldwide, particularly in urban environments where wastewater management systems play a critical role (Islam *et al.*, 2023). The discharge of wastewater greatly pollutes nearby irrigation water and contributes to the environmental pool of ARB and ARGs that pose a significant health threat (Hamed *et al.*, 2024). The previous study reported the presence of antibiotic resistance Enterobacteriaceae in Kality WWTP (Tsfaye *et al.*, 2019).

Despite growing recognition of the public health and economic consequences of AMR, considerable gaps persist in our understanding of how environmental hotspots facilitate their proliferation and spread. In targeted study area, the driving factors and AMR pattern of ESBL and CP *E. coli* in one health domain remain inadequately understood. Furthermore, heavy metals and total bacteria removal efficiency of centralized Kality WWTP not yet explored. This gap hinders the development of effective strategies to mitigate AMR, especially concerning environmental management and public health efforts (Klümper *et al.*, 2024). This research would contribute to existing knowledge by identifying heavy metals and determining AMR profile in ESBL and carbapenemase producing indicator microorganism in human, animal, and environment nexus.

1.3. Objectives of the Study

The specific objectives of this research were:

- ∅ To determine physicochemical parameters, bacteria load and heavy metals those induce selective pressure on AMR development in WWTP processing units.
- ∅ To determine AMR profiles and patterns of indicator isolates along with phenotypic detection of ESBL and carbapenemase production.
- ∅ To evaluate removal efficiency of centralized Kality WWTP.

2. LITERATURE REVIEW

2.1. Antimicrobial Resistance as Global Health Threats

Antimicrobial resistance has emerged in humans, animals, and environment as a serious global concern in the 21st century (Pulingam, 2022). In 2019, the World Health Organization included AMR as one of the threats to global health (Manai, 2022). Antimicrobial resistance is considered to be as the “*Silent Pandemic*” that needs urgent and immediate action to manage more effectively without referring as a future agenda (Founou *et al.*, 2021). If insufficient action is taken to tackle it, it is estimated that by 2050, AMR could potentially become the world’s primary cause of death mounting the combined effect of malaria, HIV/AIDS, and tuberculosis (O’Neill, 2016).

The recent studies have indicated antimicrobial involvement in agriculture and food systems may have a significant impact on driving AMR. Excessive use of antibiotics in medical, veterinary and agriculture sectors might be the root cause of worldwide AMR development (McKernan *et al.*, 2021). Besides, the discharge of antibiotics residues through feces/manure and industrial effluents into the environment has worsened the problem. These parameters generate unnatural selective pressure in clinical and natural environments that is detrimental to human and animal health (Holmes *et al.*, 2016).

2.2. *Escherichia coli* as Indicator Organism in AMR Studies

Monitoring and surveillance initiatives across human health, animal health, and environmental sectors are crucial for comprehending AMR patterns through a One Health lens (Djordjevic *et al.*, 2024). The antimicrobial resistance *E. coli* is used as a potential indicator for surveillance of the occurrence and level of antimicrobial resistance in the environment including wildlife (Anjum *et al.*, 2021). *E. coli* serves as an ideal indicator organism because it persists at low levels in the environment while demonstrating remarkable survival capabilities (van Elsas *et al.*, 2011). Furthermore, it serves as an optimal One Health indicator organism due to its dual

presence in both healthy and diseased populations as a natural gut commensal. Its multisectoral relevance stems from its ability to persist across diverse environments (Montalbano Di Filippo *et al.*, 2022). Recent studies have shown that AMR profiles of *E. coli* isolates from wastewater samples correlate to the *E. coli* AMR data from the associated populations (Huijbers *et al.*, 2020).

Antimicrobial drugs have revolutionized modern medicine, transforming infectious disease treatment and significantly extending human lifespan during 'golden age of antimicrobial chemotherapy.' However, this remarkable progress is increasingly threatened by the imprudent use and rise of AMR, which continues to escalate at an alarming rate as mentioned in Fig. 1.

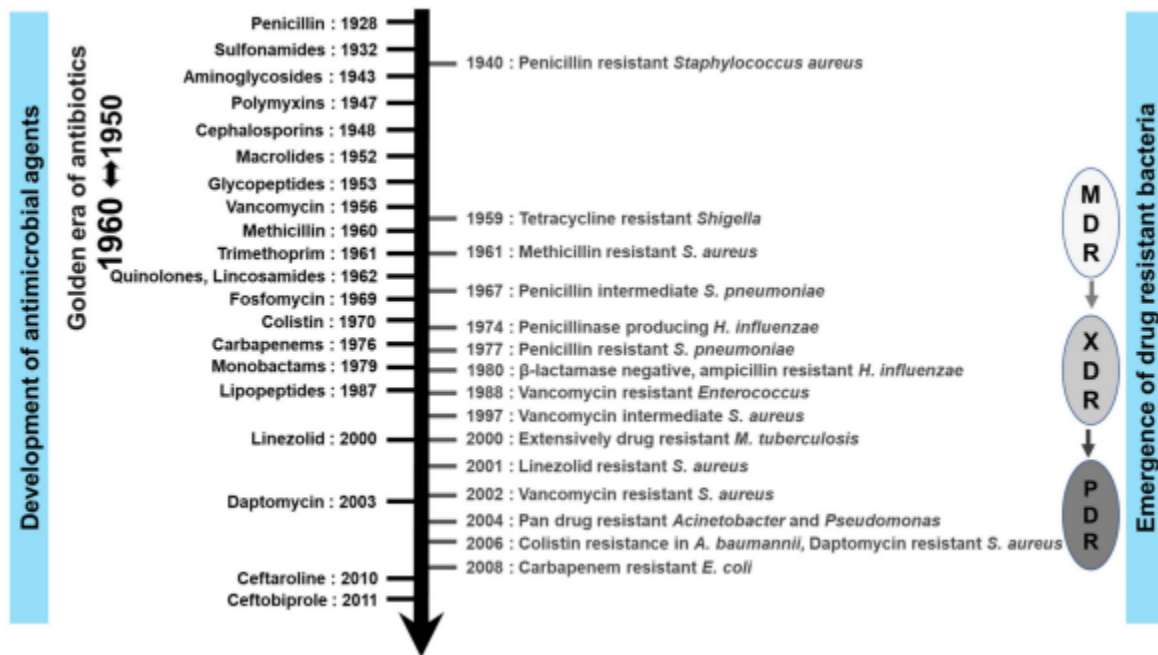


Figure 1: Timeline of antimicrobial discovery (Murugaiyan *et al.*, 2022).

2.3. Antimicrobial Resistance Mechanisms

Antimicrobial resistance in bacteria can be inherent, acquired or adaptive (Bo *et al.*, 2024). Microorganisms may evolve resistance mechanisms or acquire resistance genes through horizontal gene transfer (HGT) to survive in toxic environments (Abbas *et al.*, 2024). Acquired

resistance of microorganisms can be through either mutation or acquisition of resistance from another source. It is mainly acquired through HGT i.e. acquisition of new genetic elements from individual bacteria of the same species or even between different species (Gupta & Birdi, 2017). Inherent resistance triggered by a spontaneous gene mutation and is independent of previous antibiotic exposure (Martinez, 2014). Bacteria that lack a cell wall (Mycoplasma and related species) are intrinsically resistant to all drugs including beta lactams (Zhao *et al.*, 2019).

Some pathogens reversibly adapt and become resistant to one or more antimicrobials in response to specific environmental signals (D'Aquila, 2023). Although this adaptive response allows pathogens to quickly cope with antimicrobial threats, it is temporary; once the inducing conditions are removed, the bacteria usually revert to their original susceptibility (Lazar *et al.*, 2023).

Acquired drug resistance may cause cross resistance, MDR, pan drug resistance (PDR) and extensively drug resistance (XDR) in pathogens commonly through antimicrobial target modification, inactivation, and efflux (Belay *et al.*, 2024). The primary mechanism of β -lactam resistance is the action of β -lactamase enzymes, which break the amide bond of the beta-lactam ring, rendering the antibiotic ineffectiveness (Gaubá & Rahman, 2023). Antimicrobial resistant genes encoding ESBL are present in a diverse range of clinically significant pathogens worldwide (Azab *et al.*, 2021).

2.4. Impacts of Antimicrobial Resistances

2.4.1. Impacts on health

Antimicrobials resistant pathogens emerging as a global challenge due the resistance pattern to multiple antimicrobials “superbugs” (Algammal *et al.*, 2023). In 2019 alone, AMR was the direct cause of 1.27 million deaths and a complicit cause of 4.9 million deaths, making drug resistant infections more deadly than HIV/AIDS or Malaria (Lancet, 2022). An antimicrobial compounds has been increasingly detected in water bodies, and more recently in human serum, urine, and

breast milk of individuals not currently using the compound, potentially leading to health effects (Bever *et al.*, 2018; Weatherly & Gosse, 2017).

The prevalence of hospital-acquired infection caused by resistant bacteria underscores the importance of hospitals and extended care facilities while estimates suggest that hospitals may contribute up to 33% of environmental resistance for certain ARBs (Hocquet *et al.*, 2016). The rise of AMR is gradually reducing the effectiveness of last resort antibiotics, presenting a serious healthcare challenge (Hutchings *et al.*, 2019).

2.4.2. Impacts on food chain

Food serves as a significant vehicle for the transmission of antibiotic resistant spoilage and pathogenic bacteria, and an increase in ARB in food could adversely affect human health, although the extent of AMR spread globally through the food supply may not be fully understood (Olivier Andreoletti *et al.*, 2008). The ARB can enter the food supply at any stage of the farm-to-fork continuum, and those that contaminate products at the farm level are likely to persist on raw and undercooked foods (Teklemariam *et al.*, 2023). Fresh leafy greens and ready-to-eat foods may harbor foodborne pathogens which pose significant health risks to consumers (Losio *et al.*, 2015).

2.4.3. Impacts on environment

A significant amount of antimicrobials given to humans and animals is excreted through urine and manure, allowing residual antibiotics. It aggravates formation of AMR in environment by encouraging the selection of resistance pathogens (Hussein *et al.*, 2023). Antimicrobials can have ecotoxicological impacts that harm soil and plant health, disturb the soil microbial diversity, which is crucial for preventing the spread of AMR and is a key source for pharmaceutical innovations (Kraemer *et al.*, 2019).

The presence of antibiotics in soil can significantly disrupt microbial community structure which are vital indicators of soil health (Cycon *et al.*, 2019). Ultimately, antibiotic disruption of microbial communities can result in a higher prevalence pathogens in both soil and water, as

evidenced by antibiotic pollution in aquatic environments, which has been linked to an increased frequency of toxic cyanobacteria species that contribute to eutrophication and pose health risks to humans (Drury, 2013).

2.4.4. Impacts on economy

Antimicrobial resistance can significantly burden the economy by increasing healthcare costs from longer hospital stays and more complex treatments for resistant infections, potentially resulting in decreased productivity and a projected loss of up to 3.8% of global growth domestic product by 2050 if not addressed (McDonnell A, 2024). According to current estimations, it could cause an economic burden of up to USD 100 trillion globally by 2050 (Dadgostar, 2019). Center for disease control and prevention estimates that AMR incurs an annual cost of \$55 billion in the United States, with \$20 billion attributed to healthcare and around \$35 billion due to lost productivity, while recent research from the World Bank indicates that AMR will disproportionately raise poverty levels and impact low-income countries worldwide (Utt & Wells, 2016).

2.5. Environmental Hotspots for Antimicrobial Resistance

Antimicrobial resistant pathogens and their resistance genes enter in to the environments through various hotspots such as hospital and healthcare settings, agricultural waste or wastewater treatment plants (Samreen *et al.*, 2021). Improper management of healthcare waste can lead to the spread of diseases, as pathogens may be transmitted through improperly disposed materials, further endangering both human and animal populations (Abosse *et al.*, 2024). Furthermore, discharges of antibiotic residues, active pharmaceutical ingredients, and resistant bacteria from pharmaceutical industry into surrounding create selective pressure favoring the development of resistant microbial populations (Bjerke, 2025).

