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Antimicrobial Resistance Profile of ***Escherichia coli*** Isolated from Hospital Sewage and Polluted River: The Cases of Adama Hospital Medical College in Adama and Yerer River in Dukem

MSc. Thesis

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Declaration by student/statement of author

The undersigned declares that this document is my work. Any external sources used have been appropriately cited and referenced. I take full responsibility for the content, ideas, and conclusions presented herein.

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Approval of Thesis by Supervisor for submission

I certify that **Kajelcha Fikadu's** MSc thesis entitled "Antimicrobial Resistance Profile of *Escherichia coli* Isolated from Hospital Sewage and Polluted River: The Cases of Adama Hospital Medical College in Adama and Yerer River in Dukem" has been carried out under my direct supervision, and I recommend the thesis be accepted as the fulfilling requirement for the degree of masters of Science in health biotechnology.

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Approval Sheet after defense

We certify that **Kajelcha Fikadu's** MSc thesis entitled "Antimicrobial Resistance Profile of *Escherichia coli* Isolated from Hospital Sewage and Polluted River: The Cases of Adama Hospital Medical College in Adama and Yerer River in Dukem" is the final version.

Therefore, we, as the examining board, approved it as the final document to be accepted as fulfilling the requirement for the degree of Masters of Science in health biotechnology.

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Acronyms and Operational Definitions

AIDS	Acquired immunodeficiency syndrome
AHMC	Adama Hospital Medical College
AMR	Antimicrobial resistance
	Definition: The ability of *E. coli* isolates to survive and grow in the presence of antibiotics that would normally inhibit or kill them.
AST	Antimicrobial Susceptibility Testing
CDC	Centre of disease control
COVID-19	Corona virus disease
DDW	Double Distilled Water
EAEC	*Enteroaggregative E. coli*
E.Coli	*Escherichia Coli*
EFSA	European Food Safety Authority
EHEC	*Enterohemorrhagic E. coli*
EIEC	*Enteroinvasive E. coli*
EMB	Eosin methylene blue
EPEC	*Enteropathogenic E. coli*
ESBL	Extended-spectrum beta-lactamase
GAP	Global action plan
GDP	Growth domestic product
GLASS	Global Antimicrobial Resistance Surveillance System
NAP	National action plan
ETEC	*Enterotoxigenic E. coli*
FAO	Food and Agriculture Organization
HIV	Human immunodeficiency virus
LMICs	Low and Middle income countries
MAR	Multiple antimicrobial resistance
	Definition: *E. coli* isolates resistant to at least one agent in three or more antimicrobial categories.
SCA	Simon's citrate agar

SDGs	Sustainable development goals
STEC	Shiga toxin-producing *E. coli*
TSB	Trypton Soya Broth
WHO	World health organization
YRAI	Yere River After Industrial waste entry site/downstream
YRBI	Yerer River Before Industrial waste entry site/upstream
XR	Xadacha River

Abstract

The antimicrobial resistance profile of *Escherichia coli* is dramatically increasing across the world, particularly in low- and middle-income countries where untreated wastewater can disseminate resistant bacteria into the environment. Untreated wastewater discharged from hospitals and industries is the most known vehicle for the emergence and spread of antimicrobial resistance genes. The study aimed to evaluate the antimicrobial resistance profile of *E. coli* isolates from hospital sewage and polluted river samples in Adama and Dukem, respectively. The hospital sewage and polluted water samples were collected from Adama Hospital Medical College and two rivers in Dukem. The samples were transported to Addis Ababa University, Institute of Biotechnology Laboratory. For the purpose of isolating *E. coli*, the samples were cultivated on Eosin Methylene Blue agar, MacKonkey agar, and Nutrient agar media. Biochemical tests, including Gram staining, IMViC, TSI, and SCA, were used for the identification of *E. coli* isolates. The researchers evaluated the antimicrobial susceptibility patterns of the *E. coli* isolates using the Kirby-Bauer's disc diffusion method. The identified *E. coli* isolates were then cultured on Mueller Hinton agar media at 37°C for 24 hours, after which the inhibition zone was measured by a digital ruler. Polymerase chain reaction (PCR) was used to confirm the presence of resistance-associated genes in the *E. coli* isolates. A total of 75 presumed *E. coli* isolates were obtained via culture. Among these isolates, 50 isolates were subjected to AST. The isolates from Yerer River before the industrial waste entry site (YRBI) did not show multiple antimicrobial resistance (MAR), while the isolates from Xadacha River (XR) and Yerer River After Industrial waste entry site (YRAI) showed resistance profiles of 66.67% and 90%, respectively. This study also revealed that 86.67% of hospital sewage isolates and 65% of river water isolates showed MAR. PCR amplification confirmed the presence of *tet*A and *blaTEM* genes in 83.33% and 57.14% of the AMR isolates, respectively. In conclusion, the study showed that untreated hospital sewage and pollute river water are considered major reservoirs for the emergence of antimicrobial-resistant *E. coli* among humans, animals, and the environment. The study advocates for improved wastewater treatment, stringent regulations on antimicrobial use, and regular monitoring to curb the emergence and spread of antimicrobial resistance.

Keywords: *Antimicrobial resistance, Escherichia coli, Hospital sewage, polluted river*

1. Introduction

1.1. Background

Antimicrobial resistance (AMR) has become a major public health concern that requires an urgent intervention. All parts of the world are affected by AMR; however, the impact is disproportionately heavier in low- and middle-income countries (O'Neill, 2016). Several reports have shown that the AMR profile of pathogens is increasing dramatically, and AMR has become among the global health burdens to humans, animals, and the environment they shared. AMR is a prominent health risk that emerges and spreads through unregulated antimicrobial prescriptions, misuse of antimicrobials, lack of access to clean water, poor sanitation and hygiene, poor infection prevention and management systems, and low awareness and knowledge. Also, the COVID-19 pandemic outbreak, which has disturbed almost all healthcare systems and healthcare priority areas, worsened the spread of AMR. The pandemic increased AMR by marginalizing both national and international AMR mitigation programs, followed by the marginalizationof of healthcare priorities in general. This worldwide public health issue is predicted to claim millions of lives annually by the year 2050 if not controlled. The study by Murray *et al.* (2022) estimates that in 2019, there were 4.95 million deaths associated with bacterial AMR, including 1.27 million deaths directly attributable to AMR. This makes AMR a leading cause of death worldwide, surpassing HIV/AIDS and malaria. The study identified the top six leading pathogens that contribute to AMR burden, and *Escherichia coli* is among them (Murray *et al*., 2022).

Untreated hospital sewage, industrial waste, and livestock farms contribute to the emergence and spread of antimicrobial-resistant microorganisms. Antimicrobial drugs are considered emerging micropollutants. Even at low concentrations, their long-term exposure to the environment can have ecotoxicological effects. Antimicrobials are frequently discharged carelessly into urban wastewater treatment plants. Antimicrobials can exert selective pressure on bacteria, leading to the development of antimicrobial resistance. Tiwari *et al.* (2022) reported that MAR bacteria have been found in untreated hospital effluents and wastewater treatment plants (WWTPs). The continuous exposure of bacteria to antimicrobials contributes to the emergence of resistance.

Wastewater systems harbor a reservoir of antimicrobial resistance genes (ARGs). These genes can be inherent, exchanged among bacteria, or acquired from the reservoir (Tiwari *et al*., 2022).

Livestock products are also suspected of contributing to AMR pathogen emergence and spread among animals, humans, and the environment. According to the European Food Safety Authority (EFSA), animal meal merchandise, which includes unpasteurized (raw) milk and undercooked meat or eggs, is one of the main sources of contamination by zoonotic agents in humans (Almansour *et al*., 2023).

This might increase the risk of exposure to AMR bacteria for humans, other animals, plants, avians, and the ecosystems they share. *E. coli* is one of the most extensively distributed microorganisms in the intestinal tract of warm-blooded animals. Most of the *E. coli* isolates are not pathogenic; however, they are more pathogenic and have powerful transmission and colonization abilities, greater global distribution due to efficient dissemination, and greater resistance to various antimicrobial agents. Antimicrobials are used extensively throughout the world for the prevention and treatment of a variety of infections in both humans and animals. The sharp rise in AMR infections can also be attributed to the lack of substitutes for certain medicines. For instance, the WHO reported that fluoroquinolones and extended-spectrum cephalosporins have high levels of resistance. Thus, the WHO has declared antimicrobial as "critical importance and highest priority" for human medicine (Sevilla-Navarro *et al*., 2022). The release of raw industrial and hospital waste water is a major factor contributing to the growth of AMR bacteria. It is extremely likely that these factors are the primary sources of antimicrobial residues and antimicrobial resistance genes (ARGs). As a result, in open environments—which people, animals, and plants frequently share antimicrobial resistance genes can be broadly dispersed (Medugu *et al*., 2018).

The incidence of AMR in low- and middle-income countries (LMICs) is also rising substantially, with one of the main causes being the absence of efficient and quick molecular diagnostic methods. Therefore, diagnosis plays a key role in humans, animals, and environmental health care systems to control the spread and emergence of AMR. A well-documented and structured AMR surveillance at both the national and international levels can address this "silent pandemic". The WHO described diagnostic stewardship as "a coordinated steerage and interventions to enhance suitable use of microbiological diagnostics to manual healing decisions"

(WHO 2016). It is necessary to conduct appropriate and timely diagnostic procedures, such as specimen collection, pathogen identification, and precise, succinct communication of consequences to manually affected individuals. According to Gronthoud (2020), diagnostic stewardship at surveillance sites is among the relevant components of AMR surveillance in the overall AMR management plan (Gronthoud, 2020). In 2016, the WHO developed an important AMR data management system called the "Global Antimicrobial Resistance Surveillance System (GLASS)" for proper implementation of the Global Action Plan (GAP) and National Action Plan (NAP) for antimicrobial resistance. Planning, implementing, overseeing, and evaluating standardized worldwide AMR surveillance is made easier with the help of this technology. The main goal of diagnostic stewardship for AMR is to provide guidelines for specimen management that are applicable to front-line healthcare personnel, such as laboratory and surveillance professionals, on the spot. The other focused groups are health care managers, administrators, and policymakers at different levels. At this level, diagnostic stewardship describes the organizational and structural elements that must be in place to facilitate successful diagnostic stewardship in healthcare facilities and surveillance sites (Gronthoud, 2020). Diagnostic progress is very poor in Ethiopia due to several challenges, including limited efficient and quick diagnostic materials, peace insecurity, and natural disasters. Since there is a lack of current, high-quality data on AMR, further study is required to develop or improve the diagnostic stewardship system (**Figure 1**).

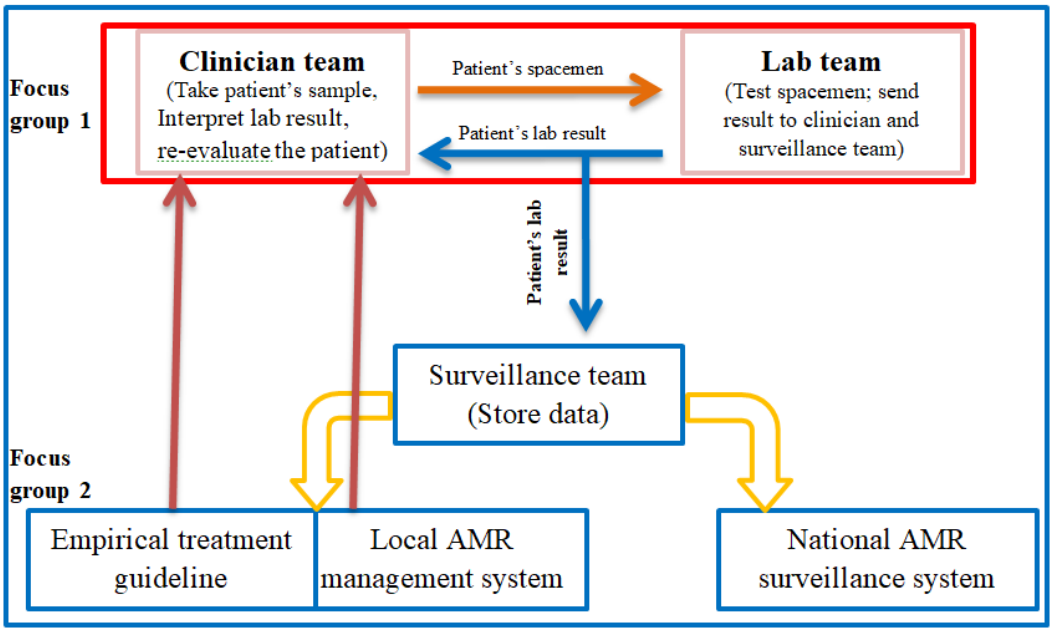


Figure 1: Diagnostic Stewardship Pathway.
An organized AMR diagnostic stewardship platform is very promising for clinicians, laboratory teams, patients, and the wide community at both the national and international levels to combat this public health burden. Efficient and rapid diagnostic stewardship provides quality AMR surveillance data, which in turn supports accurate medical decisions. Source: Adapted from (Gronthoud, 2020)

The emergence and spread of this global public health challenge, AMR, is also associated with animals and the environment. On the other hand, many public health-threatening problems, such as protozoal disease, have been linked to environmental changes and lifestyles. These factors create conducive conditions for the spread of drug resistance genes across species (Oladunjoye *et al*., 2022). As a result, antimicrobial resistance is widespread in humans, animals, and the environment, especially on organic farms where antimicrobial usage is generally limited. There is substantial concern regarding the possibility of AMR involvement in this condition due to a lack of efficient and speedy diagnostic techniques. Likewise, there is a scarcity of safe and affordable vaccinations and medications available for the prevention and treatment of life-threatening infections around the world. According to Capela *et al*. (2019), each endemic country must develop its strategies for proper management of public health problems and develop innovative policies focused on new antiprotozoal drugs (Capela *et al*., 2019).