Intensive agricultural practices are contributors to AMR as often subjected to stress from human-induced factors like agricultural chemicals and pollutants (Kaviani Rad *et al.*, 2022). Heavy metals in agricultural practice are frequently associated with the co-selection of antibiotic

resistant strains in ecosystems, with *E.coli* being common bacteria exhibiting simultaneous resistance to both heavy metals and antibiotics (Nguyen *et al.*, 2019). Starting from 1950, antimicrobials have been used in livestock for disease control and prevention, and growth promotion. However, their imprudent use raises concerns about AMR that threaten both animal and human health (Rhouma *et al.*, 2016). A lack of awareness and negative attitudes regarding AMU and AMR may influence farmers' practices indicating the need to promote better understanding of livestock disease management (Tufa *et al.*, 2023). The most common environmental hotspots that aggravate the dissemination of AMR pathogens are mentioned in Fig. 2.

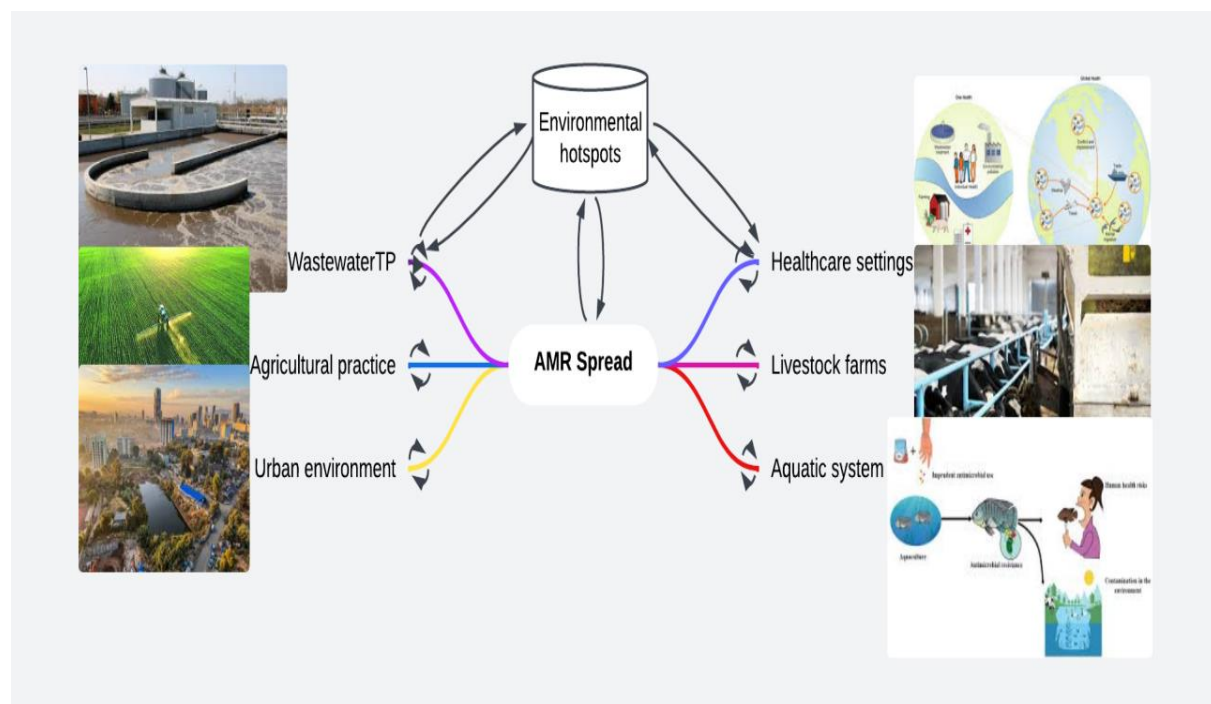


Figure 2: Environmental hotspots for emergence of AMR (Kunhikannan *et al.*, 2021).

2.5.1. Wastewater treatment plants

Antimicrobial resistance pose a significant public health threat due to their association with mobile genetic elements, which facilitate HGT among microorganisms (Sharma *et al.*, 2016). The WWTP could become major contributors to the accumulation of contaminants when the

treatment process is inadequate (Barancheshme & Munir, 2017). Wastewater flowing into WWTP contains antibiotics, heavy metals, AMR pathogens with resistance genes and microplastics (Hubeny *et al.*, 2021). The complete removal of emerging pollutants remains impossible in conventional treatment plants (Uluseker *et al.*, 2021). Consequently, it is considered as a major reservoir of CECs and resistant pathogens (Marutescu *et al.*, 2023).

The presence of antimicrobial residues, toxic heavy metals and biocides facilitates the proliferation and spread of ARGs to neighboring microbiota and environments (Krzeminski *et al.*, 2019). Also, the combination of these driving factors with the complex microbial communities and nutrients facilitates HGT mechanisms of resistant genes (Kalli *et al.*, 2023). Furthermore, it significantly alters the microbial community structure and diversity of water ecosystems (Lu *et al.*, 2022).

Human, animal and environment interfaces create chances for one or other population to be a reservoir of AMR elements which can be disseminated in any direction (Hassell *et al.*, 2017). There is a possible spread pattern for AMR genes from human to human and urban sewage, and further through WWTP to surface water (Berendonk *et al.*, 2015). The β -lactamase producing pathogens are resistant to important antimicrobial drugs and are considered important vectors in the transmission of ARGs and commonly found in inflow wastewater worldwide (Huijbers *et al.*, 2020).

2.5.2. Drivers of antimicrobial resistance in wastewater treatment plants

The rise in AMR has been triggered mainly through extensive and indiscriminate use of antimicrobials. Besides, emerging contaminants of WWTP significantly contribute to the risk of pollution in nearby receiving water bodies and AMR development due to the selection pressure (Chukwu *et al.*, 2023). The treatment technology and use of tertiary treatment methods impact the presence and concentration of CECs (Hazra & Durso, 2022). The pathogens' resistance to CECs is a global concern to public, animals and ecosystems health (Omotayo *et al.*, 2022).

Heavy metals are non-biodegradable and can exert long-term selective pressure when accumulated in the environment and sewage sludge wastewater treatment (Niu *et al.*, 2016).

Metal resistance genes are known to be genetically linked to ARGs, with plasmids, transposons, and integrons involved in the assembly and horizontal transfer of the resistance elements (Gillieatt & Coleman, 2024). Heavy metal co-resistance with antibiotics appears to be synergistic in bacterial isolates and has potential to amplify antibiotics resistance genes of area which can be transferred into different settings (Edet *et al.*, 2023).

Antibiotics are extensively used in human and animal medicine for treatment and prophylaxis purposes. However, approximately 85% of the antibiotics used in food animals are excreted partially metabolized and their residues end up in treatment plants (Mutyar & Mittal, 2014). The presence of antibiotics compounds in influent wastewater and treatment plants promote the selection and spread of ARB and ARGs, as evidenced between specific antibiotics in influent sources and their corresponding ARGs in WWTPs (Ju *et al.*, 2019).

Biocides are active compounds containing one or more active substances that can be applied to control the effect on harmful organisms, by either chemical or biological means and have been reported in WWTPs (Chen *et al.*, 2012). Despite of their advantages, biocides have been associated with the induction and co-selection of AMR through co and cross resistance mechanisms (Ruiz & Alvarez-Ordóñez, 2017).

The treatment plants are the main contributors to microplastics in the environment, as they collect and treat waste containing microplastic particles that are released when clothes are washed (Prata, 2018). This emerging contaminant is slowly degradable in the environment and have been identified as carriers of antibiotic residues and (Pham *et al.*, 2021; Sun *et al.*, 2019). In the conducive conditions of treatment plants, biofilm forms on the surface of microplastics, providing a rich niche for bacterial growth and transmission (Pham *et al.*, 2021). Different microbial communities originating from different sources interact within the wastewater matrix and may modify bacterial ecosystems (Adegoke *et al.*, 2020). The occurrence of AMR in WWTP is due to enhanced co selection pressure, co resistance, increasing mutation frequencies and HGT within colonized biofilms (Zhao *et al.*, 2023).

Different drivers of AMR along with environmental hotspots for AMR development and dissemination in wider ecosystem are elaborated in Figure 3.

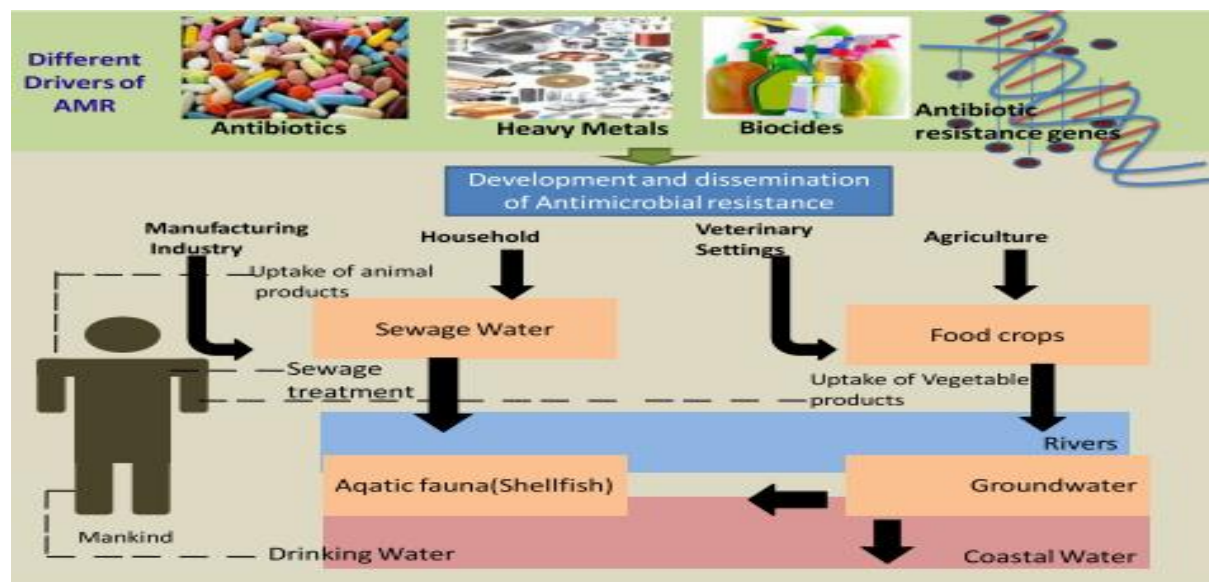


Figure 3: Scheme of AMR drivers and their dissemination (Samreen *et al.*, 2021)

2.5.3. Status of antimicrobial resistance in wastewater treatment plant

Conventional wastewater treatment is unable to completely eliminate all pollutants, which can enter surface water bodies together with reclaimed water (Godinho *et al.*, 2024). As AMR genes are potentially transferable from one microbial community to another through conjugation, transformation and transduction wastewater could be an important source of AMR in microorganisms (Sims & Kasprzyk-Hordern, 2020).

The contaminants of emerging concerns that are received by WWTPs may be the causative factor in spreading ARB in the aquatic environment (Praveenkumarreddy *et al.*, 2020). These drug resistant bacteria and genes could enter humans or animals through contaminated water or food cultivated by irrigation leading to health threats in human, animal and environment interface (Kumar *et al.*, 2020). The status of AMR in wastewater treatment plants is elucidated as mentioned in Table 1.

Table 1: Status of antimicrobial resistance in wastewater treatment plants

Authors	Country	Sources of wastewater	of Resistance records
(Praveenkumarreddy <i>et al.</i> , 2020)	South India	Domestic hospital	and Ampicillin and Cefazolin
(Mantilla-Calderon <i>et al.</i> , 2016)	Saud Arabia	Domestic wastewater	blaNDM-1
(Tegege <i>et al.</i> , 2024)	Ethiopia	Abattoir wastewater	Streptomycin & Ampicillin
(Wang <i>et al.</i> , 2023)	Northern China	Domestic wastewater	tetA, tetC, tetX and sulIII
(Rodriguez-Molina <i>et al.</i> , 2021)	Germany-Netherlands	Domestic wastewater	ESBLs <i>E. coli</i>
(Tesfaye <i>et al.</i> , 2019)	Ethiopia	Domestic hospital	and Amoxi-Clavunate and cefotaxime
(Igwaran <i>et al.</i> , 2018)	South Africa	Domestic wastewater	Erythromycin clindamycin.
(Schmiege <i>et al.</i> , 2021)	Germany	Domestic wastewater	Ceftazidime, cefotaxime and colistin
(Leopold <i>et al.</i> , 2024)	Austria	Hospital and domestic	Amoxi, ceftazidime, and cefepime

2.6. Strategies to Combat Antimicrobial Resistance

Antimicrobial resistance is a global threat with far reaching implications because of its crosscutting nature. Failure of effective control of AMR invariably jeopardizes significant progress in medicine, health and food security as well as economic growth (Fuller *et al.*, 2022). Due to the crosscutting nature, it not only affects human health but also agricultural settings and the environment health (Barbieri *et al.*, 2023).

2.6.1. Improved sanitation and waste management

Improvements in water sanitation and hygiene and solid, and liquid waste management in all sectors are critical elements of preventing infections and mitigating the spread of AMR as identified in the Global Action Plan to combat AMR (WHO, 2020). Unsafe WASH contributes to a high burden of disease and exacerbates factors that promote the development of AMR (Weets & Katz, 2024). Inadequate waste management significantly facilitates the spread of AMR by enabling the entry of antibiotics and resistant bacteria into the environment through improper disposal of human and animal waste, which can contaminate water sources, soil, and food chains, thereby promoting the transfer of ARGs between microbes and creating reservoirs for AMR bacteria (Sambaza & Naicker, 2023).

2.6.2. Infection prevention and control

Healthcare associated infections can result from invasive procedures, exposure to devices and human to human transmission in the absence of proper control measures, often caused by antimicrobial resistant pathogens that can share resistance genes. The world health organization (WHO) emphasizes that improving infection prevention and control in healthcare settings is the most effective way to reduce AMR, as it decreases infections and subsequently the need for antimicrobial treatment (WHO, 2022). These measures should be adopted in both human and animal health settings to minimize the risk of transmitting resistant organisms (Sharma *et al.*, 2022).

2.6.3. Use of alternatives to antimicrobials

Antimicrobial resistance transcends natural boundaries and has developed into a global public health threat, impacting populations in high, medium, and low-risk countries. The rising rates of resistance among key pathogens made AMR one of the most urgent public health challenges globally (Singha *et al.*, 2024). Based on the global impact of AMR and the pressing need for new antibiotics, new strategies are being implemented to combat MDR, XDR, and PDR infections due to increasingly losing of effectiveness of “last resort” antibiotics in clinical environments (Murugaiyan *et al.*, 2022).

Phytochemical constituents extracted from plants possess unique mechanisms that can effectively combat resistant pathogens while minimizing the risk of development of AMR (Hussain *et al.*, 2022). Also, probiotics are live microorganisms that produce antimicrobial metabolites and compete for nutrients. They produce inhibitory substances, competing for adhesion sites, and competing for nutrients, and modulate the immune system (Markowiak & Slizewska, 2017; Petrariu *et al.*, 2023). Furthermore, the phage therapy is being employed as innovative strategy and become more popular in the 21st century for combating AMR that utilizes bacteriophages to treat infections, especially those resistant to conventional antibiotics (Singha *et al.*, 2023).

2.6.4. Surveillance and monitoring

Basic health interventions particularly good hygiene practices and antimicrobial stewardship is particularly challenging in developing countries due to human resource limitations and weak health infrastructures, which hinder effective surveillance and management of AMR (Aryee & Price, 2015). European countries have taken initiatives to reduce the incidence and spread of AMR by ensuring the prudent use of antimicrobials. Recently, the food and drug administration has released plans to monitor AMR issues (Huang *et al.*, 2019). Judicial use of antibiotics, monitoring of antibiotics, progress in healthcare systems, development of health insurance policies, limited drug promotion, coherent disease control policy and community stewardship plans were key factors implemented (Laxminarayan, 2013).

2.6.5. One health adoption

Humans, animals and environment are all part of the AMR ecosystem, intricately connected with one another through direct contact, food chains, disposal of manure, sewage effluent and pollution (Woolhouse, 2024). One Health approach could be a very effective tool to nexus-based monitoring and designing novel screening. Its solid foundation nature relied on the mechanisms of AMR transmission between all components of single health (Cantón *et al.*, 2012). Antimicrobial stewardship is a crucial aspect of the One Health approach to managing AMR, focusing on the responsible use of antimicrobials in all domain, necessitating the implementation of stewardship programs in all health sectors (Musoke *et al.*, 2020).

The world health organization (WHO), food and agricultural organization (FAO) and world animal health organization (WOAH) have developed comprehensive action plans in 2015 to address the AMR crisis (Gu *et al.*, 2021). They collaboratively launched an AMR-related management program known as the Global Action Plan focusing on AMR (GAP-AMR). This plan aims to ensure that infectious disease can always be successfully treated and prevented by preserving antimicrobials in a responsible manner (Berman *et al.*, 2023).

3. MATERIALS AND METHODS

3.1. Description of Study Area

The study was conducted in Akaki Kality sub city of Addis Ababa particularly in and around Kality waste water treatment plant (WWTP) which is situated in the industrial area covering around 40 hectares of land as shown in Figure 4. It is located in Addis Ababa's Kality neighborhood on the east side of the little Akaki River with coordinates $8^{\circ}54'52''\text{N}$ and $38^{\circ}45'18''\text{E}$. It is capable of handling a maximum flow rate of $100,000\text{m}^3$ per day. However, it currently operates at about 70% of that design capacity, due to the incomplete connectivity of the city's sewage infrastructure to the plant. It is under the administration of the Addis Ababa Water and Sewerage Authority (AAWSA). It was designed for the treatment of domestic wastewater in most of Addis Ababa's cities. However, certain industrial wastes are being discharged from point and non-point sources. The final effluent wastewater is discharged into little Akaki River also used for irrigation purposes by smallholder farmers of the Akaki-Kality sub-city.

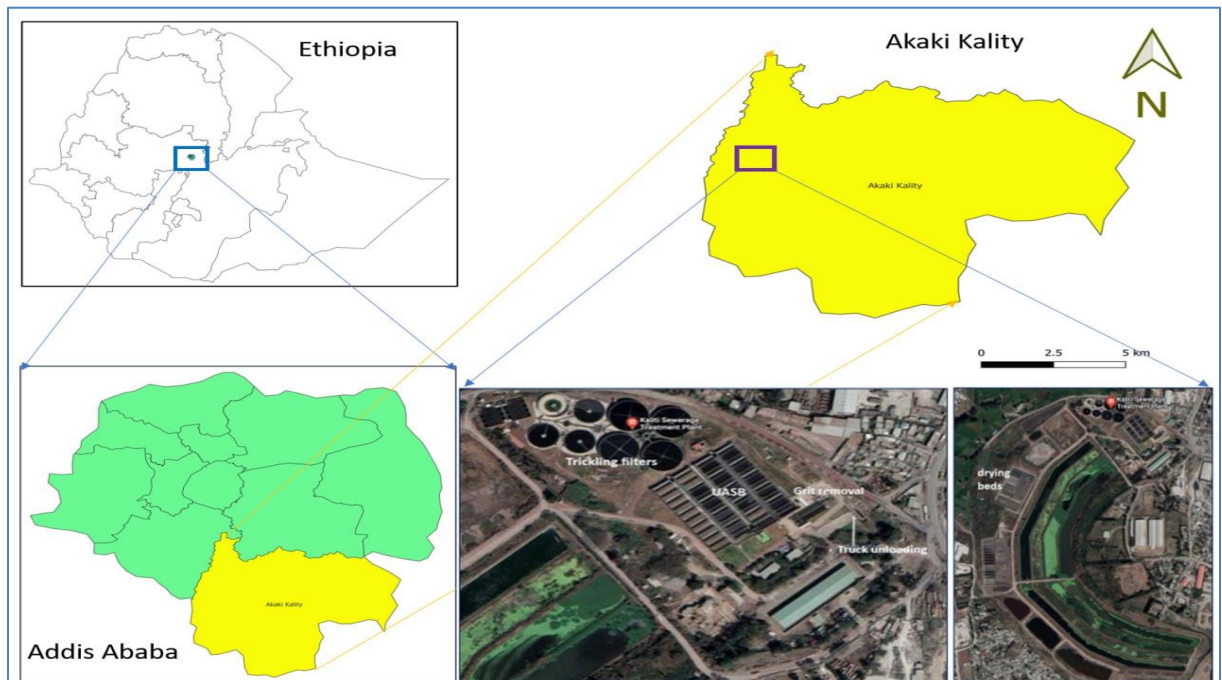


Figure 4: Kality centralized wastewater treatment plant.

3.2. Study Settings and Sample Collection

Study domains

Environmental domain

The wastewater samples from WWTP units processing (UASB reactor inlet, UASB reactor outlet, Trickling filter outlet, secondary clarifier outlet, and effluent) were collected to determine the presence and concentration of selected heavy metals (Lead (Pb), Cadmium, Chromium (Cr), Zinc (Zn), Copper (Cu) and Manganese).

Also, water samples from little Akaki River (adjacent points <1000m and distal points <1000m) were collected to determine *E. coli* carriage rate, antimicrobial resistance profiles and patterns along with ESBL and carbapenemase production. Furthermore, removal efficiency of COD, BOD₅, total bacteria and selected toxic heavy metals by centralized Kality WWTP was evaluated. The purposive sampling method was employed to collect LAR samples from designed measuring points. 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 (ISO 19458: 2007) (Pascual-Benito *et al.*, 2020).

Human and Animal Domain

The expected human population with and without minimal exposure to the effluent of wastewater discharge (WWTP workers, nearby residents living within and outside a radius of <1000m from the WWTP) were targeted based on the inclusion criteria. A semi-structured questionnaire was conducted to avoid confounding variables on inclusion and exclusion criteria. The *E. coli* carriage rate of ESBL and CP *E. coli* was determined from nearby and distant residents of WWTP. The midterm urine samples were collected from eligible and volunteered participants of both adjacent (<1000m radius) and distant (>1000m radius) residents. The adjacent and distant residents' radius is defined based on previous study (Rodriguez-Molina *et al.*, 2021).

3.3. Study Design

A cross-sectional study that adopted a One Health approach was employed to investigate heavy metal contamination and AMR profiles of ESBL and CP *E. coli* isolates from human, cattle and wastewater samples. The research was conducted from November 2024 to May 2025. All heavy metal analyses, total bacteria load enumeration and physicochemical parameter determinations were performed in triple independent replication sets to ensure analytical precision and data reproducibility. Microbiological specimens were maintained in cold-chain conditions during transport to the microbiology laboratory at Addis Ababa University, College of Veterinary Medicine and Agriculture. These samples were processed for total bacteria count, *E. coli* isolation and confirmation, AMR profiling, and screening of ESBL and carbapenemase production. Concurrently, the heavy metal analysis and physicochemical parameter determination of water samples were conducted at ACEWM-core water laboratory, Addis Ababa University.

3.4. Sample Size determination

A total of five wastewater samples from WWTP units processing of different sampling points (UASB inlet, UASB outlet, Trickling filter, Secondary clarifiers and effluent) were collected to determine the presence and concentration of selected heavy metals, bacteria enumeration, antimicrobial resistance profiles and patterns of indicator pathogen along with ESBL and CP *E. coli*. Moreover, two water samples from little Akaki River (adjacent points <1000m and distal points <1000m) were purposively collected to determine *E. coli* carriage rate, antimicrobial resistance profiles and patterns along with ESBL and carbapenemase production.