Several investigations have revealed numerous problems, including psychiatric, neurological, renal, cardiac, respiratory system, hematological, and even SDG-related disorders. These complications pose a significant risk of hospitalization and secondary bacterial infections, where global health systems require timely access to active antimicrobial and vaccines to treat or prevent disease (Silver, 2022). As a result, the development of new and effective antimicrobial and vaccines remains a key groundwork for national and international public health systems to control AMR. The current global crisis, caused by inefficient One Health implementation, may have exacerbated antimicrobial consumption. In addition, the pandemic has marginalized many public fitness priorities and applications, resulting in the interruption of offerings and applications inclusive of AMR surveillance. As a result, complicated AMR mechanisms are evolving and spreading worldwide. This complication is challenging in the treatment of infectious diseases, which can lead to prolonged illness, disability, and death. These issues, taken together, pose a danger to AMR's recent earnings (Silver, 2022).

E. coli can be found in humans, animals, plants, and the environment, where they are highly likely to exchange AMR genes. As a result, understanding the dynamics of AMR through the perspective of one health strategy, which has shown great promise in treating AMR and other public health challenges, is critical. Furthermore, vaccines teach the immune system to make antibodies that fight non-self cells and infections. The process of delivering a vaccine to a target organism is known as vaccination. Vaccination is a form of preventative health care that is easier, safer, and more effective than medications. Several studies have confirmed that antimicrobial consumption is among the key drivers of AMR emergence and spread among the environment, animals, and humans. Vaccines of advanced nature are preferable to other drugs. 1) They are intended to prevent disease rather than cure it after exposure to a specific pathogen.2) They can work for a long period once administered to the target organism. Their nature tends to lead to biological products rather than chemical products, as with most drugs. 4) They are cost-effective, as marginalized groups and populations that may have limited access to other types of health care can receive vaccines (*WHO* 2020).

Therefore, controlling AMR through vaccination is an opportunity to embed the response to this threat in primary health care. Vaccines can function in a variety of ways, including directly inhibiting the transmission of vulnerable and resistant strains and preventing widespread exposure to certain illnesses. Indirectly, this reduces the chance of a pathogen developing drug resistance, preventing the pathogen from spreading within a community and preventing disease-related complications by reducing the incidence of secondary infections. As a result, vaccines minimize the burden of AMR on the capacity of the global public health care system to control infections in humans, animals, and the environment. Generally, vaccines are the first choice for decreasing the risk of both the emergence and spread of AMR in line with the reduction in antimicrobial consumption. This approach is very promising for promoting healthier populations and retaining the effectiveness of antimicrobials (Vekemans *et al*., 2021).

AMR viruses, bacteria, protozoa, and fungi are common contaminants in the environment that are also present in humans, animals, and plants. Excrement and wastewater from humans and animals have been identified and reported as major sources (WHO, 2014). *E. coli* is the best AMR indicator. The reason for this is that it can be found in humans, animals, and the environment in which they live. The *E. coli* strains serve as indicators due to their prevalence,

genetic diversity, and association with both AMR and virulence factors. Pathogenic *E. coli* causes different health problems, such as sepsis and urinary tract infections. Infections with *E. coli* are associated with an increased length of hospital stay, a higher cost of care, drainage of limited resources, high rates of morbidity and mortality, and increased antimicrobial resistivity. Thus, worldwide health experts are highly concerned about the forthcoming incompetence of managing bacterial infections due to increasing rates of AMR across animals, humans, and the environment (Medugu *et al.*, 2022). The misuse of antimicrobials with an increased level of pathogen adaptability at the molecular level has resulted in severe AMR (Sevilla-Navarro *et al*., 2022). To reduce and overcome this global threat, the concepts of One Health and planetary health must be put into practice. The study's goal is to examine *E. coli's* AMR profile and determine the magnitude and degree of resistance, which is critical for monitoring antimicrobial resistance.

1.2. Statement of the Problem

There is widespread worry about AMR, which is one of the leading health hazards in poor nations due to their reliance on agricultural goods. The current economic crisis adds to the global public health burden, exacerbating the AMR dilemma. These two challenges and climatic change are the major driving forces for the spread of antimicrobial-resistant pathogenic bacteria, fungi, protozoans, and viruses. Likewise, antimicrobial-resistant pathogens and their genes have been found in streams, rivers, lakes, and seas. These are commonly traced back to releases from hospitals, farms, or sewage systems. Even properly functioning wastewater treatment systems may not fully remove resistant pathogens and their genes.

Additionally, reducing the likelihood of conducting research has put an enormous burden on health workers and researchers, and it is important to work together with scholars worldwide. Moreover, as a result of the One Health crisis, the globe underwent a significant transformation, and it became evident that intricate difficulties were behind the worrying rise in AMR. The pandemic took center stage, severely disrupting national action plans (NAPs) and global action plans (GAPs) for antimicrobial resistance. This has created the chance of using a single wide-spectrum antimicrobial repeatedly, and the more antimicrobial we use, the greater the likelihood of becoming resistant to the antimicrobial.

AMR poses a significant public health challenge in Ethiopia. Although there is no comprehensive summary of existing AMR data in the country, recent research sheds light on the prevalence of antimicrobial resistance. A systematic review analyzed data from original studies conducted between January 2009 and July 2019 in Ethiopia. Among 48,021 study participants, 15,845 isolates were tested for antimicrobial resistance. Gram-positive bacteria showed resistance to Vancomycin, ranging from 8% (Enterococcus species) to 20% (*S. aureus*). Gram-negative pathogens, including *E. coli*, were resistant to key antimicrobial agents described in the national treatment guidelines. These resistant pathogens were associated with various clinical conditions such as urinary tract infections, diarrhea, surgical site infections, and the like. The prevalence of AMR in Ethiopia is high, emphasizing the need for evidence-based treatment guided by local susceptibility patterns (Berhe *et al*., 2021).

Moreover, there is no rapid and efficient molecular diagnostic tool available for LMICs. These are among the serious issues that lead to a scarcity of quality national and global AMR surveillance data. To the best of our knowledge, there has been no previous research in Adama and Dukem in this country, despite the fact that multiple studies on the molecular characterization of AMR in *E. coli* have been conducted; the majority of AMR-focused research has been limited to Ethiopia's capital city, Addis Ababa. As a result, the likelihood of AMR spreading and developing has increased. These challenges are most prevalent in emerging and developing countries, such as Ethiopia. Likewise, untreated industrial sewage water and hospital wastewater are highly contaminating environments. According to Dadi *et al.* (2017), the local environment, people, and livestock in and around Dukem are exposed to highly contaminated effluents (Dadi *et al*., 2017). As a result, the goal of this study was to assess the current AMR profile of *E. coli* and provide recommendations for addressing antimicrobial resistance. Moreover, the study emphasized the evaluation of antimicrobial susceptibility patterns of *E. coli* in hospital sewage water and polluted rivers.

1.3. Hypothesis

We hypothesized that untreated hospital sewage water and industrial waste water discharged into the environment are more likely to serve as channels for the establishment and spread of antimicrobial resistance genes. *E. coli* isolates from the Yerer River after the Industrial waste site were more resistant than isolates from the same river Before the Industrial waste entry point that did not receive industrial wastewater. Furthermore, the study hypothesized that the *E. coli* isolates from the selected sites belong to the high-risk group and are resistant to the majority of the commonly used antimicrobials.

1.4. Objectives of the Study

1.4.1. General Objective

The general objective of the study was to assess the AMR profiles of *E. coli* isolates from Adama hospital medical college sewage and the two rivers, Yerer and Xadacha, in Dukem.

1.4.2. Specific Objectives

- To isolate and identify *E-coli* strains from hospital and river water samples
- To characterize AMR *E-coli* isolated from each sample
- To examine the antimicrobial resistivity patterns of the *E-coli* isolates

2. Literature Review

2.1. Overview of *E. coli* pathogenesis

E. coli is a commensal and intestinal bacterium that can cause both inflammatory and non-inflammatory diarrhea. It is the most common antimicrobial-resistant indicator organism aligning with the One Health approach. The reason is that *E. coli* is highly prevalent in both humans, animals, and the environment. In addition, it is a fast-growing bacteria, allowing scholars to test the AMR patterns in a short period (Murray *et al*., 2022).

There are several *E. coli* pathotypes, which include enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (EPEC), and enteroinvasive *E. coli* (EIEC). These conditions are commonly associated with noninflammatory diarrhea. Enterotoxigenic *E. coli* (ETEC) causes non-bloody, watery diarrhea and belly cramping. Travelers's diarrhea is often caused by an overabundance of contaminants. ETEC, for example, produces two types of toxins: one that is heat-labile, releasing chloride when cAMP is activated, and another that is warm-tolerant, causing watery diarrhea in the small intestine that is unlikely to be allergic. Shiga toxin-producing *E. coli* is also known as enterohemorrhagic *E. coli* (EHEC) (STEC). Symptoms of these illnesses include inflammation, bloody diarrhea, stomach cramps, fever, and vomiting. Hemolytic uremic syndrome is one example of how this might cause a severe headache. This infection can spread through the fecal-oral route and by eating infected foods. *E. coli* O157:H7 causes approximately 50% of EHEC infections. Non-O157:H7 *E. coli*, which causes much less dysentery than the other STEC strains, is more generally visible within the southern 1/2 of the globe. When the intestines get infected, germs cling to the wall and form sores inside the mucosal cells, compromising the mucosa's integrity. Pollutants can penetrate through intestinal epithelial cells to endothelial cells, which line blood vessels. These toxins can reach the gut and other visceral organs, such as the kidney, leading to complications such as hemolytic uremic syndrome (HUS), which can occur when damage or inflammation occurs in the kidney’s small blood vessels, leading to pallor, lethargy, and decreased urination. In general, antimicrobial/antimotility medications are not advised for EHEC (Lee *et al*., 2021).

2.2. The Concept of Antimicrobial Resistance

AMR is now recognized as a global public health burden and emergency. It occurs when bacteria, viruses, fungi, and parasites evolve mechanisms to protect themselves from the effects of antimicrobial drugs, rendering these drugs less effective. AMR makes infections harder to treat, jeopardizing medical procedures like surgery, caesarean sections, and cancer chemotherapy. AMR jeopardizes advancements in contemporary medicine. Antimicrobial abuse and misuse are the main causes of drug-resistant diseases in people, animals, and plants. Poverty and inequality disproportionately affect low- and middle-income countries, exacerbating the effects of AMR. According to World Bank forecasts, AMR could result in 3.4 trillion USD in annual GDP losses by 2030 and an additional $1 trillion in healthcare expenses by 2050. This leads to an inability to ensure universal access to quality diagnosis and appropriate treatment. To resolve these challenges, it needs to focus on strategic information and innovation, including surveillance of AMR, research, and development for new vaccines, diagnostics, and medicines (World Health Organisation, 2021).

Furthermore, Murray *et al*., (2022) found that antimicrobial resistance was associated with about four million out of five million fatalities worldwide, as well as a number of other causes of sickness and HIV/AIDS. It is far more than the 700,000 annual passes that the World Health Organization (WHO) and the United Nations have recommended. By 2050, it is projected that the number will increase by three million (Trotter *et al*., 2019). However, the reality that emerged immediately revealed that we are much closer to that figure than we had previously imagined (Irfan *et al*., 2022). AMR flows widely between nations and frequently face a variety of significant challenges. As routine antimicrobial use increases, so will the possibility of AMR (Savin *et al.*, 2020).

Furthermore, research shows that AMR-related infections would continue to reduce global GDP by 2% to 3.5% by 2050, costing USD 60-100 trillion (Neill, 2014). *E. coli* is a colonist of the gastrointestinal tract and feces of warm-blooded mammals and reptiles (Leekitcharoenphon *et al.*, 2021). More than 2 million people are annually infected by *E. coli*, a widespread and weak commensal bacterium that can cause both secondary and supplementary gastrointestinal ailments. Different hosts and physical components unite to form the ancestral structure of

commensal *E.coli*. Ancestral factors also complicate bacterial pathogenicity by indicating changes in commensal environments (Leekitcharoenphon *et al*., 2021).

2.3. Mechanism of antimicrobial resistance development

AMR is the capacity of a bacteria, virus, or fungus to prevent an antimicrobial from working normally. AMR is a growing concern in the medical community. *E. coli* is one of the most common bacteria that cause infections in humans. The emergence of AMR in *E. coli* is a complex process involving several mechanisms. *E. coli* can develop resistance to antimicrobials through mutations in its DNA. These mutations can alter the structure of the bacterial cell wall, making it more difficult for antimicrobials to penetrate and kill the bacteria. They can also acquire resistance genes from other bacteria through horizontal gene transfer. This approach includes the switch of genetic cloth from one bacterium to another. This can occur through several mechanisms, including conjugation, transduction, and transformation. *E. coli* can develop resistance to antimicrobials by producing efflux pumps. These pumps are proteins that pump antimicrobials out of the bacterial cell before they can cause any damage. They may develop resistance to medications by forming biofilms, in which the matrix prevents drugs from reaching the bacterium. Furthermore, by producing enzymes that break down antimicrobials, *E. coli* may become resistant. These enzymes can break down antimicrobials before they can cause any damage. When these mechanisms work together, they can increase the resistance of *E. coli* to multiple antimicrobials, making it difficult to treat infections caused by this bacterium (Galindo-Méndez, 2020). The mechanisms of AMR in *E. coli* have been well documented. *E. coli* uses various enzymes to modify drug recognition sites and break down the β-lactam ring in the structure of drugs. For example, in fluoroquinolones, the pathogen uses efflux pumps and DNA *gyrase* mutations as mechanisms of resistance. The continuous use of drugs induces an increase in resistance mechanisms, which is why there is a need for continuous research on drug effectiveness and the discovery of new and better medications to fight against *E. coli* pathogens (Figure 2) (C. Jariremombe, 2023).