The sample size was determined to investigate *E. coli* carriage rates in adjacent and distant residents. The formula was applied with a previously reported result of 29% and 7% of carriage rate of *E.coli* for both adjacent and distant residents respectively (Rodriguez-Molina *et al.*, 2021). The desired level of statistical significance and the power of the test were set at 5% and 80% respectively and employed to compute

the required total sample size of 88 (44 samples for each adjacent and distant) residents based on the formula given by (Bolarinwa, 2020).

$$N = \frac{(Z_{\alpha/2} + Z_{\beta})^2 \times (P_1(1 - P_1) + P_2(1 - P_2))}{(P_1 - P_2)^2}$$

Where,

N= required sample size per sub-group population

Z_{α/2}=desired significance level at 5% (1.96)

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

P₁ = previous prevalence report of nearby residents

P₂ = Previous prevalence report of distant residents

Urine samples were obtained from 88 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, without prior proportion report, with an effect size of 20% (Kim, 2016). For the pilot study, a total of 78 fecal samples (39 from each group) were collected from adjacent and distal resident cattle of the WWTP.

Inclusion and exclusion criteria for the study

Detailed information was obtained from the targeted human population on confounding variables. The sociodemographic characteristics, residents-vicinity, work history, hospital visits and contact with patients, international travel, and health conditions all in the past months according to Annex 2. Therefore, all participants who had a history of working at slaughterhouses and hospitals, contact with patients, international travel, chronic health problems, too young and old (<18 and >67year) were excluded from target populations. Cattle with a history of purchase from other areas and poor body condition were excluded based on Annex 3. Slaughterhouse and animal farms can be a risk factor for *E. coli* carriage (Dohmen *et al.*, 2017).

3.5. Determination of Physicochemical Parameter

Wastewater samples were collected from each treatment unit of the WWTP and determined with standard procedures as mentioned in Annex 4. The physicochemical parameters measured in this study were temperature, pH, electrical conductivity (EC), total dissolved solids (TDS), salinity, dissolved oxygen (DO), chemical oxygen demand, and biological oxygen demand (BOD₅). To determine pH, temperature, and TDS, a multiparameter ion-specific meter (HANNA portable meter) was utilized. The parameters, specifically DO and BOD₅, were assessed based on Winkler's standard protocol (Aniyikaiye *et al.*, 2019).

3.6. Detection of Heavy Metals

Wastewater samples were collected during peak operational capacity of the treatment plant to ensure representative sampling. Samples were obtained from predetermined sampling locations at Kality WWTP using grab sampling methodology. The collected samples were subsequently analyzed for the presence and concentration of lead, chromium, cadmium, copper, zinc, and manganese as stated in Annex 5. Water samples were prepared using microwave-assisted acid digestion with concentrated nitric acid (HNO₃) before elemental quantification through inductively coupled plasma-optical emission spectrometry (ICP-OES) (Element, 2007). The acidification of water samples serves to maintain metal ions in solution by preventing precipitation, and minimizes adsorption losses of metal cations to container surfaces (Dagne, 2020).

3.7. Total Bacteria Load Enumeration

Wastewater samples were analyzed for the total bacterial load using the total plate count (TPC) method (Emmanuel *et al.*, 2016). A five-fold serial dilution was performed. From each dilution, an aliquot of 1 mL was transferred to sterile Petri dishes containing plate count agar (HIMEDIA-M091, India). The mixture was gently swirled to ensure an even distribution of bacteria throughout the agar. The plates were then incubated at 37 °C for 24 hours. After incubation, colonies that formed on the agar were counted, with plates

containing between 30 and 300 colony-forming units (CFU/mL) considered suitable for enumeration (Sanders, 2012). The counted colonies were multiplied by the dilution factor. For ease of data interpretation, numeric scales were subsequently converted to log CFU/mL for analysis as illustrated in Annex 6.

3.8. Isolation and Confirmation of *E. coli* from One Health Domains

Midstream human urine collected with the help of medical professional, cattle fecal samples, and water samples were pre-enriched in buffered peptone water (OXOID-CM0509, UK) and incubated at 37°C for 18–24 hours. Pre-enriched and diluted samples were first plated on MacConkey agar (OXOID-CM0115B, UK) and incubated at 37°C for 24 hours. Colonies exhibiting pink color (lactose fermenting) were subcultured onto Eosin Methylene Blue agar (Becton Dickinson, France) for further differentiation. On EMB agar, presumptive *E. coli* colonies displaying a characteristic metallic green sheen were selected for biochemical confirmation using standard protocols.

The presumptive *E. coli* isolates were subjected to a series of standard biochemical tests for definitive identification. The IMViC tests (Indole production, Methyl Red, Voges-Proskauer, and Citrate utilization tests) were performed along with supplementary tests of Triple Sugar Iron agar (OXOID-CM0277, UK) and urease tests to confirm the isolates as *E. coli*. A reference strain *E. coli* ATCC 25922 served as a positive control throughout the process. Confirmed isolates were preserved in Brain Heart Infusion broth (OXOID-CM1135B, UK) with 20µl of 25% glycerol and stored for further analysis as mentioned in Annex 7.

3.9. Antibiotic Susceptibility Testing of *E. coli* Isolates

The Kirby-Bauer disk diffusion method was employed to evaluate antibiotic susceptibility patterns of confirmed *E. coli* isolates according to standard procedure as mentioned in Annex 8 (Bauer *et al.*, 1966). The turbidity of inoculum suspension was adjusted to 0.5 McFarland standards (1.5×10^8 CFU/mL) and dispensed evenly onto a MHA. The test isolates were challenged with nine different antimicrobial agents of major

classes (Cefotaxime (CTX)-30µg, Ceftazidime (CAZ)-30µg, Amoxicillin clavulanic acid (AMC) -20µg, Meropenem (MEM) -10µg and Trimethoprim-Sulfamethoxazole (STX)-25µg (Oxoid, UK) and Tetracycline (T30C) -30µg, Doxycycline (DXT)-30µg, Gentamycin-10µg and Ciprofloxacin (CIP)-5µg (Mast Group discs).

Inhibition zone were measured and interpreted according to Clinical and Laboratory Standards Institute performance standards (CLSI, 2022). The reference *E. coli* ATCC 25922 was used for validation of test. Test isolates that are not susceptible to at least one agent of at least three antimicrobial classes were considered as MDR (Sweeney *et al.*, 2018). Multiple antimicrobial resistance index (MARI) of the resistant isolates was determined as the number of antimicrobials to which an isolate resistant divided by total number of antimicrobials to which isolates challenged (Ejikeugwu *et al.*, 2022). In this study, while performing the AMR profiles, all isolates exhibiting intermediate or resistant AST profiles were categorized as non-susceptible based on the previous study suggestions (Egwu *et al.*, 2024; Jardine *et al.*, 2012).

3.10. Phenotypic Detection of ESBL and Carbapenemase Production

The Double Disc Synergy Test (DDST) was used to phenotypically confirm ESBL production in *E. coli* isolates, following the method stated in Annex 9 described by (Wayne, 2017). A bacterial suspension was prepared in sterile saline and adjusted to a 0.5 McFarland standard (1.5×10^8 CFU/mL). This suspension was then uniformly spread on Mueller-Hinton agar (OXOID-CM0337, UK) dispensed plates. Then, amoxicillin clavulanic acid and third-generation cephalosporins (cefotaxime and ceftazidime) disks were seeded on the agar surface and incubated for overnight at 37°C. A positive ESBL result was indicated by the presence of enhanced inhibition zone between the amoxicillin-clavulanic acid and the cephalosporin discs (John-Onwe *et al.*, 2022). The *E. coli* ATCC 25922 used as the reference strain

The Modified Carbapenem Inactivation Method (mCIM) was employed to detect carbapenemase production in suspected *E. coli* isolates, following the protocol stated in Annex 10 described by (van der Zwaluw *et al.*, 2015). A 10 µg meropenem disc was

incubated for 4 hours in tryptone soya broth (OXOID-CM0129B, UK) containing test isolate. After incubation, the disc was removed and placed on MHA plate inoculated with a meropenem-susceptible *E. coli* ATCC 25922 reference strains. Following overnight incubation, the zone of inhibition diameter was measured with electronic caliper. The absence or <15mm zone of inhibition indicated carbapenemase production. Conversely, a visible (>19mm) inhibition confirmed that meropenem remained active.

3.11. Removal Efficiency of Wastewater Treatment Plant

The treatment technology and use of tertiary treatment methods impact the presence and concentration of CECs (Hazra & Durso, 2022). The removal efficiency of each wastewater treatment processing units, which implies the percentage ratio of the concentration of selected contaminants removed during the treatment process to the total amount present in the influent water before the treatment was estimated using Equation below (Agoro *et al.*, 2020).

$$\text{Removal efficiency \%} = \frac{\text{Concentration in influent} - \text{Concentration in effluent}}{\text{Concentration in influent}} \times 100$$

3.12. Data Analysis

Microsoft Excel data was exported to STATA software version 17.0 for statistical analysis. Descriptive summary statistics were determined as frequencies and proportions for categorical variables. One way ANOVA and Tukey's post hoc were employed to evaluate the existence of statistical mean difference between groups and within groups. The logistic regression model with OR was used to evaluate the strength of association between different variables. The Pearson chi-square test (χ^2) was used to compute association between variables. A *P-value* of < 0.05 was considered significant.

3.13. Ethical Considerations

Ethical clearance of animal aspect was taken from the Institutional Animal Research Ethical Committee of College of Veterinary Medicine and Agriculture, Addis Ababa University (*Certificate Ref No.: VR/ERC/04/73/17/2025*). Procedures involving human samples was reviewed and approved by Aklilu Lemma Institute of Pathobiology Institutional Research Ethics Review Committee, Addis Ababa University (*Certificate Ref No.: ALIPB IRERC/173/2017/25*). The study was adhered to the principles outlined in the Declaration of Helsinki (Shrestha & Dunn, 2020). Any communication with them was based on their voluntary consent. 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. The certificates were attached for details in Annex 11 and Annex 12.

4. RESULTS

4.1. Physicochemical Parameter Analysis

Physicochemical analysis of the centralized Kaliti WWTP showed significant variations among treatment stages (all parameters $p < 0.001$) as elucidated in Table 2. The UASB reactor exhibited raised electrical conductivity (1.12 ± 0.02 to 1.39 ± 0.04 mS/cm, $p < 0.001$) and TDS (559.0 ± 7.7 to 692.7 ± 18.7 mg/L, $p < 0.001$) and lowered pH (7.39 ± 0.01 to 6.99 ± 0.04 , $p < 0.001$). The temperature showed a gradual decrease from the inlet ($20.20 \pm 0.10^\circ\text{C}$) to the trickling filter ($19.63 \pm 0.15^\circ\text{C}$, $p = 0.0003$), followed by a partial recovery in the effluent ($19.77 \pm 0.06^\circ\text{C}$). BOD demonstrated a distinctive trend, maintaining stability during UASB reactor (0.42 ± 0.05 to 0.44 ± 0.02 mg/L, $p = 0.999$) but reaching a peak at clarifiers (4.20 ± 0.08 mg/L).

Aerobic treatment units served to effectively moderate the impacts of UASB, particularly the trickling filter diminished conductivity to 1.04 ± 0.03 mS/cm ($p < 0.001$ compared to UASB outlet) and elevated pH levels to alkaline conditions (7.84 ± 0.06). Clarifiers reached the highest TDS reduction (486.8 ± 3.8 mg/L, $p < 0.001$). The parameters of the final effluent were similar to those of the influent regarding conductivity (1.09 ± 0.04 mS/cm, $p = 0.633$ vs inlet) and pH (7.70 ± 0.10), however, TDS (545.0 ± 21.1 mg/L) demonstrated that removal was not complete. The COD removal was the most effective, dropping to 192.3 ± 17.5 mg/L ($p < 0.001$) after the trickling filter, and achieving total removal (0.0 mg/L) after clarifiers.

Table 2: Determination of physicochemical parameters of treatment process units

Sample Points	Temp	P ^H	EC	TDS	Salinity	DO	COD	BOD ₅
UASB inlet	20.2±0.10 ^a	7.3±0.01 ^a	1.12±0.02 ^a	558.9±7.7 ^a	2.17±0.06 ^a	0.82±0.04 ^a	496.3±3 ^a	0.42±0.05 ^a
UASB inlet	20.03±0.05 ^b	6.98±0.04 ^b	1.38±0.03 ^b	692.7±18.7 ^b	2.7±0.10 ^b	0.83±0.02 ^a	54±4 ^b	0.44±0.02 ^a
Trickling outlet	19.63±0.15 ^c	7.84±0.06 ^c	1.04±0.03 ^c	521.7±14.3 ^c	2.03±0.06 ^a	3.5±0.04 ^b	192±18 ^c	3.14±0.07 ^b
Clarifiers outlet	19.71±0.11 ^d	7.69±0.04 ^d	0.97±0.01 ^d	486.8±3.9 ^d	1.83±0.12 ^c	4.55±0.05 ^c	0±0.00 ^d	4.2±0.08 ^c
Effluent	19.7±0.05 ^d	7.7±0.10 ^d	1.08±0.04 ^a	544.9±21.1 ^{a,c}	2.13±0.56 ^a	4.94±0.04 ^d	BDL	2.79±0.2 ^d

Physicochemical parameter values with different subscripts in the same column are significantly different ($P < 0.05$)

Key: UASB=Upflow anaerobic sludge blanket, Tem=Temperature (°C), EC=Electrical conductivity (mS/cm), TDS=Total dissolved oxygen (mg/L), Do=Dissolved oxygen (mg/L), COD=chemical oxygen demand (mg/L O₂) and BOD₅=Biological oxygen demand after 5-days (mg/L)

4.2. Detection of Heavy Metals

Analysis of occurrence and concentrations of heavy metals throughout the wastewater treatment process showed clear patterns (Table 3). The levels of cadmium revealed progressive increase from 2.17 ± 0.12 mg/L at the UASB inlet to 2.54 ± 0.02 mg/L at the trickling filter outlet ($p < 0.05$), and then decreased slightly to 2.32 ± 0.03 mg/L in the final effluent ($p < 0.05$). The concentrations of manganese showed a steady increase across the treatment stages, rising from 2.08 ± 0.03 mg/L at the UASB outlet to 2.53 ± 0.02 mg/L in the effluent ($p < 0.05$ for all sequential comparisons). Lead was found only in the outlet of the secondary clarifiers (4.33 ± 0.03 mg/L), whereas chromium was present solely in the outlet of the trickling filter (2.62 ± 0.03 mg/L). Throughout the treatment process, copper and zinc levels stayed below detection limits.

Table 3: Heavy metals concentration in different sampling points (ppb)

<i>Heavy metals</i>	Pb	Cr	Cd	Cu	Mn	Zn
UASB inlet	BDL	BDL	2.17 ± 0.12^a	BDL	BDL	BDL
UASB outlet	BDL	BDL	2.44 ± 0.02^b	BDL	2.08 ± 0.03^a	BDL
Trickling filter	BDL	2.62 ± 0.03	2.54 ± 0.02^c	BDL	2.22 ± 0.05^b	BDL
Clarifiers outlet	4.33 ± 0.03	BDL	2.56 ± 0.03^c	BDL	2.50 ± 0.05^c	BDL
Effluent	BDL	BDL	2.32 ± 0.03^d	BDL	2.53 ± 0.02^c	BDL

The means in the same column with different letters are significantly different ($P < 0.05$)

Key: *ppb-parts per billion, UASB-Upflow anaerobic sludge blanket, BDL-below detectable level, Pb-Lead, Cr-Chromium, Cd-Cadmium, Cu-Copper, Mn-Manganese, Zn-Zinc*

4.3. Total Bacteria Enumeration

The treatment process at Kality WWTP showed a progressive decrease in bacterial load (log CFU/mL) across successive treatment units, as demonstrated by microbiological analysis (ANOVA, $F = 807.6$, $p < 0.001$) in Table 4. The UASB reactor demonstrated a significant 1.17 log reduction (7.37 ± 0.05 to 6.14 ± 0.06 log CFU/mL, $p < 0.001$). A subsequent aerobic treatment in the trickling filter led to a slight increase (6.14 ± 0.06 to 6.26 ± 0.03 log CFU/mL, $p = 0.178$). Additional treatment via secondary clarifiers resulted in a final effluent of 5.85 ± 0.07 log CFU/mL, which indicates a statistically significant overall reduction of 1.46 log CFU/mL from the influent ($p < 0.01$). It was found through a comparative analysis with the water quality of LAR that the bacterial concentrations in the plant effluent were significantly higher than those at both adjacent and distant ($p < 0.001$) sampling points of the WWTP.

Table 4: Total bacteria count in treatment stages and nearby river

<i>Sampling sources</i>	<i>Bacteria load (log cfu/mL)</i>	<i>Ranges</i>	<i>Statistical values</i>
UASB reactor inlet	7.31 ± 0.05^a	7.32-7.42	
UASB reactor outlet	6.14 ± 0.06^b	6.08-6.20	
Trickling filter outlet	6.26 ± 0.03^c	6.23-6.29	($F=807.6$,
Clarifiers outlet	6.04 ± 0.07^d	5.98-6.11	$P < 0.001$)
Effluent	5.85 ± 0.07^e	5.78-5.92	
LAR adjacent-WWTP	4.86 ± 0.05^f	4.81-4.90	
LAR distal-WWTP	4.80 ± 0.04^f	4.78-4.85	

The means with the different letters in the same column differ statistically ($p < 0.05$).

Key: LAR-Little Akaki River, cfu/mL-Colony Forming Units per milliliter, WWTP-Wastewater treatment Plant

4.4. Isolation and Confirmation of *Escherichia coli* Isolates

The chi-square test indicated a statistically significant association between sampling sources and the *E. coli* carriage rate (Pearson $\chi^2=20.38$, $P=0.002$). The finding revealed that the *E.coli* carriage rate for nearby and distant resident humans to WWTP was 18.2% (8/44) and 13.64% (6/44) and 20.5% (8/39) and 17.9% (7/39) for cattle respectively in Table 5. The odds of *E. coli* carriage rate across different sampling sources and distance from WWTP were illustrated in (Table 6).

Table 5: Association between sampling sources and *E. coli* carriage (N=173)

Sampling sources	Total	Negative (N=138)	Positive (N=35)	Positive %
WWTP (Reference)	5	1	4	80
Adjacent cattle-WWTP	39	31	8	20.5
Adjacent human-WWTP	44	36	8	18.2
Distal cattle-WWTP	39	32	7	17.9
Distal human-WWTP	44	38	6	13.6
LAR adjacent-WWTP	1	0	1	100
LAR distal-WWTP	1	0	1	100

Key: Pearson $\chi^2 (6) =20.38$, $p=0.002$, LAR=Little Akaki River

Table 6: Multivariate logistic regression analysis for *E. coli* carriage rate across variables

Variables	Level	OR	95% CI	P value
Sampling sources	Human	Ref		
	Cattle	1.36	0.56 - 2.81	0.57
	Wastewater	24.1	2.4 - 242	0.007
Distance from WWTP	Point source	Ref		
	Adjacent	1.27	0.57 - 2.88	0.54
	Distant	0.17	0.08 – 0.34	0.00

Key: OR- odds ratio, CI-confidence interval, WWTP-wastewater treatment plant

4.4.1. Antimicrobial susceptibility test

The antimicrobial susceptibility testing of *E. coli* (N=35) isolates showed concerning resistance patterns, particularly with high rates of resistance to tetracycline (80%), ciprofloxacin (71.4%) and trimethoprim-sulfamethoxazole (54.3%). Whereas gentamicin (14.3%) and meropenem (11.4%) showed lower yet significant resistance. In contrast, amoxicillin-clavulanate exhibited outstanding activity (91.4% susceptibility) as mentioned in the (Table 7).

Table 7: Antimicrobial susceptibility profile of *Escherichia coli* (N= 35)

Antimicrobial agents	Antimicrobial classes	R%	I%	S%
Tetracycline	Tetracycline	28(80)	1(2.86)	6(17.14)
Gentamicin	Aminoglycosides	5(14.29)	3(8.57)	27(77.14)
Doxycycline	Tetracycline	19(54.29)	0(0.00)	16(45.71)
AMC	Penicillin	0(0.00)	3(8.57)	32(91.43)
Cefotaxime	Cephalosporin	16(45.71)	17(48.57)	2(5.71)
Ceftazidime	Cephalosporin	2(5.71)	24(68.57)	9(25.71)
Meropenem	Carbapenem	4(11.43)	15(42.86)	16(45.71)
STX	Sulfonamide	19(54.29)	3(8.57)	13(37.14)
Ciprofloxacin	Fluoroquinolone	25(71.43)	9(25.71)	1(2.86)

Key: N-total *E. coli* isolates, STX- Trimethoprim-Sulfamethoxazole, AMC-Amoxicillin clavulanate, R-resistant, I-intermediate, S-susceptible

4.4.2. Antimicrobial resistance profile of *E. coli*

The analysis of AMR profiles in *E. coli* isolates from various sampling sources revealed considerable differences in resistance patterns (Table 8). However, none of the tested antimicrobials exhibited statistically significant source-dependent variations ($p>0.05$). While resistance to tetracycline, ciprofloxacin and cefotaxime resistance was prevalent, no significant sampling source-specific resistance pattern was observed ($p>0.05$). Notably, isolates were susceptible to amoxicillin-clavulanate across all sampling sources, except for those collected from humans adjacent to the WWTP.

Table 8: Antimicrobial resistance profile of *E. coli* isolates across sampling sources

Antimicrobials	WWTP	AC	AH	ALAR	DC	DH	DLAR	Overall	χ^2, P value
Tetracycline	75(3/4)	62.5(5/8)	87.5(7/8)	100(1/1)	85.7(6/7)	100(6/6)	100(1/1)	82.9(29/35)	4.3, 0.63
Gentamicin	50(2/4)	12.5(1/8)	25(2/8)	0(0/1)	14.3(1/7)	16.7(1/6)	100(1/1)	22.9(8/35)	6.3, 0.39
Doxycycline	75 (3/4)	50(4/8)	62.5(5/8)	0(0/1)	28.6(2/7)	66.7(4/6)	100(1/1)	54.3(19/35)	5.2, 0.51
AMC	0(0/4)	0(0/8)	37.5(3/8)	0(0/1)	0(0/7)	0(0/6)	0(0/1)	8.6(3/35)	11.1, 0.09
Cefotaxime	100(4/4)	100(8/8)	87.5(7/8)	100(1/1)	85.7(6/7)	100(6/6)	100(1/1)	94.3(33/35)	2.9, 0.83
Ceftazidime	100(4/4)	75(6/8)	62.5(5/8)	100(1/1)	71.4(5/7)	83.3(5/6)	0(0/1)	74.3(22/35)	5.5, 0.48
Meropenem	100(4/4)	75(6/8)	50(4/8)	100(1/1)	14.3(1/7)	50(3/6)	0(0/1)	54.3(19/35)	11.4, 0.08
STX	25(1/4)	62.5(5/8)	75(6/8)	0(0/1)	71.4(5/7)	83.3(5/6)	0(0/1)	62.9(22/35)	7.6, 0.26
Ciprofloxacin	100(4/4)	100(8/8)	100(8/8)	100(1/1)	85.7(6/7)	100(6/6)	100(1/1)	97.1(34/35)	4.1, 0.66

Key: WWTP-Wastewater treatment plant, AC and AH (Adjacent Cattle and human to WWTP), DC and DH (distal Cattle and human to WWTP), ALAR and DLAR (adjacent and distal Little Akaki River), AMC-Amoxicillin clavulanic acid

4.4.3. Antimicrobial resistance patterns of *E. coli* isolates

Out of 35 confirmed *E. coli* isolates, 34 (97.1%) exhibited MDR patterns (Table 9). The predominant resistance combinations included T30C, CTX, CAZ, STX, and CIP, with several isolates showed resistance to three or more antimicrobial classes. Interestingly, 8.8% of isolates showed resistance to MEM and STX with T30C, CIP, and CAZ/CTX. Higher multiple antibiotic resistance index (MARI) values ranged from 0.33 to 0.89, with 88.2% of isolates showing high-risk MARI values (≥ 0.55), implying resistance to five or more antimicrobials, including combinations of AMC, GM, and DXT.