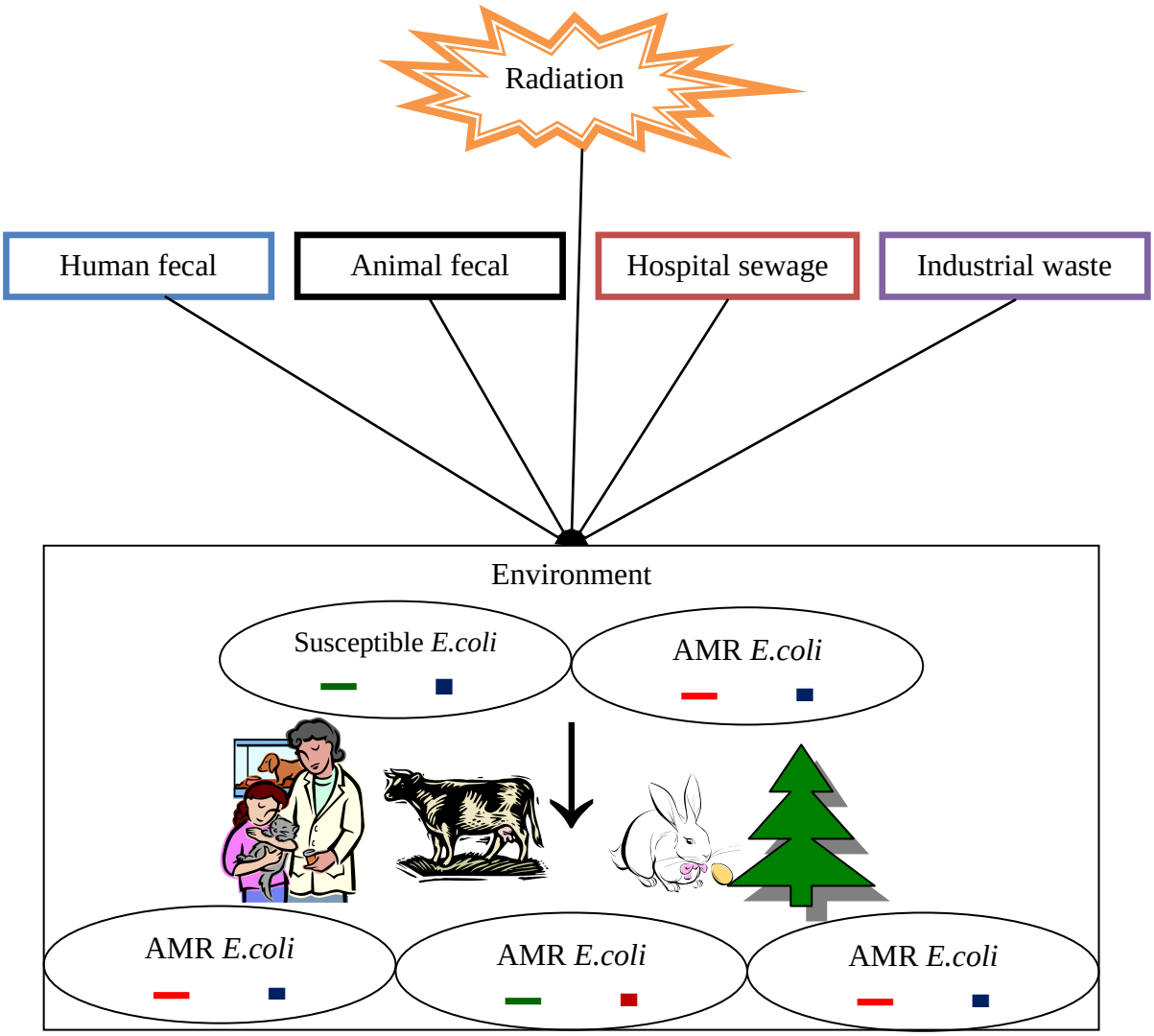


Figure 2: Reservoirs of AMR genes and their possible spreading and emerging mechanism. Human and animal feces, untreated hospital sewage, and industrial waste all contribute to the emergence and spread of AMR. Discharging these wastes into the environment can cause mutation and horizontal gene transfer, resulting in AMR *E. coli*.

2.4. Potential Threats from AMR

Since the protocol's inception and Alexander Fleming's 1928 discovery of antimicrobials, great breakthroughs have been made in the research and manufacture of antimicrobials for medicinal applications. However, the current slowdown in AMR research and development, combined with inappropriate antimicrobial use for both humans and animals, has contributed to the continued discovery of novel antimicrobial agents and the growth in resistance to them. AMR poses a significant threat to public health, economic stability, and medical advancements. Because humans and animals have such close contact, there is a strong concern that *E. coli* found in animals may transmit AMR to humans. Furthermore, the origins and spread of AMR are complicated by interactions between humans, animals, and the environment (Chowdhury & Chakraborty, 2017).

2.5. Factors promoting the emergence and spread of AMR

Understanding the determinants of AMR should come first in order to effectively address the warning that AMR represents. *E. coli* can help spread AMR by creating around 1 million progenies in a matter of hours, resulting in genetic change because of the opposing animals' rapid copy cycles. According to sources, AMR is a natural process (Oladunjoye *et al.*, 2022). The rise of AMR AMR is one of the greatest well-being warnings worldwide. Furthermore, antimicrobial misuse is widely acknowledged as the leading cause of AMR in humans and animals. Commensal microbiota, particularly stomach bacteria, is known to contribute to the development of AMR (Leekitcharoenphon *et al*., 2021).

2.5.1. Lack of rapid and efficient diagnostic facilities

In this era, rapid and efficient diagnostic tools are the main focus area in public health management for preventing or reducing global public health challenges such as AMR. The global One Health issues have given rise to a greater opportunity to pay more attention to the development, distribution, and proper utilization of rapid and efficient molecular diagnostic tools. In 2023, Kapona reported that almost all emerging and developing countries are suffering from the inaccessibility of modern diagnostic facilities, which is very promising for the management of AMR. Clinicians who work in developed countries have access to sophisticated, expensive, automated systems and molecular methods that can provide rapid pathogen

identification and antimicrobial susceptibility testing (AST) directly from patient samples. On the other hand, many hospitals in low- and middle-income countries (LMICs) use traditional culture-based practices. These approaches necessitate the growth or culture of bacteria extracted from patient samples in order to identify the specific pathogen and conduct antimicrobial susceptibility testing. The process may take up to three days. As a result, it is challenging to identify the specific bacterium that causes the infection, and clinicians are treating patients based on their symptoms and their own clinical experience. The most common treatment is broad-spectrum antimicrobials, which are effective against a wide range of bacteria but may also promote resistance (Kapona, 2023).

AMR surveillance at the local, national, and international levels is a key prerequisite for improving human, animal, and environmental health systems and managing health emergencies by providing early warnings of emerging AMR pathogens. Despite the disease's potentially substantial impact, the challenges of getting and assessing high-quality data on resistant infections in low- and middle-income countries severely limit national AMR monitoring statistics. The inability to access modern and rapid molecular diagnostic facilities is the leading challenge for obtaining poor AMR surveillance data across developing countries such as Ethiopia (Iskandar *et al*., 2021).

2.5.2. Improper utilization of antimicrobials

Several studies have indicated that Alexander Fleming in the 1940s warned the public about the high demand for antimicrobial in the future, which could lead to their improper utilization. Overuse, inaccurate prescription, and self-medication of antimicrobial cause selection pressure on bacteria, promoting the establishment and spread of AMR and making infections more difficult to treat. In 2017, the WHO reported that many global public health surveys indicate that many patients utilize antimicrobial regardless of their specifications. For instance, they use antibacterial agents for treating viral diseases such as the common cold (WHO Geneva, 2017). Although AMR is a naturally occurring mechanism, any improper use of antimicrobial can alarmingly accelerate its emergence and spread over time. AMR can be delayed over time as organisms adapt their metabolism to changing environmental conditions, but it cannot be totally avoided. Consequently, combating this global public health burden, AMR, requires holistic interdisciplinary teamwork and collaboration between governments, nongovernment agencies,

industries, policymakers, higher institutions, researchers, and the wider community (Dadgostar, 2019).

2.5.3. Low economic status and unequal funding

AMR is a growing concern in low- and middle-income countries (LMICs). The World Health Organization (WHO) has been working to scale up support to LMICs to address AMR through support. Ethiopia is one of the first seven countries—Egypt, Ethiopia, Jordan, Indonesia, Nigeria, Pakistan, and Sudan to receive a comprehensive technical package to guide the implementation of national AMR movement plans that are primarily customized to the specific needs of the United States. Several researchers have reported that global antimicrobial use alarmingly increased by 65% in five years from 2000–2015. The dramatic upward thrust in international antimicrobial use is predominantly due to the overconsumption of antimicrobial in emerging and developing countries, which is the direct result of rising incomes (Klein *et al*., 2018; Mbchb *et al*., 2018). To put it another way, there is evidence correlating antimicrobial use to increased GDP and housing demand in low- and middle-income countries (LMICs) (Dadgostar, 2019). Low economic status and unequal funding drive AMR by promoting antimicrobial misuse, inadequate healthcare, poor sanitation, and limited research, allowing resistant strains to emerge and spread, especially in low-income regions. This lack of funding can impede progress in turning plans into action. Furthermore, the bulk of people in developing countries rely on animals and the products they produce, which may necessitate adding more antimicrobial to livestock feed (Boeckel *et al.*, 2015).

2.5.4. Poor antimicrobial regulatory framework in developing countries

Weak regulatory frameworks and inadequate enforcement are significant factors that contribute to the spread of AMR. Many countries have limited or outdated antimicrobial use regulations, leading to overuse and misuse. Regulations often fail to address prescription, over-the-counter availability, and agricultural use. In countries where antimicrobial may be purchased without a prescription, self-medication is common. Even with regulations, enforcement is often weak. Regulatory bodies lack resources, capacity, and political will to monitor and enforce guidelines. Penalties for non-compliance are inadequate (Ayukekbong *et al*., 2017).

Regulatory frameworks vary across regions and jurisdictions, creating loopholes for misuse, especially in areas with weaker regulations. Cross-border movement further undermines local regulations. Vested interests, like pharmaceutical and agricultural lobbyists, pressure policymakers to maintain lax regulations, hindering stronger policies. Regulatory bodies, particularly in resource-constrained environments, lack the necessary budget, manpower, and experience to conduct effective monitoring and enforcement. This results in poor data, inadequate surveillance, and ineffective AMR interventions. Therefore, addressing these challenges requires strengthening regulations, improving enforcement, enhancing coordination, increasing agency resources, and minimizing vested interests. Robust, well-enforced regulations are crucial to curb antimicrobial misuse and mitigate AMR (Anderson *et al*., 2019).

2.6. One Health approach in the fight against AMR

One Health is an integrated approach that recognizes the interconnectedness of human, animal, and environmental health. The approach addresses AMR by integrating human, animal, and environmental health, fostering coordinated efforts to reduce antimicrobial misuse and mitigate resistance across all sectors. If the One Health concept is not properly implemented, the close relationship between humans and animals may play a significant role in the development and spread of antimicrobial resistance. A lack of shared One Health perspectives and information on AMR can result in a rapid rise in economic difficulties and AMR mortality. Only five of the forty-three studies examined AMR through the lens of a single Ethiopian health system, according to a 2022 study by Fujita and colleagues (Fujita *et al*., 2022).

Since 2011, Ethiopian authorities have been part of a global network for mitigating AMR risk within a national plan framework. The basic strategy for combating and managing AMR has been in place since 2015. Ethiopia now embraces a concerted, collaborative, and integrated One Health system to provide a sustainable solution to the global planetary fitness problem and AMR threats (Fujita *et al*., 2022). In 2019, around 50,000 people died as a result of AMR infections, making it difficult to compile AMR data. Despite Ethiopia's early start and substantial advances in AMR prevention and management, the government concedes that the country still lags behind in terms of the hazards and risks connected with AMR, which remain serious. This is due to inadequate multisector harmonizing, insufficient and low-quality data on antimicrobial use and

resistance status, a lack of sustainable financing, suboptimal practices, and an inadequate number of trained professionals in one health system (Ibrahim *et al*., 2019).

The World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the World Organization for Animal Health (OIE) evolved the Global Action Plan on AMR in 2015. The World Health Assembly subsequently adopted it. Later, the World Health Assembly accepted it. The Global Framework for Streamlining Collaboration among Unique Sectors incorporates the collaborative, multizone, transdisciplinary One Health concept, which is underlined in the Global Action Plan. It employs AMR in this case and is designed to keep you safe (WHO, 2015; Fujita *et al*., 2022). Ethiopia is committed to the One Health approach, supported by the National One Health Platform and multiple stakeholders. Despite early efforts in AMR prevention and progress, challenges remain, including inadequate multisector coordination, insufficient data, lack of sustainable financing, suboptimal practices, limited trained professionals, and insufficient laboratory facilities. The government has endorsed policies like the Public Health Policy (1993), Animal Diseases Prevention Proclamation (2002), and the National One Health Strategic Plan (2018–2022) to address these issues (Erkyihun *et al*., 2022).

3. Materials and Methods

3.1. Study Area

The study was carried out in the vicinity of Adama and Dukem in the eastern Oromia region of Ethiopia's. Hospital effluent was collected from Adama Hospital Medical College, and contaminated river water samples were collected from the Yerer River After Industrial Waste Entry Site (YRAI). Samples from the Yerer River Before Industrial Waste Entry Site (YRBI) and the Xadacha River (XR) were collected for use as controls. The river samples were all from Dukem. The XR is located 3 km away from Dukem town and known to be free from the point, industrial, pollution. Several untreated industrial sources have polluted the Yerer River, which flows through Dukem. Ethiopia is a country in northeastern Africa and part of the Horn of Africa. Sudan and South Sudan lie to the west, Eritrea and Djibouti to the northeast, Somalia to the east and southeast, and Kenya to the south. Ethiopia has a national government system involving 10 regional states, 2 city administrations, and 840 districts. Ethiopia, which covers an area of 1.1 million km2, has a diverse climate and landscape. The country's climate can be divided into three zones: the recent zone, which includes all tropical and arid areas with temperatures ranging from 27°C to 50°C; the alpine vegetated cool zones at more than 2600 meters above sea level, where temperatures range from near freezing to 16°C; and the temperate zones, where the majority of the population lives.