Table 9: Antimicrobial resistance pattern of MDR *Escherichia coli* isolates (N=34)

AMR patterns	MARI	Isolate n(%)	AMR patterns	MARI	Isolate n(%)
MEM, STX, T30C	0.33	1(2.9)	CIP, CTX, DXT/T30C*, GM	0.56	1(2.9)
CIP, DXT/T30C*, STX	0.44	1(2.9)	CAZ/CTX*, CIP, MEM, STX, T30C	0.67	1(2.9)
CAZ/CTX*, CIP, STX	0.44	1(2.9)	CAZ/CTX*, CIP, DXT/T30C*, MEM	0.67	2(5.9)
CAZ/CTX*, CIP, DXT	0.44	1(2.9)	CAZ/CTX*, CIP, DXT/T30C*, STX	0.67	2(5.9)
CAZ/CTX*, CIP, MEM, STX	0.55	1(2.9)	CIP, CTX, DXT/T30C*, MEM, STX	0.67	2(5.9)
CIP, CTX, DXT, MEM, STX	0.55	1(2.9)	CIP, CTX, DXT/T30C*, GM, MEM	0.67	1(2.9)
CAZ/CTX*, CIP, GM, MEM	0.55	1(2.9)	CAZ/CTX*, CIP, DXT/T30C*, MEM, STX	0.78	1(2.9)
CAZ/CTX*, CIP, GM, STX	0.55	1(2.9)	AMC, CAZ/CTX*, CIP, DXT/T30C*, STX	0.78	1(2.9)
AMC, CIP, CTX, STX, T30C	0.55	1(2.9)	CAZ/CTX*, CIP, GM, MEM, STX, T30C	0.78	1(2.9)
CAZ/CTX*, CIP, MEM, T30C	0.55	3(8.8)	CAZ/CTX*, CIP, DXT/T30C, GM, MEM	0.78	1(2.9)
CAZ/CTX*, CIP, STX, T30C	0.55	3(8.8)	CAZ/CTX*, CIP, DXT/T30C, GM, STX	0.78	1(2.9)
CIP, CTX, MEM, STX, T30C	0.55	1(2.9)	CAZ/CTX, CIP, DXT/T30C, GM, MEM, STX	0.89	1(2.9)
CAZ/CTX*, CIP, DXT/T30C*	0.55	2(5.9)	AMC, CAZ/CTX*, CIP, DXT/T30C* MEM, STX	0.89	1(2.9)

Key: “*” indicates antimicrobials from the same classes, T30C-Tetracycline, CTX-Cefotaxime, CIP-Ciprofloxacin, DXT-Doxycycline, STX-Sulfamethoxazole-Trimethoprim, GM-Gentamicin, MEM-Meropenem and CAZ-Ceftazidime

4.4.4. Phenotypic detection of ESBL and carbapenemase-producing *E. coli*

A total of 35 isolates of *E. coli* that showed resistance and intermediate to cefotaxime, ceftazidime and meropenem were screened phenotypically for ESBL and carbapenemase production using double disc synergy test (DDST) and mCIM respectively. The findings of the result revealed that 20% (7/35) and 5.7% (2/35) of isolates were tested as ESBL and carbapenemase producers. Co-production of the ESBL and carbapenemase 2.9% (1/35) was observed in *E. coli* isolated from Tricking filter outlet unit of WWTP (Figure 5).

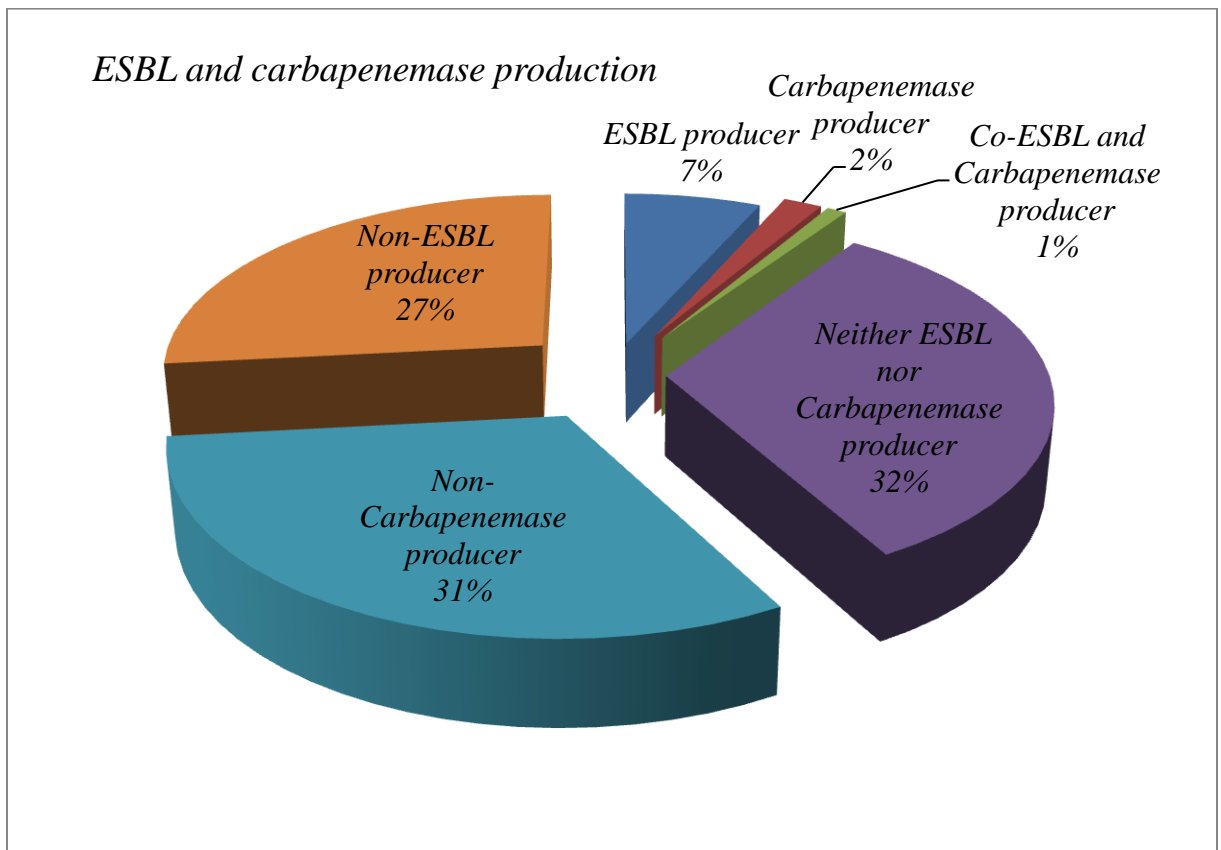
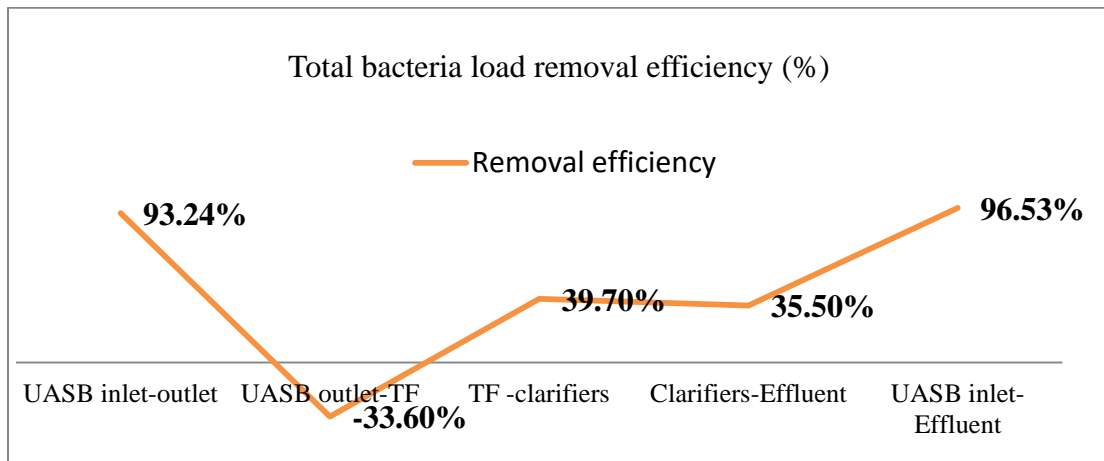


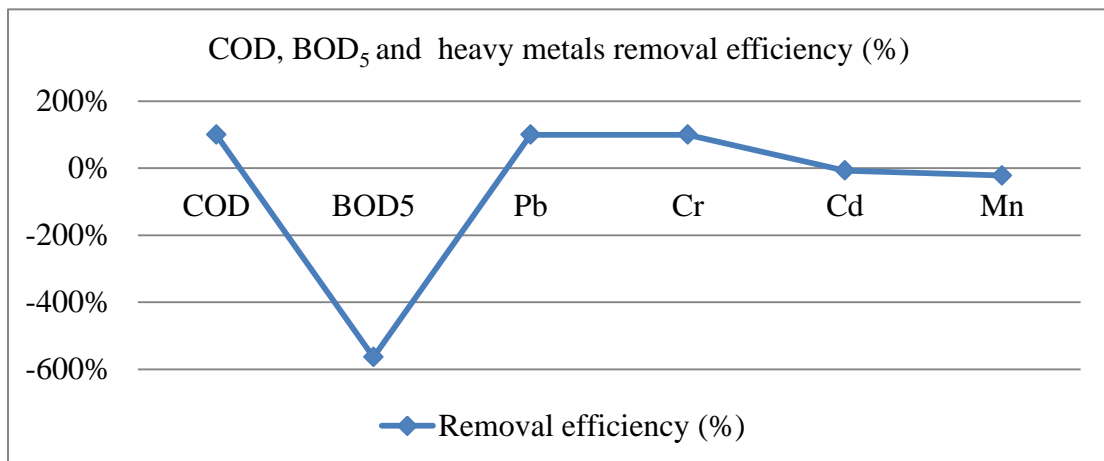
Figure 5: Phenotypic detection of ESBL and carbapenemase production by *E. coli* isolated from WWTP, animals and humans.

4.4.5. Contaminant removal efficiency of wastewater treatment plant

The present finding indicated that WWTP performed significant bacterial removal, with the UASB reactor serving as the primary treatment unit (93.2% reduction) (Figure 6 (A)). However, the trickling filter showed a 33.6% increase in bacterial load. Subsequent treatment processing units particularly the secondary clarifiers (39.7%) and effluent (35.5%) further reduced the total bacterial counts to the entire WWTP removal efficiency of 96.5%. The WWTP demonstrated complete removal of heavy metals Pb and Cr. However, 6.9% and 21.6% increment of Cd and Mn level in effluent. The system achieved 100% COD removal and 564% BOD₅ rise as graphically shown in Figure 6 (B).



(A)



(B)

Figure 6: Removal efficiency of centralized Kality wastewater treatment plant. (A) indicates bacteria load and (B) indicates physicochemical and heavy metals.

5. DISCUSSION

Wastewater treatment plants are vital for municipal water recycling and agricultural irrigation. However, it can represent a significant environmental concern due to their role in proliferation and dissemination of superbugs (Uluseker *et al.*, 2021). The discharge of treated or partially treated wastewater to environments and creates a pathway for MDR pathogens to enter the food chain and ecosystem, posing risks to human, animal, and environmental health (La Rosa *et al.*, 2025). This study, focusing on the Kality WWTP, revealed a high combined prevalence of MDR *E. coli* in cattle, humans, and reclaimed water interface. Besides, the trickling filter of treatment process unit exhibited the co-existence of ESBL and CP *E. coli*. Furthermore, the finding indicated the occurrence and concentrations of selected heavy metals beyond permissible limit across different stages of WWTP processing units. This indicates WWTP serves as hotspot for AMR pathogens.

5.1. Physicochemical Parameter Analysis

Based on the present study results, the physicochemical parameter analysis of the Kality WWTP revealed significant variations across treatment stages, highlighting the effectiveness and limitations of different treatment units. The result demonstrated that the treatment plant's Upflow Anaerobic Sludge Blanket (UASB) reactor effectively reduced COD by 89.12% (496.3 ± 3 to 54 ± 4 mg/L) consistent with the previous studies reporting 80.5% COD removal in the same plant's UASB-trickling filter system (Tessema *et al.*, 2024). According to this research findings, the mean COD concentration level in the final treated effluent was below detectable level that contradicted with the study result report that showed (53 ± 2.21 mg/L) (Shuralla *et al.*, 2024).

The mean COD and BOD concentration of the treated effluent was less than 250 mg/L and 5 mg/L respectively, which are below the EEPA discharge limit (EEPA, 2003). The finding showed the stability of BOD during the UASB treatment processing unit along with its peak at the clarifier stage. The subsequent decrease in BOD suggests effective treatment, aligning with findings that aerobic processes typically yield better removal efficiencies for organic compounds (Khan *et al.*, 2011). This finding disagrees with the

report that showed incomplete removal of BOD in effluent (Olabode *et al.*, 2020). The presence of BOD in the effluent of a WWTP, despite undetectable levels of COD, can be attributed to short hydraulic retention times (HRT) that limit complete microbial degradation of biodegradable organics (Muhammad Al Kholif, 2025).

The increase in electrical conductivity (1.12 to 1.39 mS/cm) and total dissolved solids (TDS) (559 to 693 mg/L) obtained during anaerobic treatment reflects concentration effects resulting from anaerobic digestion process similar to those reported in (Arthur *et al.*, 2022; Dessie *et al.*, 2022). The reduction in pH is consistent with the findings from previous studies, indicating that anaerobic processes tend to produce organic acids (Zieliński *et al.*, 2022). Temperature variations across treatment stages indicated decrease from the inlet to the trickling filter, followed by a modest recovery in the effluent. This variation can influence microbial activity, which is critical for efficient wastewater treatment (Liang *et al.*, 2021).

5.2. Heavy Metals Analysis

Based on this study finding, heavy metal analysis revealed selective occurrence and concentration patterns across different treatment stages. The observed progressive increase in Cd (2.17 to 2.32 mg/L) and Mn (2.08 to 2.53 mg/L) concentrations across treatment stages contrasts with studies reporting declining trends (Abd-El-Kader *et al.*, 2020). This apparent contradiction is likely due to sludge retention time, wastewater composition, and concentration effects from organic matter degradation. The concentration of both Cd (2.32 mg/L) and Mn (2.53 mg/L) in the effluent significantly exceeds established standards (0.01 and 0.2 mg/L) indicating potential risks for environmental and human health (EPA, 2003). The presence of Mn in the UASB outlet, despite its absence at the inlet, can be attributed by internal recirculation, residue accumulation and bound or undetectable forms (Hu *et al.*, 2018; Sylwan & Thorin, 2021).

The result revealed the exclusive detection of Pb and Cr in specific stages of treatment process units of secondary clarifiers (4.33 mg/L) and trickling filters (2.62 mg/L). This random detection may indicate selective retention of particulate Pb or Cr (III) oxidation

to more soluble Cr (VI). The current study did not detect Cu, Zn, Pb, and Cr in the effluent whereas the previous study reported the accumulation of these metals in the effluent (Johnny Girma, 2023). This divergence might be attributed due to seasonal variation, sample preparation and industrial discharge input fluctuation. The observed Cu and Cr result in this study align closely with recent findings comparable performance in similar plant system (Shuralla *et al.*, 2024).

5.3. Total Bacteria Count

The results demonstrated a significant reduction in bacterial load across the treatment stages, with UASB reactor achieving a notable decrease of 1.17 log CFU/mL. The mean bacterial load in the effluent was recorded at 5.86 log CFU/mL. This finding is consistent with previous studies which reported average bacterial concentrations in the effluent of wastewater treatment plants ranging from 4 to 8 log CFU/mL (López *et al.*, 2019). The measured total bacteria count in the effluent (5.85 log CFU/mL) significantly exceeds the established limits (3 log CFU/mL) for irrigation purposes (Al-Gheethi *et al.*, 2018). This indicates effluent still contains a measurable bacterial load that may pose health risks if not adequately managed.

5.4. *Escherichia coli* Carriage Rate

5.4.1. Antimicrobial susceptibility test

The statistical analysis of *E. coli* carriage rates revealed significant relationships between sampling sources and *E. coli* presence. The result illustrated that most of *E. coli* isolates were resistant to tetracycline (80%), ciprofloxacin (71.4%), and sulfamethoxazole-trimethoprim (54.3%). The intermediate resistance to ceftazidime (68.6%) and meropenem (42.9%) is concerning, indicating emerging resistance to last-resort antibiotics. This carbapenems resistance pattern of wastewater based isolate of *E.coli* coincides with the result report that showed resistance of isolate to imipenem (Bessa *et al.*, 2014). The high susceptibility to amoxicillin-clavulanate (91.4%) suggests it remains effective. However, these results appear to contrast with reports of widespread resistance

of *E.coli* isolated from raw, treated wastewater and urine to β -lactam or β -lactamase inhibitor combinations (Emurotu *et al.*, 2024; Kotlarska *et al.*, 2015). This is likely due to spatio-temporal variations, and methodological differences in susceptibility testing.

5.4.2. Antimicrobial resistance pattern

The high rate of overall MDR *E. coli* 97.1% in this study indicates the significant possible transmission and dissemination pathways for AMR. This finding supports the previous study result that similarly elucidated resistant *E. coli* in reclaimed water across multiple antibiotic classes (Papajova *et al.*, 2022). The MARI values for MDR *E. coli* isolates in this study (0.33–0.89) reflect substantial antibiotic selection pressure in the studied wastewater-based epidemiology of indicator organism. This result aligns with finding (0.33–0.73) reported by (Emurotu *et al.*, 2024). Notably, 88.2% of isolates in our study exhibited high-risk MARI values (≥ 0.55) indicating a high risk of contamination where antimicrobials often utilized (Khan *et al.*, 2015).

5.4.3. Phenotypic detection of ESBL and carbapenemase producing *E. coli*

Extended-spectrum β -lactamase and carbapenemase-producing *E. coli* have emerged as significant health threats globally. In this study, *E. coli* isolates exhibiting resistant-intermediate resistance patterns were phenotypically assessed for ESBL and CP *E. coli* using DDST and mCIM, respectively. The results indicated that 20% (7/35) of the isolates tested positive for ESBL production, 5.7% (2/35) for carbapenemase production, and 2.9% (1/35) for co-production of both. Notably, the co-production of ESBL and CP *E. coli* was identified in isolates from the trickling filter system. This finding suggests the escalating issue of the growing concern of AMR in wastewater systems. This result contrasts with previous reports, which indicated a significantly higher prevalence of ESBL *E. coli* isolated from wastewater (63.64%) (Ahmad Zahra *et al.*, 2025). This difference might be due to potential variations in AMR patterns in different environments.

5.5. Contaminant Removal Efficiency of Wastewater Treatment Plant

The contaminant removal efficiency assessment of centralized Kality WWTP demonstrated significant effectiveness in wastewater treatment, achieving an overall bacterial reduction of 1.46 log units, with the UASB process contributing a 93.2% removal rate. This result in agreement with the finding report showed bacterial log removal rate at effluent was positive (0.27 to 2.81) log CFU/mL (Haileselassie *et al.*, 2025). However, the trickling filter showed a concerning 33.6% increase in bacterial levels, due to biofilm formation. Despite substantial pathogen reduction, the final effluent levels of 5.85 log CFU/mL exceeded acceptable limits (EEPA, 2003).

Additionally, the WWTP successfully reduced heavy metals, particularly Pb and Cr, as well as COD indicating effective management of organic loads and metal contaminants. The findings revealed elevated levels of Cd at 6.9% and Cr at 21.6% in the effluent. In contrast, another study reported that the total concentration of heavy metals in the effluent was below standard guidelines, achieving a removal efficiency of 94% or higher (Onchoke & Franclemont, 2022). This difference is likely due to variations in geography, season and analytical methods.

Study limitations

While this study provides valuable insights within a One Health framework, certain limitations should be acknowledged. Resistance gene detection using molecular techniques (metagenomic sequencing) of circulating ESBL and carbapenemase resistance genes (*bla_{CTX-M}*, *bla_{SHV}*, *bla_{KPC}* and *bla_{NDM}*) was not performed due to lack of resources in the laboratory. Additionally, potential co-resistance patterns between antimicrobials and heavy metals were not investigated. Furthermore, seasonal variations in *E. coli* carriage rates were not assessed, as this could help us understand the effects of different seasons on *E. coli* occurrence. These gaps warrant the need for future research incorporating genomic sequencing (metagenomic analysis), co-selection studies, and longitudinal sampling to enhance the understanding of AMR emergence and spread in WWTP influenced environments within one health approach.

6. CONCLUSSIONS AND RECOMMENDATIONS

The present study highlights the occurrence of selected heavy metals and ESBL and carbapenemase producing antimicrobial resistant *E. coli* within one health framework, focusing on Kality WWTP. The treatment plant exhibited high efficiency in bacterial load reduction, achieving an overall removal rate of 96.5%. However, concerning levels of Cd and Mn persisted in the effluent. Additionally, a high prevalence of MDR *E. coli* was detected across One Health domains, with significant resistance to tetracycline, ciprofloxacin and cefotaxime. The incomplete removal of heavy metals, coupled with the detection of MDR isolates, ESBL and CP *E. coli*, underscores the WWTP's role as a hotspot for AMR emergence and dissemination. Adopting a One Health approach in wastewater-based AMR surveillance could provide deeper insights into AMR dynamics and inform more effective intervention strategies. Based on the above concluding remarks, the following recommendations are proposed:

- Implement regular surveillance programs for wastewater-based AMR pathogens monitoring within One Health framework.
- Routinely assess and monitor the removal efficiency of Kality WWTP.
- Strengthening public health awareness regarding AMR risks and antimicrobial stewardship.
- Establish and implement AMR surveillance systems focused on food safety in the studied area.
- Conduct further research employing seasonal variability, antimicrobial and heavy metal co-resistance patterns using meta-genome sequencing.

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8. APPENDICES

Annex 1: Participants informed consent form

PARTICIPANT INFORMED CONSENT FORM

Name/ID of study participant: _____ Age _____ Sex _____

Investigator name: Tesfaye Fatalo Site: Kality Wastewater Treatment Plant

I agree to participate in a study that plans to be conducted on “*Heavy Metals and Antimicrobial Resistance Profiles of Beta-Lactamase and Carbapenemase Producing E. Coli in One Health Paradigm: Kality Wastewater Treatment Plant, Addis Ababa, Ethiopia*”, which will provide insights into the occurrence and concentration of heavy metals, and the AMR profiles of ESBL and carbapenemase producing *E coli* in one health framework.