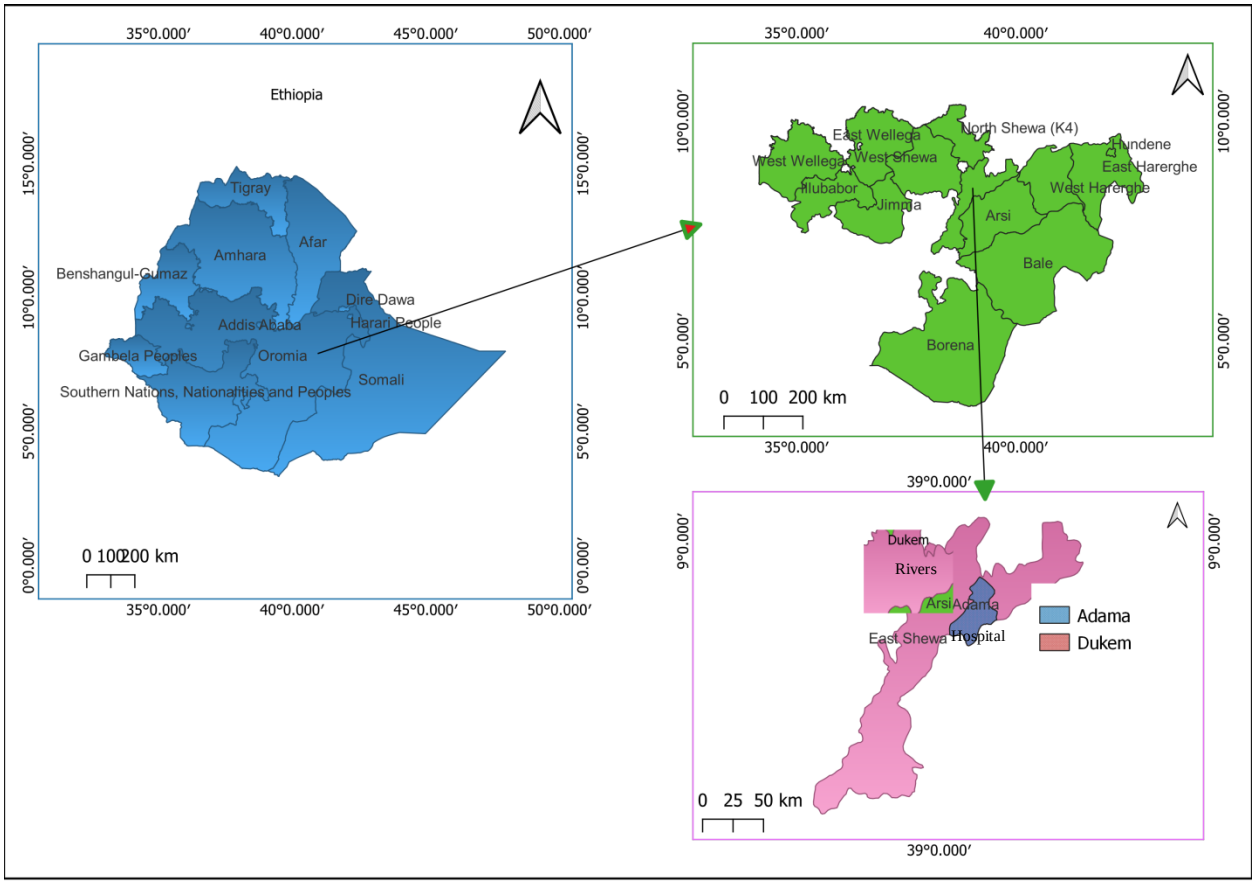


Figure 3: A map of sample sites (Adama Hospital Medical College, YRBI, YRAI, and XR) generated using QGIS version 3.24 software

3.2. Research Design

The research involves a cross-sectional study. This approach was used to examine the AMR profiles of *E. coli* isolates from river water in Dukem and hospital sewage water in Adama. The cross-sectional study design was chosen because it is both quick and affordable. The presence or absence of AMR in *E. coli* would be investigated. Data collection involved a mix of qualitative and quantitative data. The widely used statistical program SPSS version 26 was used to investigate the prevalence and AMR of *E. coli* isolates across the selected antimicrobials. Likewise, it was used to compare AMR profiles among the isolates from different sampling sites.

3.3. Sample Collection and Transportation

The samples were gathered from the YRAI, YRBI, and XR in Dukem, as well as the Adama Hospital Medical College. Purposive sampling was used to gather liquid samples following the

wastewater sampling method (Eaton, 2005). Every sample from the hospital was collected over the course of five days using a sterile leakproof stool cup. Three sampling locations in Dukem were used to collect the river water samples: two were from Yerer River and one from XR. After mixing, some samples were taken from stagnant water, while the remaining samples were taken from the surface. Ten samples were taken from XR at 30-meter horizontal intervals. The samples were taken upstream to prevent disturbing the water, beginning close to the main road to Addis Ababa and Dukem. In the case of the Yerer River, samples were collected from two sampling sites at the site before and after the industrial waste entry sites. Similarly, a total of 10 water samples were collected from downstream site of the industrial waste discharge point. Five water samples were collected before the industrial waste entry site at 50 meters upstream of the industrial waste entry point of the same river. The samples were subsequently shipped in an icebox within a day of collection to Addis Ababa University's Institute of Biotechnology and stored at refrigeration temperature (+4°C) till the study began. The sampling sites were chosen to fill a gap in research evaluating the AMR profile of *E. coli* isolates from hospitals and contaminated waterways in Adama and Dukem, respectively. A cross-sectional study was carried out to determine the AMR of the *E. coli* profile. It was also aimed to characterize AMR patterns and confirm common resistance genes in *E. coli* strains between February 2023 and June 2024. Furthermore, the isolates are extremely questionable since they were exposed to a variety of human activities, including animal waste, untreated chemical residues from industry, healthcare facilities, pharmaceuticals, and other potential sources of pollution.

Table 1: List of sample sites and the number of each sample

Sample sites	**Sample sub-sites**	**Amount of samples**
Hospital	Adama Hospital Medical College	n = 15
River	Yerer River Before Industrial Waste Entry Site	n = 5
	Yerer River After Industrial Waste Entry Site	n = 10
	Xadacha River	n = 10
		Total = 40

3.4. Isolation, identification and characterization of the *E. coli* isolates from the collected samples

Phenotypic characterization approaches were used to isolate and identify *E. coli* from hospital sewage and river water samples. Each sample was enriched in Trypton Soya Broth (TSB) and incubated overnight at 37°C. Then, 0.5 mL of each cultivated sample was inoculated on EMB agar and incubated at 37°C for 24 hours. Spread plate and streak plate techniques were used to isolate and identify *E. coli* isolates using various selective and differential media, including Eosin Methylene Blue (EMB) agar, Maconkey agar, and nutrient agar media. After one day of incubation, the streak plate method was employed to purify the overnight culture by extracting a single colony. Presumptive *E. coli* isolates were defined as overnight colonies with typical dark red to purple red coloring and a metallic sheen. To determine whether the colonies were pink (showing lactose fermentation) or pale (representing non-lactose fermentation), the separated colonies from each sample with a greenish metallic sheen color were picked up, streaked on MacConkey agar, and incubated at 37°C for 24 hours (Panchalingam *et al*., 2012). Next, well-separated colonies were cultivated on nutrient agar media to retain pure *E. coli* cultures for future research, such as biochemical tests or molecular analysis. The phenotypic and genotypic characteristics of the *E. coli* isolates were determined using standard bacteriological methods such as colony structure determination and biochemical tests, including IMViC and TSI. The putative *E. coli* isolates were identified using the standard diagnostic microbiologic procedures outlined by Panchalingam *et al*., (2012).

3.4.1. Biochemical tests

Each isolate was exposed to several biochemical tests, including methyl red synthesis, the Indole test, the *catalase* test, triple sugar iron utilization, and Simmons' citrate utilization. These biochemical tests identified the isolates at the genus level.

Triple sugar iron (TSI) utilization test

It was determined whether the isolates could survive solely on sugars (glucose, lactose, and sucrose). The experiment was performed using three sugar-enriched TSA agar mediums. Sterilized test tubes were used to culture the isolated colonies on TSI agar slants for an entire day

at 37°C. The following day, positive outcomes were noticed, including the formation of gas bubbles and a golden tint.

Methyl red test

The methyl red test was used to determine the isolates' ability to oxidize glucose and create low-pH acidic compounds. After preparing the MR-VP broth, the isolates were injected into separate test tubes. To promote proper growth, the inocula were incubated at 37°C for the entire day. Both the control and inoculum-containing tubes received four to five drops of methyl red indicator reagent and were incubated for five to ten min each. A crimson color developed on the top layer of the positive isolates, except for the control tube, indicating a successful test.

Voges–Proskauer test

The goal of this test was to see if the isolates could convert acetyl methyl carbinol into neutral end products that weren't acidic. The isolates were grown in MR-VP broth at 37°C for one day. After shaking for 30 seconds, each test tube, including the control tube, received 4-5 drops of 40% KOH and alpha-naphthol and was left open for at least 15 min. There was no color change, indicating that the target isolates did not have a beneficial outcome.

Catalase test

Catalase tests were performed to assess the isolates' ability to convert hydrogen peroxide into oxygen and water. A loop of overnight inoculums was applied on a glass slide and thoroughly mixed with a drop of hydrogen peroxide (3%). In the case of the isolates, a bubble appeared after 5–10 seconds, indicating a positive outcome.

Simmon's citrate test

Simmon citrate agar uses citrate as a carbon source and ammonium as an energy source. To determine whether the isolates could use these components, they were cultured on a slant citrate agar medium for 24 hours at 37 °C. The medium did not change color, indicating a negative outcome consistent with E. coli.

Indole test

To find out if the isolates could hydrolyze tryptophan to indole, an indole test was conducted. A loop of isolates was incubated at 37°C for the entire night in a test tube filled with peptone water (which also contained sodium chloride and peptone). When 3–4 drops of Kovac's reagent were added the next day, a cherry-red coating formed within 10–15 min, indicating a favorable outcome. This proved that the isolate produced indole from tryptophan.

The subsequent flow of the study is shown in the following diagram (**Figure 5**).

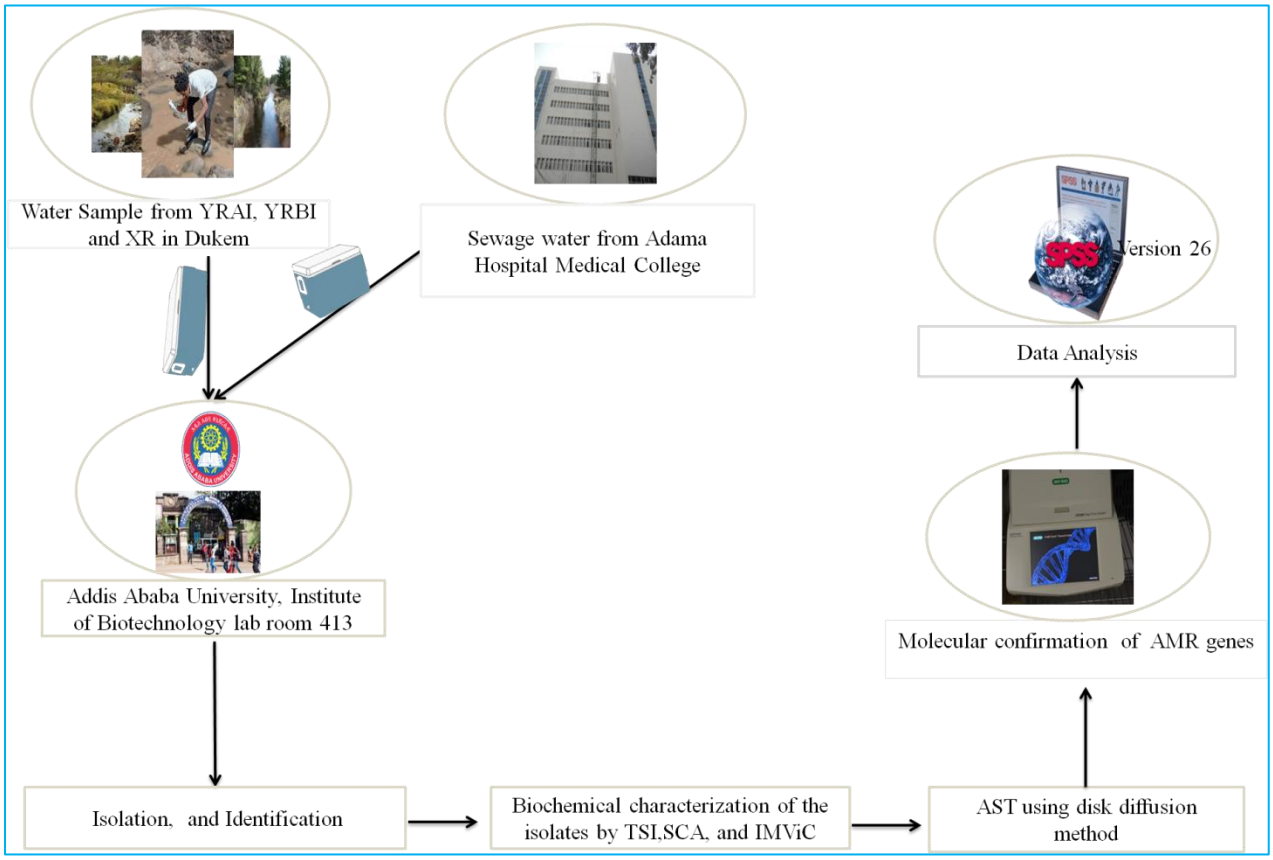


Figure 4: The general workflow diagram of the laboratory procedures for the isolation, identification, and characterization of potential *antimicrobial E. coli isolates* from hospital sewage in Adama and the river water in Dukem.

3.5. Antimicrobial Susceptibility Testing (AST)

The AMR of the *E. coli* profile from hospital sewage and river water samples was determined using the *Kirby Bauer disc diffusion* method, which is the most widely used and economical method for determining antimicrobial susceptibility on Mueller Hinton agar. The test was

conducted based on the Clinical and Laboratory Standards Institute's (CLSI) guidelines. *E. coli* isolates were tested for resistance to the most widely used antimicrobial agents, such as Gentamycin (10µg), Norfloxacin (10µg), Ciprofloxacin (5µg), Tetracycline (30µg), Compound sulphonamides (300µg), Ampicillin (10µg), Vancomycin (10µg), Chloramphenicol (30µg), Streptomycin (10µg), Erythromycin (10µg), Naldic Acid (5µg) (Thermo Scientific™ Oxoid™, United Kingdom) (Daoud *et al*., 2020). The antimicrobial disks were carefully placed on the surface of the inoculation plates at a distance to avoid overlapping the inhibition zones, and then incubated at 37°C for 24 hours. The findings of the overnight inoculation were evaluated by the Clinical and Laboratory Standards Institute protocol. Multiple antimicrobial resistance (MAR) refers to pathogenic bacteria' resistance to three or more antimicrobials (Wolfensberger *et al*., 2019).