For this study I have been requested to give information as residents of wastewater treatment plant, on watering and grazing habits of the animals, whether animals had been sick, treated and purchased from other place or not in past months. Besides, I have been requested to give my urine along with fecal sample of the cattle.

The researcher has clearly briefed me that there are no serious risks associated with the research procedure. I have been informed that there is no direct benefit provided to me. I have the right to decide or refuse, withdraw from participating in the research without penalty. He also informed that all the results would be kept confidential. I have been given enough time to think over and even discuss it with others if I wish, before I signed this informed consent. It is therefore, with full understanding of the situation that I gave my informed consent and cooperate at my will in the course of the conduct of the study.

Name (participant) _____ Signature _____ Date _____

Name (investigator) _____ Signature _____ Date _____

Annex 2: Semi-structured questionnaire for confounding variables-Participants

Semi-structured questionnaire- Participants

Section 1: Demographic Information

1. In which age groups are you categorized?

A) Young (<18) B) Adult (18-67) C) Old (>67)

2. Distance from wastewater treatment plant

A) >1000m B) <1000m C) WWTP worker

Section 2: Work History

3. Have you worked in or visited the slaughterhouses in past months?

A) Yes, I worked B) Yes, I visited C) No, not yet

4. Have you traveled internationally in the past months?

A) Yes, I travelled to abroad B) Not travelled

Section 3: Health and Medical Information

5. Have you used antibiotics in the past months?

A) Yes, I used it B) No, I haven't used it

6. Have you visited hospital or had contact with patients in the past months?

A) Yes, I visited B) Yes, I had contact with patients C) No

7. Do you have any chronic health problem like Urinary Tract Infections?

A) Yes, I have it B) No, I haven't

Annex 3: Semi-structured questionnaire to avoid confounding variable-Cattle

Semi-structured questionnaire-Cattle

1. Age Category of Cattle:

- A) Calf (< 6 months) B) Young Stock (7-12 months) C) Heifer (1-2 years)
D) Adult (2-5 years) E) Old Cattle (> 5 years)

2. Have the cattle been diagnosed and received treatment in the past months?

- A) Yes B) No

3. What is the overall body condition of the cattle?

- A) Excellent B) Good C) Fair D) Poor

4. What is the primary source of water for the cattle?

- A) Pond B) Well C) Little Akaki River

5. What is the distance from the nearest wastewater treatment plant?

- A) > 1000 m B) < 1000 m

6. Were the cattle purchased from another area within the past months?

- A) Yes B) No

7. Do the cattle have access to grasses and other green forages grown using irrigation?

- A) Yes B) No

8. Are there any nearby poultry, dairy, beef, or swine farms?

- A) Yes B) No

Annex 4: Procedure for wastewater physicochemical parameter analysis

1. Materials used

- HANNA multiparameter device, DO meter, Photometer

2. Procedures

- HANNA multiparameter device calibrated
- Probe immersed into the beaker containing sample
- Stable reading ensured and result recorded for Temperature, Power of Hydrogen, salinity, Total dissolved solids (TDS) and Electrical conductivity (EC).
- Do meter calibrated using zero oxygen solution, then probe submerged and stirred gently. Finally, result recorded for DO in mg/MI
- BOD bottles were filled with water sample and initial DO measure with DO meter
- BOD bottles containing sample were incubated at 20 degree celsius for 5-days
- Then, final DO measured. And, BOD5 calculated as DO initial – DO final.
- 2ml of wastewater sample was added onto prepared dichromate solution
- Samples were digested in COD reactor at 150 degree celsius for 2Hr
- Cooled and result for COD was recorded (mg/mL) by photometer

Annex 5: Heavy metals analysis

1. Materials and reagents

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

2. Procedures

- 10 ml of conc (65% HNO₃) was added to 5ml of wastewater sample vessel
- Samples were digested in microwave digester at 200 degree celsius for 15min
- Cooled and diluted with 35ml of 5% HNO₃
- The digestate was transferred to 50ml tube for metals analysis
- ICP-OES was calibrated with multi elements standards and blankets used
- Through Autosampler device, ICP-OES, recognize and quantify the metals

Annex 6: Total bacteria enumeration

1. Method

- Total plate count with serial dilutions

2. Procedures

- 1ml of fresh sample was transferred to 9ml of sterile buffered peptone water
- Mixed with vortex and fivefold serial dilution repeated
- 1ml of each dilution poured on plate count agar plates in triplicate
- Incubate for overnight and colony counted (30-300 colonies)
- Expressed as CFU/mL, counted colony “X” dilution factor
- Finally, converted into log₁₀ scale

Annex 7: *Escherichia coli* isolation and confirmation

1. Procedures

- Samples were collected aseptically and transferred to buffered peptone water
- Streaked on MacConkey agar media and incubated for 24hr at 37⁰C
- Pure pink colony subcultured on EMB media and incubated for overnight
- Pure colony with green metallic sheen, cultured on nutrient agar media
- Presumptive isolates confirmed with biochemical and supplementary tests
- Biochemical test (IMVIC) and supplementary tests (TSI and Urease) applied
- Confirmed isolates preserved with BHI media for further analysis

Annex 8: Antimicrobial susceptibility testing

1. Procedures

- 3-5 confirmed colonies from overnight incubated fresh nutrient agar taken.
- Suspension turbidity adjusted to 0.5 McFarland standard
- Suspension streaked evenly on MHA plate with sterile swab
- Different antimicrobial disks aseptically placed on dried plate with dispenser
- Zone of inhibition diameter was recorded after overnight incubation by caliper
- Result interpreted based on CLSI 2022 as R, S and I.
- Through the procedure, *E.coli* ATCC 25922 used as Quality control strain

Annex 9: Phenotypic detection of ESBL producing *E.coli* isolates

1. Procedures

- Turbidity suspension in sterile saline was adjusted to 0.5 McFarland standard
- Sterile swab inoculum evenly streaked onto MHA plate
- Cefotaxime and ceftazidime placed 24mm apart Amoxicillin clavulanic acid
- After incubation, observed enhanced zone of inhibition towards AMC
- If shows enhanced zone of inhibition towards AMC, isolate is ESBL producer

Annex 10: Phenotypic detection of carbapenemase producing *E.coli* isolates

1. Procedures

- 3-5 pure colonies of *E.coli* isolate inoculated to 2ml of TSB agar
- Meropenem disk was added onto it and incubated for 4hr at 37⁰c
- *E.coli* ATCC 25922 suspension in sterile saline adjusted to 0.5 McFarland
- Suspension evenly streaked on MHA plate and let to dry
- Meropenem disk from TSB culture placed on MHA plate
- After overnight incubation, Zone of inhibition recorded and interpreted
- Isolate with no or <15mm of diameter inhibition, Carbapenemase producer
- If isolates exhibited zone of inhibition >19mm, non-carbapenemase producer

Annex 11: Ethical statement for animal based research activities

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

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/73/17/2025

Name of Applicant: **Tesfaye Fatalo** (DVM, MSc student)

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

Title of the project: *Unraveling heavy metals and AMR profiles of ESBL and Carbapenemase producing E. coli within one health paradigm: Scenario of Kality Waste water treatment plant, Addis Ababa, Ethiopia*

Date of application: **December, 2024**
 Nature of the project: **Field animal and environmental investigation**
 Target animal species: **Cattle**
 Number of animals involved: **78**
 Study area: **Addis Ababa, Ethiopia**

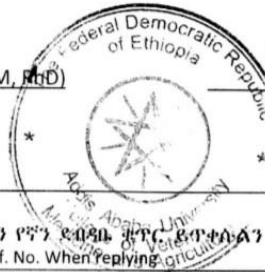
Minutes No. and date of review: **VM/ERC/04/17/025, 25/02/2025**

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 Tesfaye Fatalo.

Professor Getachew Terefe (DVM, PhD)

Chairman

Signature



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Annex 12: Ethical statement for human based research activities

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አዲስ አበባ ፣ ኢትዮጵያ
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ADDIS ABABA UNIVERSITY
Akilu Lemma Institute of Pathobiology (ALIPB)
Addis Ababa, ETHIOPIA
☎ 251-11-276-30-91/213-57-25
e-mail: akilu.lemma@aau.edu.et

Akilu Lemma Institute of Pathobiology Institutional Research Ethics Review Committee (ALIPB-IRERC)

Ethical Clearance Certificate

Ref. No.: ALIPB IRERC/173/2017/25

Date: February 10, 2025

Title of the project: "Unravel of toxic heavy metals and antimicrobial resistance profiles of esbl and carbapenemase-producing *E. coli* In One Health Paradigm: scenario of Kality wastewater treatment plant, Addis Ababa"

PI: Tesfaye Fatalo,
Recommendation of the ALIPB-IRERC

Dear: Tesfaye,

The ALIPB-IRERC has reviewed your above mentioned Research Proposal and noted its merit. The IRERC would like to remind you as the 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 ALIPB-IRERC ahead of time any amendments or modifications in the protocol or premature suspension or termination of the study.

STATUS: **Approved**

Needs **NRERB** clearance:

Yes: ___ No: x

IRERC Chairperson: Berhanu Erko, Prof.

IRERC Secretary: Esayas Akilu, PhD.

Signature: Berhanu Erko

Signature: Esayas Akilu

Approval

Name: Professor Mengistu Legesse, Director

Signature: Mengistu Legesse

Date: Feb 10, 2025



Cc// IRERC office

Annex 13: Some photos credited during research activities



Secondary clarifiers

Chlorination stage

Effluent

Some of sampling points of WWTP



HANNA portable meter

COD digester

DO and BOD measuring

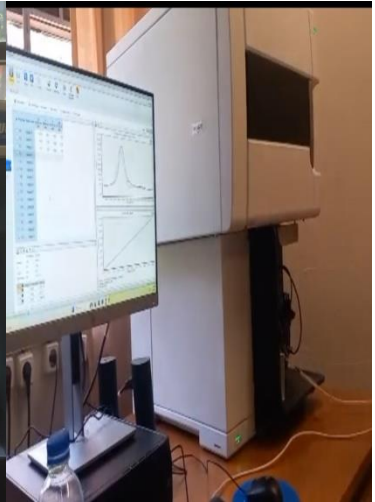
Physicochemical parameters determination



Sample preparation



Microwave digestion

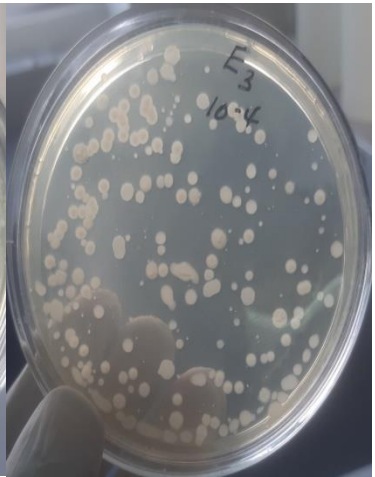


ICP-OES

Heavy metal analysis



Samples collected



Total bacteria load enumeration



TSI test



Urease test



Confirmed E.coli isolate



Antimicrobial susceptibility test



Double disk synergy test (DDST)

Annex 14: Plagiarism check report



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File size: **3.02M**
Page count: **90**
Word count: **19,393**
Character count: **118,680**
Submission date: **09-Jun-2025 11:32AM (UTC+0300)**
Submission ID: **2695326103**



PHYSICOCHEMICAL, HEAVY METAL AND MICROBIOLOGICAL
EVALUATION OF KALITY WASTEWATER TREATMENT PLANT:
IMPLICATIONS FOR ANTIMICROBIAL RESISTANCE AND
TREATMENT EFFICIENCY WITHIN A ONE HEALTH NEXUS

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