Percentage of MAR *E. coli* = $\frac{Number\ of\ MDR\ E.coli\ isolates}{Total\ number\ of\ E.coli\ isolates\ tested} \frac{x100}{1}$

For MAR *E. coli* isolates, the MAR index was calculated as follows:

MAR index $= \frac{a}{b}$, Where **"a"** is the number of antimicrobials to which the isolate resists and **"b"** is the number of antimicrobials to which the isolate was exposed. The MAR index is a straightforward and informative measure of AMR in bacterial isolates. It helps in understanding the extent of resistance and assessing the potential risks associated with the spread of AMR bacteria. A low-risk category corresponds to a MAR index less than 0.2, while a high-risk category corresponds to a MAR index greater than 0.2. Therefore, organisms with MAR indices above 0.2 indicate the presence of MAR genes, often originating from environments with antimicrobial abuse (Dela Peña *et al*., 2022).

Kirby–Bauer disk diffusion

The disc diffusion method was utilized to determine the antimicrobial susceptibility (AST) of the selected antimicrobial medications against the alleged *E. coli* isolates. The resistance breakpoint was established according to CLSI recommendations. The major goal of the Kirby-Bauer disk diffusion AST is to identify the sensitivity or resistance of *E. coli* isolates to various antimicrobial drugs, which can help a clinician choose the best treatment options for a patient. The *E. coli* isolates were inoculated onto Mueller-Hinton agar in the presence of several

antimicrobial-impregnated filter paper disks and incubated for 24 hours at 37 °C. Log phase growth is strongly suggested for obtaining a good result. As a result, subcultures of the conserved isolate to be tested must be prepared the day before the AST test day. The overnight inoculums were streaked on Mueller-Hinton agar, and the selected antimicrobial-impregnated disks were placed on the agar surface using sterile forceps, one at a time. Next, the disks were incubated at 37°C for 24 hours, and the zone of inhibition was measured with a digital ruler. The growth condition of the disks on the plate demonstrated the antimicrobial ' capacity to suppress *E. coli*. The interpretation followed the Zone Diameters interpretative standards for *E. coli* and other enteric gram-negative rods developed by the Clinical Laboratory Standards Institute (CLSI, 2023).

3.6. Molecular confirmation of the isolates for resistance genes

The anticipated *E. coli* isolates were inoculated in Trypton Soya Broth (TSB) and incubated at 37°C for 24 hours. The cells were then extracted from the overnight broth culture by centrifugation at 14,000 rpm for five min. The pellet was rinsed with phosphate-buffered saline (1x PBS) to eliminate inhibitors. The pellet was mixed with 500 liters of nuclease-free water and heated at 100 °C on a block heater for 15 min to extract DNA and lyse the cells. The supernatant was discarded. After being boiled and chilled for more than half an hour, the *E. coli* suspension was thawed and centrifuged for 15 min at 14,000 rpm to remove cell debris (Reischl *et al*., 2004). The supernatant was collected and transferred to sterile Eppendorf tubes. DNA quality was examined using gel electrophoresis under UV light. To make the gel, 0.45% agarose was dissolved in 50 ml of 1xTAE buffer. The dissolved agarose was allowed to cool to 55 °C before adding 1 µL of ethidium bromide. It was then placed on a gel tray with the combs on top and left to set for 30 min. The gel was then placed in the gel tank device, which was filled with 1xTAE buffer. Following the addition of 1 µL of loading dye, each DNA sample and ladder were loaded into separate wells. The gel electrophoresis was run for 60 min at 100 volts and then visualized using UV light. The target DNA bands were examined by comparing them to a standard DNA ladder (Lee *et al*., 2012). Moreover, the quality and quantity of the extracted DNA were checked using a nanodrop spectrophotometer (Thermo Scientific TM, USA) at an A260/280 absorbance ratio as described previously by (Do & Better, 2022).

The AMR *E. coli* isolates were characterized using molecular and phenotypic methods. The molecular characterization was carried out using the usual polymerase chain reaction (PCR) technique. Phenotypic AMR characterization studies evaluated *E. coli's* ability to thrive in the presence of a specific antimicrobial , whereas molecular AMR characterization testing identified resistance genes in *E. coli* isolates conferring resistance to one or more antimicrobial classes. Together, molecular and phenotypic AMR diagnostics aid in better understanding the level of resistance in a specific situation, as well as the underlying mechanisms that cause resistance (WHO, 2013). Molecular AMR testing entails a variety of complicated methodologies that necessitate specific laboratory settings and professionals. AMR *E. coli* was discovered, and its molecular features were investigated using a PCR amplification molecular assay (Anjum *et al.,* 2017).

The DNA extracted from antimicrobial-resistant (AMR) *E. coli* was amplified by polymerase chain reaction (PCR) (Almeida *et al*., 2001; Manage *et al*., 2019; Persson *et al*., 2007). The amplification was performed to determine the presence of two target AMR genes: *tet*A and *blaTEM*. To do this, a set of primers was created utilizing previously developed forward and reverse primer sequences. *tet*A FW 5'GGCGGTCTTCTTCTTCATCATGC3' and *tet*A RV 3'CGGCAGGCAGAGCAAGTAGA5' were amplified to detect the *tet*A gene. The *tet*A gene is a membrane-associated protein that confers resistance to Tetracycline in *E. coli*. It encodes an efflux pump that expels the antimicrobial from the cell, allowing the bacteria to survive in the presence of Tetracycline (Gholami-Ahangaran *et al*., 2022). The presence of the *blaTEM* gene was identified using the primer pairs *blaTEM* FW 5'TCGCCGCATACACTATTCTCAGAATGA3' and *blaTEM* RV 3'ACGCTCACCGGCTCCAGATTTAT5'. The *blaTEM* gene is one of the most well-known genes that confer AMR in bacteria, specifically *E. coli.* This gene encodes the TEM *beta-lactamase* enzyme, which breaks the beta-lactam ring of antimicrobial such as ampicillin and penicillin, which is required for their antibacterial activities (Fang *et al*., 2008).

For each *blaTEM* and *TertA*, the PCR was performed in a total reaction volume of 50 µL containing 0.5 µL each of the forward and reverse primers, 2.5 µL standard buffer B and MgCl2 each, 0.5 µL dNTPs, 0.25 µL Taq DNA polymerase (Delta Biotechnology PLC, Ethiopia), and 1 µL of DNA template. The amplification was performed under the following modified conditions:

initial denaturation at 94°C for 5 min and 94°C for 30 seconds for denaturation; annealing at 62°C for 30 seconds; and extension at 72°C for 30 seconds for 30 cycles, followed by a final extension at 72°C for 10 min (Suzuki *et al*., 2023). The PCR products were then run on a 1.3% agarose gel electrophoresis gel, which was stained with (10mg/ml) Ethidium bromide in the presence of a negative control (no DNA) to check for possible contamination in the master mix. The gel was subjected to 100 V to run for 50 min in 1xTAE buffer of pH 8. The buffer was prepared from three chemical compositions namely Tris hydrochloride (40mM), EDTA (1mM), and Glacial acetic acid (20mM). The PCR products were then visualized under UV light using a gel documentation machine (BioRAD USA). The bands' sizes were estimated and recorded in the presence of a 1000bp DNA ladder (Soli BioDyne).

Table 2: A set of primers used for targeting various resistance genes

Targeted genes	5' – 3'; 3' – 5'	Primer Sequence	Number of bases	References
*tet*A	*tet*A FW	GGCGGTCTTCTTCTTCATCATGC	23	(Gholami-Ahangaran et al., 2022)
	*tet*A RV	CGGCAGGCAGAGCAAGTAGA	20	
blaTEM	blaTEM FW	TCGCCGCATACACTATTCTCAGAATGA	27	(Fang et al., 2008)
	blaTEM RV	ACGCTCACCGGCTCCAGATTTAT	23	

3.7. Data analysis

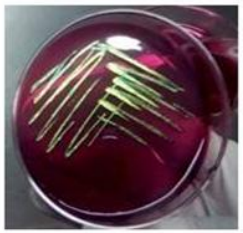
The combination of qualitative and quantitative data analysis provided a full view of the AMR *E. coli* profile, allowing for extensive phenotypic and genetic characterization of resistant *E. coli* isolates and their prevalence. Among the many statistical software systems available today, IBM Corporation's Statistical Package for the Social Sciences (SPSS) version 26 was used. The SPSS software is commonly used, and it generates a full report on the computer screen that may be copied and pasted into another document.

4. Results

4.1. Isolation and identification of *E. coli* isolates

The study included 40 liquid samples obtained from three separate locations: Adama Hospital Medical College in Adama City, the YRAI, YRBI, and the XR near Dukem. Twenty-five of these originated from the two Rivers in Dukem. Fifteen samples were collected from the Adama Hospital Medical College. The gathered samples were subsequently taken to Addis Ababa University Institute of Biotechnology's Molecular Biotechnology Laboratory, where they were maintained in a refrigerator at 4°C until the experiment began. Each sample was subjected to enrichment on a TSB and incubated overnight at 37°C by the spread plate technique. Then, 0.5 mL of each overnight sample was added to EMB agar and incubated at 37°C for 24 hours.

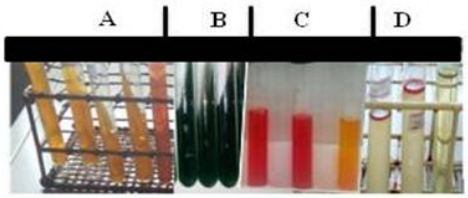
The next day, dark green metallic sheen colonies were observed, and individual colonies that showed a green metallic sheen color were safely picked and streaked on MacConkey agar and on nutrient agar media for further isolation and purification. The selected colonies, which were subsequently cultured on MacConkey agar media, converted the media color from red to pink. The colonies were then inoculated in TSB broth for 24 hours, after which Gram staining and a series of biochemical tests were performed on the purified isolates. The TSI, SCA, IMViC (++--), and Gram staining tests were the biochemical tests used to identify the *E. coli* isolates. The isolates were positive according to the Gram staining, *catalase* test, triple sugar iron methyl red test, and Indole test but negative according to the Voges-Proskauer test and Simmon's citrate agar test (Figure 6). In this study, a total of 75 isolates were identified as presumptive of *E. coli.*



I. Overnight culture on EMB agar

II. Overnight culture on MacConkey agar

III. Overnight culture on NA



IV. Biochemical characterization: a) Triple Sugar Iron, b) Citrate c) Methyle red d) Indole

Figure 5: Isolation and identification of *E.coli*. **I)** After 24 hours, a green metallic sheen appeared on the EMB, indicating that the colonies were *E. coli.* Eosin Methylene Blue Agar is a deferential medium that only supports gram-negative bacteria. **II)** The colonies were then cultivated for a full day on MacConkey agar. The presence of *E. coli* isolates was confirmed by the pink color produced when lactose was broken down into glucose and galactose in an acidic medium. **III)** Additional purification was performed on nutrient agar (NA). Tick grayish white colonies were formed, revealing that the isolates were E. coli. **IV)** Biochemical assays, including TSI, SCA, and IMViC test results**.**

Table 3: The result of the biochemical tests of the 75 suspected *E. coli* isolates from the hospital sewage and river water samples.

Isolate code	Lactose fermentation	Glucose fermentation	Sucrose fermentation	Gas production	H_2S production	Citrate utilization	Indole test	Methyl red test	*Voges-prokauer test*	*Catalase* test	Remark
AHMC1	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC2	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas production, Indole positive, no acetoin producer, no H_2S Production, not citrate user.
AHMC3	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC4	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC5	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC6	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC7	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC8	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC9	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC10	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production,

											no H_2S Production, not citrate user.
AHMC11	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC12	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC13	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC14	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC15	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC16	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC17	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC18	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC19	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC20	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC21	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC22	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production,

											no H_2S Production, not citrate user.
AHMC23	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC24	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC25	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC26	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC27	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC28	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC29	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC30	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC31	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC32	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC33	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC34	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production,

											no H_2S Production, not citrate user.
AHMC35	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC36	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC37	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC38	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC39	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC40	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC41	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC42	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC43	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC44	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC45	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC46	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production,

											no H_2S Production, not citrate user.
AHMC47	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC48	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
AHMC49	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC50	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
AHMC51	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI1	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI2	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI3	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI4	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI5	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI6	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI7	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production,

											no acetoin production, not citrate user.
YRAI8	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI9	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI10	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI11	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRAI12	+	+	+		-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
YRBI1	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRBI2	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRBI3	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRBI4	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRBI5	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
YRBI6	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
XR1	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production,

											no H_2S Production, not citrate user.
XR2	+	+	+	-	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, no gas production, Indole positive, no H_2S Production, no acetoin production, not citrate user.
XR3	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
XR4	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
XR5	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.
XR6	+	+	+	+	-	-	+	+	-	+	Glucose, lactose and sucrose fermenter, gas producer, Indole positive, no acetoin production, no H_2S Production, not citrate user.

Where AHMC = Adama Hospital Medical College; YRAI = Yerer River after industrial waste entry site; YRBI = Yerer River before the Industrial waste entry site; and XR = Xadacha River. The numbers represent isolates. The table above shows the results of key biochemical assays used to describe the isolates. The first column represents the isolate from the selected sites, and the first row reflects the biochemical tests used in this study. The "+" sign represents positive result for the test, whereas the "-" sign denotes negative result for the test. The last column shows remark of each isolates.

Table 4: The distinctive color of *E.coli* on each biochemical test

Lactose	**Glucose**	**Sucrose**	**Gas**	**H2S**	**Citrate**	**Indole**	**Catalase test**	**Grams staining**	**Methyl red**	**Voges-prokauer**
Yellow slant	Yellow bottom	Yellow slant	Bubbles	No color change	No color change	Red ring	Bubbles	Pink	Red color	No red color

4.2. Antimicrobial Susceptibility Test (AST)

This study concentrated on an antimicrobial-resistant *E. coli* profile in river water and untreated hospital sewage water samples. The 50 (66.67%) of the 75 biochemically verified *E. coli* isolates were selected at random based on time and resource constraints. They tested for antimicrobial susceptibility to eleven antimicrobials using the Clinical and Laboratory Standard Institute guideline (CLSI, 2023). These includes Gentamycin (10µg), Norfloxacin (10µg), Ciprofloxacin (5µg), Tetracycline (30µg), Compound sulphonamides (300µg), Ampicillin (10µg), Vancomycin (10µg), Chloramphenicol (30µg), Streptomycin (10µg), Erythromycin (10µg), and Naldic Acid (5µg) (Thermo Scientific™ Oxoid™, United Kingdom). Most of the *E. coli* isolates were resistant to multiple antimicrobial , and the most frequent resistance was to Erythromycin (76%), followed by Tetracycline and Vancomycin (72%) each, Streptomycin (60%), Ampicillin (56%), Naldic Acid (54%), Compound sulphonamides (52%), Chloramphenicol (30%), Ciprofloxacin and Gentamycin (28% each) and Norfloxacin (8%) (Figure 7 and Table 4). The resistance pattern of the *E. coli* isolates to the first three antimicrobial Erythromycin, Tetracycline, and Vancomycin was much stronger than the resistance patterns of the remaining eight antimicrobial. The term "multiple antimicrobial resistance" refers to a pathogenic microorganism's resistance to three or more antimicrobial (Wolfensberger *et al*., 2019). In this study, the MAR profile of *E. coli* was evaluated by calculating the percentage of isolates resistant to three antimicrobial: Erythromycin, Tetracycline, and Vancomycin. The MAR index evaluates the level of antimicrobial resistance in an isolate, and the mean MAR index was 0.27 when determined according to Osundiya *et al.*'s (2013) procedure (Osundiya *et al*., 2013).

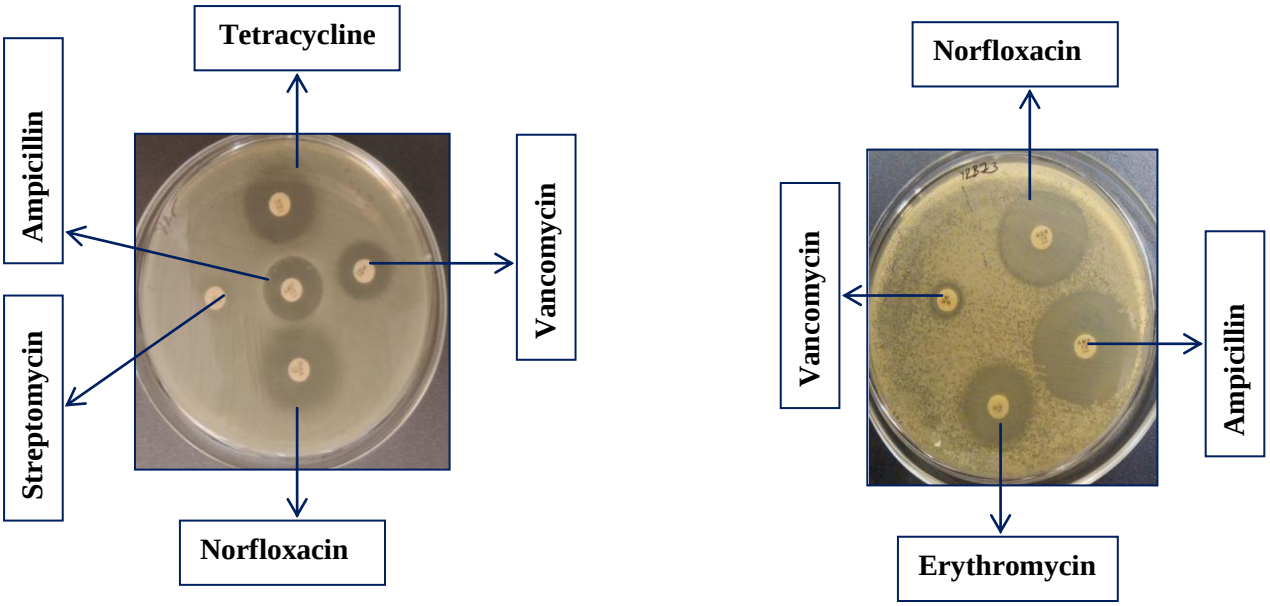


Figure 6: Antimicrobial susceptibility test by *Kirby's Disk diffusion* method. The image above shows the inhibition zone of the selected antimicrobials against the 50 *E.coli* isolates.

Table 5: Antimicrobial susceptibility patterns of *E. coli* isolates against the selected antimicrobial

List of Antimicrobial	Number of Resistance isolates	Number of Intermediate isolates	Number of Susceptible isolates
Ampicillin	28 (56.0%)	11 (22.0%)	11 (22.0%)
Chloramphenicol	15 (30.0%)	10 (20.0%)	25(50.0%)
Ciprofloxacin	14 (28.0%)	8 (16.0%)	28 (56.0%)
Compoundsulphonamides	26 (52.0%)	19 (38.0%)	5 (10.0%)
Erythromycin	38 (76.0%)	8 (16.0%)	4 (8.0%)
Gentamycin	14 (28.0%)	8 (16.0%)	28 (56.0%)
NaldicAcid	27 (54.0%)	18 (36.0%)	5 (10.0%)
Norfloxacin	4 (8.0%)	16 (32.0%)	30 (60.0%)
Streptomycin	30 (60.0%)	12 (24.0%)	8 (16.0%)
Tetracycline	36 (72.0%)	7 (14.0%)	7 (14.0%)
Vancomycin	36 (72.0%)	9 (18.0%)	5 (10.0%)

The table above summarizes the quantity and percentage of resistant, intermediate, and susceptible *E. coli* isolates from each of the eleven antimicrobial disks chosen for the AST. The majority of the isolates were sensitive to Norfloxacin, Gentamycin, and Ciprofloxacin, yielding 60%, 56%, and 56%, respectively. Of the total AST, 48.73% were resistant, while 22.91% and 28.36% were intermediate and susceptible, respectively.

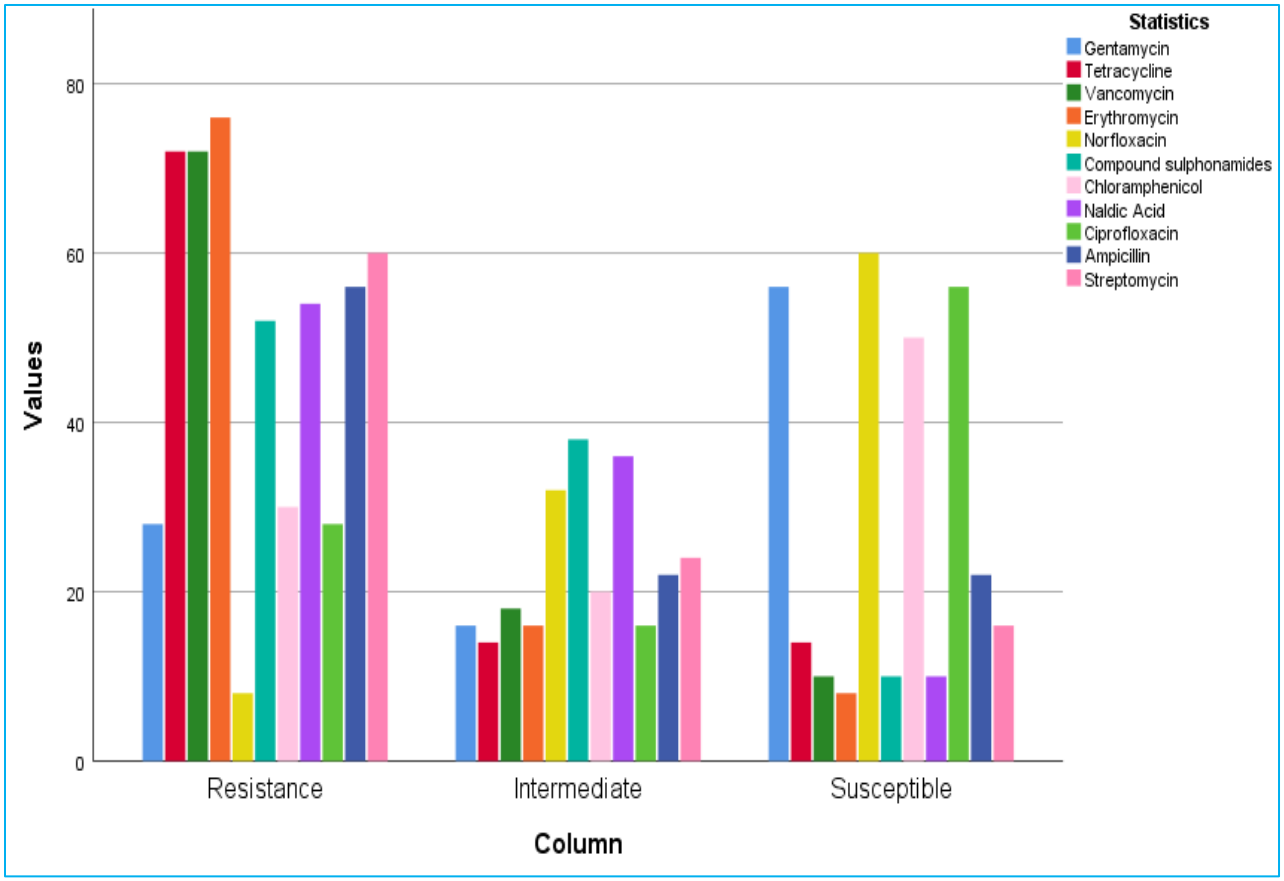


Figure 7: AMR pattern of *E. coli* Isolates against the selected antimicrobial. The *E. coli* isolates from hospital sewage and river waters in Adama and Dukem, respectively, were subjected to eleven antimicrobial. Namely: Ampicillin, Chloramphenicol, Ciprofloxacin, Compound Sulphonamides, Erythromycin, Gentamycin, NaldicAcid, Norfloxacin, Streptomycin, Tetracycline, and Vancomycin.

Table 6: AST patterns of *E. coli* isolates among the six sample sites

Sample sites	Sample sub-sites	Resistance	Intermediate	Susceptible
Hospital	AHMC	174 (64.94%)	78 (59.55%)	78 (51.66 %)
River	YRAI	66 (24.63%)	25 (19.08%)	19 (12.58%)
	YRBI	6 (2.22%)	28 (7.63%)	10 (18.56%)
	XR	22 (8.21%)	18 (13.74%)	26 (17.2%)

Table 5 shows the AST patterns of each *E. coli* isolate exposed to the specified antimicrobial disks. Of the total resistant isolates, 64.94% came from AHMC, followed by YRAI (24.63%), XR (8.21%), and YRBI (2.22%).

Table 7: Sample site * Antimicrobial Resistant Patterns Crosstabulation

Count					
		Antimicrobial Resistant Patterns			Total
		Resistant	Intermediate	Susceptible	
Sample site	Hospital	174	78	78	330
	YRAI	66	25	19	110
	YRBI	6	28	10	44
	XR	22	18	26	66
Total		268	149	133	550

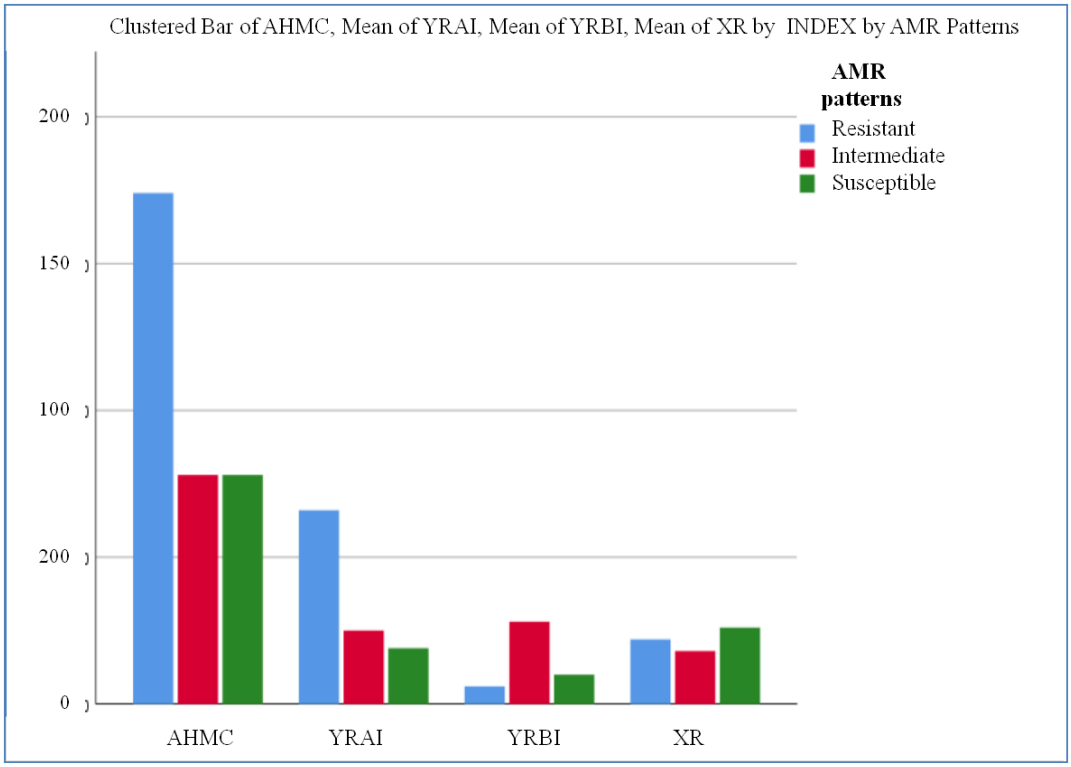


Figure 8: Resistance patterns of *E. coli* isolates among the four sample sites. This figure represented the order of sample sites from the largest AMR isolates source to the smallest: AHMC →YRAI→ XR→YRBI.

Table 8: Multiple Comparisons among the selected sample sites

Dependent Variable: Antimicrobial Resistant Patterns						
Tukey HSD						
(I) Sample site	(J) Sample site	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Hospital	YRAI	.136	.088	.413	-.09	.36
	YRBI	-.382*	.129	.017	-.71	-.05
	XR	-.352*	.108	.007	-.63	-.07
YRAI	Hospital	-.136	.088	.413	-.36	.09
	YRBI	-.518*	.143	.002	-.89	-.15
	XR	-.488*	.125	.001	-.81	-.17
YRBI	Hospital	.382*	.129	.017	.05	.71
	YRAI	.518*	.143	.002	.15	.89
	XR	.030	.156	.997	-.37	.43
XR	Hospital	.352*	.108	.007	.07	.63
	YRAI	.488*	.125	.001	.17	.81
	YRBI	-.030	.156	.997	-.43	.37
*. The mean difference is significant at the 0.05 level.						

From table 8 above, the comparison among AHMC and YRAI there is no significant difference in AMR patterns between *E. coli* from the Hospital and YRAI samples. But there is a significant difference in AMR patterns in the case of YRAI and YRBI, showing higher AMR in YRAI. Likewise, there is statistical difference between YRAI and XR where XR accounts lower AMR *E.coli* profile.

Table 9: Multiple antimicrobial resistances (MAR) index result of each AMR E. coli isolates

Hospital Isolates	MAR index	Remark	River Isolates	MAR index	Remark
AHMC3	0.9	High risk category	YRAI8	0.82	High risk category
AHMC20	0.9	High risk category	YRAI9	0.82	High risk category
AHMC22	0.82	High risk category	YRAI10	0.73	High risk category
AHMC12	0.82	High risk category	YRAI2	0.73	High risk category
AHMC24	0.82	High risk category	YRAI7	0.64	High risk category
AHMC13	0.64	High risk category	YRAI3	0.64	High risk category
AHMC1	0.64	High risk category	YRAI1	0.54	High risk category
AHMC19	0.64	High risk category	YRAI4	0.45	High risk category
AHMC23	0.54	High risk category	YRAI6	0.45	High risk category
AHMC21	0.54	High risk category	YRAI5	0.18	Low risk category
AHMC11	0.54	High risk category	XR1	0.45	High risk category
AHMC10	0.54	High risk category	XR6	0.45	High risk category
AHMC9	0.54	High risk category	XR4	0.36	High risk category
AHMC16	0.54	High risk category	XR5	0.36	High risk category
AHMC5	0.54	High risk category	XR3	0.27	High risk category

AHMC2	0.54	High risk category	XR2	0.1	Low risk category
AHMC30	0.45	High risk category	YRBI2	0.27	High risk category
AHMC25	0.45	High risk category	YRBI1	0.1	Low risk category
AHMC26	0.45	High risk category	YRBI3	0.1	Low risk category
AHMC27	0.45	High risk category	YRBI4	0.1	Low risk category
AHMC28	0.45	High risk category			
AHMC14	0.45	High risk category			
AHMC4	0.45	High risk category			
AHMC17	0.45	High risk category			
AHMC7	0.36	High risk category			
AHMC29	0.36	High risk category			
AHMC18	0.27	High risk category			
AHMC15	0.27	High risk category			
AHMC6	0.27	High risk category			
AHMC8	0.1	Low risk category			

The table 9 above summarized the MAR index of the *E. coli* isolates and most of the isolates' MAR index was greater than 0.2. From the total 50 isolates, 44(88%) have more than 0.2 while only 6(12%) have less than 0.2 MAR index.

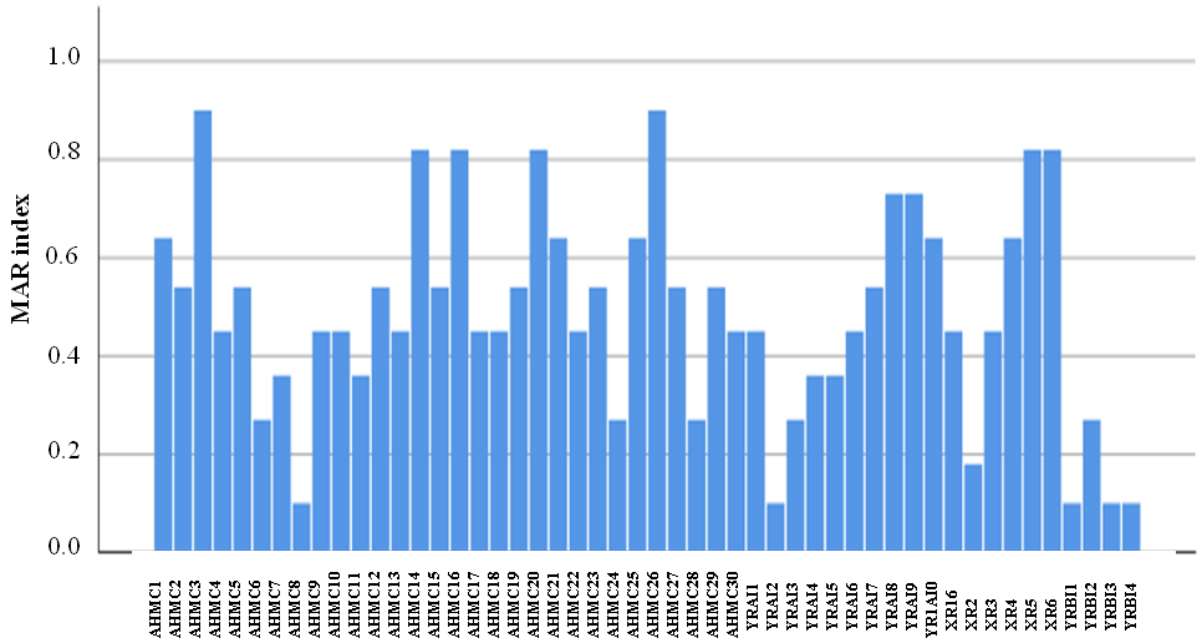


Figure 9: Multiple antimicrobial resistance (MAR) index of each identified AMR *E. coli* isolates.

The average MAR index values for the hospital sample, the YRAI (downstream from the industrial waste discharge point), XR, and the YRBI (upstream from the industrial waste

discharge point) are 0.53, 0.6, 0.33, and 0.14, respectively. In this study, both the hospital and YRAI had the highest MAR index.

4.3. Molecular analysis of AMR genes

The anticipated *E. coli* isolates were stored in glycerol (25%) at -20°C until the next step is started. These were then thawed at room temperature and inoculated on fresh EMB agar media at 37°C for 24 hours to activate the isolates. Then, three to five colonies were inoculated in Trypton Soya Broth media for resuscitation. The culture conditions were adjusted to 37°C, and the cells were incubated for 24 hours. The boiling and freezing DNA extraction method was applied as described previously by Momtaz *et al*. (2012), with slight modifications (Momtaz *et al*., 2012). After 24 hours, 1 mL of culture broth was transferred to sterilized 2 mL microcentrifuge tubes and centrifuged at 1400 rpm for 5 min. The supernatant was then removed, and the pellet was re-suspended in 500 µL of phosphate-buffered saline (PBS). The supernatant was again discarded, and nuclease-free water (500 µL) was mixed with the pellet and boiled on a block heater at 100 °C for 15 min. The step was then frozen for more than 30 min at -20°C. The suspension was thawed and centrifuged at 1400 rpm for 15 min to break open the cells to release the DNA into the solution. The supernatants containing the DNA were thereafter collected into other sterilized and nucleic acid-free microcentrifuge tubes. Agarose gel electrophoresis (1.3%) and Nanodrop (A260/280 absorbance ratio) spectrophotometers were used to evaluate the quality and quantity of the extracted DNA. Gel electrophoresis was used to check DNA quality, where clear and distinct bands were observed under UV visualization. This was done after the DNA extract was checked by the NanoDrop machine. Extracts whose 260/280 absorption ratios ranged from the standard values (1.8-2.0) were stored at -20°C. The remaining supernatants were then stored at -20% for further analysis by PCR amplification.

The extracted DNA of the isolates with an A260/A280 absorbance ratio of 1.7–1.9 was further analyzed via polymerase chain reaction (PCR) to confirm the presence of AMR genes in the *E. coli* isolates. From all samples, 75 *E. coli* isolates were identified via morphological and biochemical tests, while 50 of the isolates were subjected to AST due to resource and time constraints. Molecular confirmation took place using PCR amplification in the presence of specific primers targeting the *tet*A and *blaTEM* genes. From the 36 isolates that showed resistance to Tetracycline under the phenotypic test, only 30 (83.33%) isolates harbored the *tet*A

gene. Likewise, 28 isolates that were phenotypically confirmed Ampicillin-resistant were subjected to the *blaTEM* gene test, and only 16 (57.14%) of them harbored the gene.

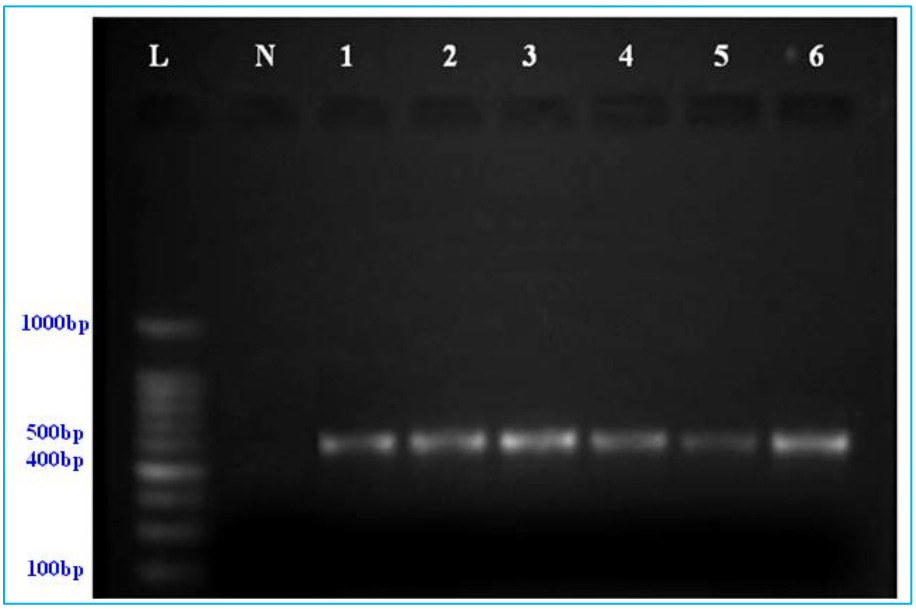


Figure 10: Gel electrophoresis results of PCR products for *tetA* gene: This picture showed the confirmed molecular gel image of the amplified product of *tet*A gene of some of the identified positive *E. coli* isolates. Where: L = 1 kb ladder, N= Negative control, Lane3-Lane8 represents positive isolates with the estimated bands size of 550 bp.

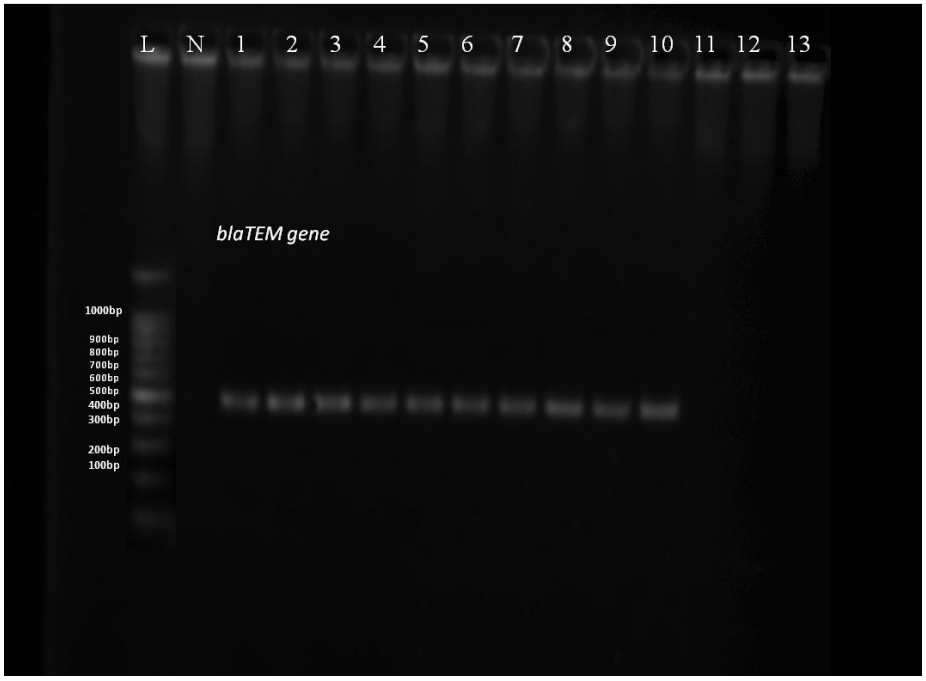


Figure 11: Gel electrophoresis results of PCR products for *blaTEM* gene. The above picture indicated the gel image of the PCR product of some of the identified of *blaTEM* gene positive *E.*

coli isolates. Where: L = 1 kb ladder, N= Negative control, Lane3 up to Lane10 represents positive isolates with the estimated bands size of 534 bp while Lane 11, 12, and 13 were *blaTEM* gene negative.

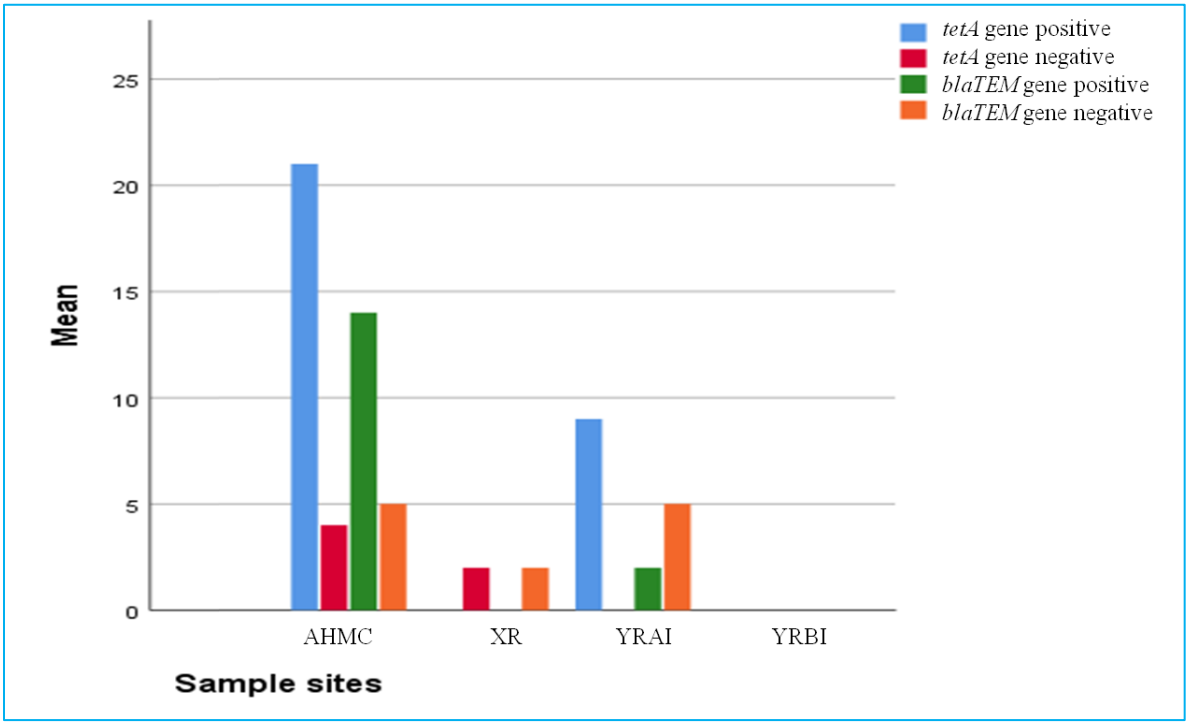


Figure 12: The different sample sources and the antimicrobial resistant genes, *tetA* and *blaTEM* genes, distribution patterns among the sample sites. Clustered Bar Mean of *tetA* Gene Positive, Mean of t*etA* Gene Negative, Mean of *blaTEM* Gene Positive, Mean of *blaTEM* Gene Negative by Sample sites by INDEX

Molecular validation of the isolates confirmed the distribution of AMR *E. coli* across the sample sites. This study looked at the AMR profiles of *E. coli* isolates from Adama Hospital Medical College, as well as YRAI, YRBI, and XR water from Dukem. After carefully isolating and identifying the *E. coli* isolates and determining the *E. coli* burden, the phenotypic AMR profiles and distribution of resistance genes were assessed. The prevalence of *E. coli* in liquid samples was examined to determine the most likely pathways for AMR gene propagation. The study discovered a considerable difference in the AMR profile and quantity of *E. coli* between sampling sites. As indicated in the Table 8, this study confirmed there is no statistical significant difference in AMR patterns among YRAI and AHMC, assuming the null hypothesis is true (p=0.413). Likewise, the AMR *E. coli* profile was higher at YRAI where the untreated industrial sewage discharged compared to those from the same river where the industrial wastes did not discharge (YRBI) (p = 0.002) (Table 8).

5. Discussion

This study focused whether antimicrobial -resistant *E.coli* isolates are highly prevalent and disperse in rivers polluted with drug residues, biocides, various business pollutants, and untreated hospital sewage water. Several rivers in Ethiopia, particularly those that flow through Adama and Dukem, are heavily contaminated by a range of contaminants, both from factors (industrial discharge, city wastewater discharge, storage waste, clinical waste, etc.) and nonfactor sources (e.g., one-of-a-kind sewage runoff). Almost all of the assessed sites along the various rivers had poor water quality and no longer met the river water first-class requirement (Assegide *et al.*, 2022). These polluted rivers can contribute to the spread of AMR genes between humans, animals, and the environment they share. That is why monitoring and regulating the discharge of wastewater treatment agents and other diffuse pollution sources are essential for preventing the spread of AMR genes. To the level of our knowledge, no previous studies have investigated the AMR profile of *E. coli* isolates from polluted rivers in Dukem and hospital wards in Adama Hospital Medical College in Adama City.

Both hospital sewage and river water samples were inoculated on EMB agar, which has dual characteristics. It is both a selective and differential medium. EMB agar is a selective and differential way of life medium used for the isolation of fecal coliforms. Lactose and sucrose are sources of fermentable carbohydrates that encourage the growth of some gram-negative bacteria, especially fecal and non-fecal coliform bacteria. The presence of eosin Y and methylene blue dyes in the medium inhibits the growth of most gram-positive organisms and helps in the differentiation of enteric bacteria. Lactose-fermenting gram-negative microorganisms acidify the medium, which reduces the pH, and the dye produces a darkish red complex generally related to an inexperienced metal sheen. This metal-inexperienced sheen shows likely lactose and/or sucrose fermentation potential of a bacterium. *E. coli*, a fecal coliform, produces colonies with a distinct green metallic sheen on EMB agar. Only gram-negative bacteria can grow on EMB agar, and after 24 hours of incubation, a green metallic sheen color was developed, depicting *E. coli* (Tankeshwar, 2022). A series of biochemical tests were applied for further identification of the isolates. Biochemical tests were used to identify *E. coli* bacteria. These tests are based on the ability of *E. coli* to produce certain enzymes or metabolize specific compounds. (Aryal, 2022). (A) The TSI contains three sugars: glucose, lactose, and sucrose. This test is used to

presumptively identify Enterobacteriaceae, including *E. coli*. The TSI test is based on the fermentation of these carbohydrates, which produces gas and hydrogen sulfide. The TSI media's reddish-orange color changed to yellow, indicating that the isolates fermented the sugars. The presence of phenol red in the media showed a color change, which turned yellow at a low (acidic) pH. The indole test was used to determine the presence of tryptophanase, an enzyme generated by *E. coli* but not other bacteria. Following the addition of the reagent, p-dimethylaminocinnamaldehyde (DMACA), a red ring developed at the top of the overnight culture (Sagar, 2019). The Methyl Red test was used to determine whether *E. coli* produces large amounts of acid from glucose. The Voges-Proskauer test and the citrate test were negative for the isolates.

Antimicrobial susceptibility tests are used to determine the effectiveness of antimicrobial against bacterial isolates from different sources (Gaşpar *et al*., 2021). According to this study, among the 75 total *E. coli* isolates, 51 (68%) were from hospital sewage samples and 24 (32%) were from river water samples. The 50 isolates were randomly selected due to resource constraint and subjected to an antimicrobial susceptibility test (AST) using *Kerby's disc diffusion* method according to the European Committee on Antimicrobial susceptibility Testing (EUCAST) methodology. Among the 50 *E. coli* isolates, 30 (60%) were from hospital samples, while 20 (40%) were from river water samples.

Of the total isolates, 66.67% (50/75) were subjected to an antimicrobial susceptibility test (AST). From the 50 isolates, 60% (30/50) were from the hospital samples while the rest 40% (20/50) isolates were from river samples. The MAR index was more than 0.2, which is similar to a study conducted in Nigeria (Ayandele *et al*., 2020). The MAR index of all samples indicated that *E. coli* isolates were in the high-risk category except the YRBI, which had a MAR score of 0.14. MAR isolates are those that resist several antimicrobial , three or more in this case, during AST (Kwak *et al*., 2015). This study found that 90% (45/50) of *E. coli* isolates were resistant to MAR, which is higher than Tanzania's reported 86.76% (177/204) (Kiiti *et al*., 2021).

This study discovered a significant increase in resistance to Erythromycin (76%), the first most resistant, indicating regular antimicrobial usage around the sample sites. Tetracycline 36 (72.0%) was the second most resistant. The first result was lower than what Yitayew *and his colic's*

reported in 2022 (Yitayew *et al*., 2022) while the second was greater than the report from Mequanint Belete and his colic's (Belete *et al*., 2022). Tetracycline is among the most frequently used antimicrobial in African countries where agriculture is the main source of income (Kimera *et al*., 2020). Streptomycin had the third highest resistance rate (60%), which was lower than the Kenyan study's finding (98%) (Ayandele *et al*., 2020). This study found that 56% of the isolates were resistant to Ampicillin, which is greater than the 39.6% reported in a study conducted in the Philippines (Dela Peña *et al*., 2022). The *E. coli* isolates were highly susceptible to Norfloxacin 30 (60%) and Ciprofloxacin 28 (56%). These results were lower compared to the study conducted in Bahir Dar city (Belete *et al*., 2022) where 50 (92.6%) and 39 (72.2%) of the isolates were resistant to Norfloxacin and ciprofloxacin, respectively. The observed differences between the current study and prior findings could be attributable to many reasons. These could include variations in collection methodology and sample size, pathogen evolution, culturing techniques, and environmental variables.

Furthermore, this study showed that the *E. coli* isolates from the YRAI more resistant (24.63%) to the antimicrobials than were those from the same river before the industrial waste discharge, which accounted for only 2.22% ($p = 0.002$). The study's finding that the antimicrobial -resistant *E. coli* profile was higher at the YRAI site where untreated industrial sewage is discharged compared to areas of the same river without such discharges showed the significant impact of industrial pollution on the development and spread of antimicrobial resistance. Industrial sewage often contains antimicrobial, heavy metals, and other pollutants that create a selective environment favoring antimicrobial -resistant bacteria. The higher AMR profile at the polluted river site suggests that industrial pollution is a key driver of resistance in the river environment. Populations relying on the Yerer River for drinking water, agriculture, or recreation face heightened risks of exposure to antimicrobial -resistant bacteria. Polluted rivers facilitate the transfer of resistance genes between bacteria, enhancing the spread of resistance. The spread of resistant bacteria from environmental sources to humans can lead to infections that are harder to treat and manage (Le *et al*., 2023).

The isolates from the XR and YRBI exhibited significantly greater susceptibility to the 11 antimicrobial. In this study, it was observed that the isolates from YRBI showed the least MAR result profile (0%) compared to the rest of the river water sample sources, followed by XR

(66.67%) and YRAI (90%), respectively. This study also revealed that 86.67% of hospital isolates showed MAR, and the isolates from river water showed 65% MAR. The isolates that showed phenotypic resistance were subjected to a molecular test to confirm the presence of the two selected genes, namely the *tetA* gene and the *blaTEM* gene. The genes were selected based on the phenotypic resistance pattern of the isolates to the Tetracycline and *beta-lactamase* antimicrobial families. The PCR test confirmed that 83.33% of the isolates were found to harbor the *tetA* gene, which was higher than the result reported by Melese *et al.* (2017), where only 65.1% of the isolates were found to harbor the *tet*A gene (Messele *et al*., 2017) in Ethiopia. The disparity may result from variations in the techniques used for sampling, regional variations, environmental conditions, or the emergence of new bacterial strains.

This finding revealed that *E. coli* had a high AMR profile in hospital sewage water and Yerer River water after the discharge of industrial sewage waste. The study's findings ($p = 0.04$) suggested that there were few statistically significant differences between the two. *E. coli* from YRAI isolates exhibited a significantly higher AMR profile than YRBI isolates.

6. Conclusion and recommendation

The study focused on AMR patterns of *E. coli* strains isolated from hospital sewage and polluted rivers in Adama and Dukem, Oromia, Ethiopia. The following are the key findings and conclusions drawn from the study:

- The *E. coli* isolates from both sources showed high levels of AMR to the selected antimicrobial, particularly Erythromycin, Tetracycline, Ampicillin, and Streptomycin.
- Hospital sewage was identified as a major contributor of antimicrobial resistance, followed by YRAI, to the spread of resistant *E. coli* to the environment.
- The MAR index result depicted that the presence of AMR *E. coli* in the targeted sample sources poses significant health risks to the surrounding communities.
- Based on the rising level of MAR *E.coli*, the study advocates urgent needs for improved wastewater treatment, stringent regulations on antimicrobial use, and regular monitoring to control the spread of antimicrobial resistance.

Researchers have recommended further study to better understand the mechanisms of AMR and the AMR profiles of *E. coli* bacteria. This research should take a holistic "One Health" approach, examining the links between resistant *E. coli* in human, animal, and environmental settings. Additionally, the researchers suggest exploring alternative treatment options for *E. coli* infections as well as conducting studies on the impact of resistant *E. coli* on local ecosystems, animal populations, and human health. Understanding these broader implications is crucial for developing effective strategies to combat the growing threat of antimicrobial resistance.

The findings highlight the need for a collaborative, multidisciplinary effort to address this critical public health issue. Further research in this area could lead to important advancements in preventing and managing antimicrobial -resistant *E. coli* infections.

Ethical statement

This study analyzed the diversity and AMR profile of *E. coli* isolates from hospital sewage and river water in Adama and Dukem, respectively. A variety of bacteriological procedures were used to isolate, identify, and assess the diversity and AMR profile of *E. coli*. The study matched the findings to those of earlier studies conducted in other parts of Ethiopia, adhering to the American Society for Microbiology's ethical norms and criteria (Wise, 2006).

The study posed minimal danger to participants, researchers, animals, and the environment. Human and animal participants were indirectly involved due to the presence of *E. coli* in sewage and river water. The researchers obtained no personal or identifiable information from the individuals, nor were they contacted for any purpose. Prior to sample collection, researchers provided informed consent through hospital laboratory administration. The confidentiality and privacy of the participants and management were protected. This study was approved by the Addis Ababa University Institutional Research Board, College of Natural and Computational Sciences, and Institute of Biotechnology. The Institute of Biotechnology Research Ethics Review Board (IRB) granted permission to collect samples of hospital sewage water and river water.

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Ref.No. CNCSDO/161/15/2023

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Date: September 08, 2023

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To Whom It May Concern

The College of Natural and Computational Sciences Institutional Review Board (**CMS-IRB**) Committee in its meeting held on **31/08/2023, Minute No. IRB/06/2015/2023** has examined die project proposal entitled "Antimicrobial Resistance Profile of Escherichia coli Isolated from Hospital Sewage and Polluted Rivers: The Cases of Adama and Dukem" by **Kajelcha Fikadu** from the Addis Ababa University.

The proposal is approved for implementation for one year, effective September 8, 2023.

With Regards!

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Annexes

Annex 1: Antimicrobial Resistant Patterns

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Resistant	268	48.7	48.7	48.7
	Intermediate	149	27.1	27.1	75.8
	Susceptible	133	24.2	24.2	100.0
	Total	550	100.0	100.0	

Annex 2: Descriptive statistics of the E.coli isolates' MAR index

Descriptive Statistics						
	N	Minimum	Maximum	Mean	Std. Deviation	Variance
MAR index	50	.10	.90	.4858	.21738	.047
Valid N (listwise)	50					

Annex 3: Chi-Square Tests

Chi-Square Tests			
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	100.000[a]	98	.425
Likelihood Ratio	94.222	98	.589
N of Valid Cases	50		
a. 150 cells (100.0%) have expected count less than 5. The minimum expected count is .16.			