Functional and molecular characterization of potential bacterial pathogens in Akaki River and its toxicogenomics effects on model organisms, Ethiopia

BY

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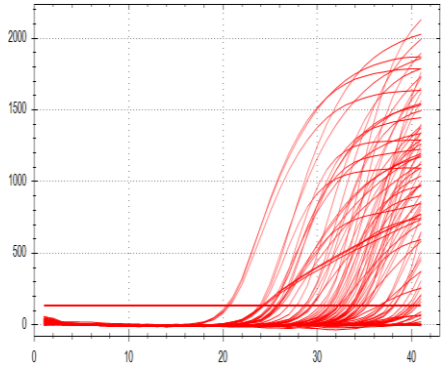

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ABBREVIATIONS

ABC……...ATP-binding cassette superfamily

AMR……..Anti-Microbial Resistance

CARD……Comprehensive Antibiotic Resistance Database

CDS……...Coding Sequence

CFU……...Colony Forming Unit

CGE……...Center for Genomic Epidemiology

DAEC……Diffusely Adherent *E. coli*

DNA……..Deoxyribonucleic Acid

EAEC……....Enteroaggregative *E. coli*

EDHS…….Ethiopian demographic and health survey

EHEC……..Enterohemorrhagic *E. coli*

EIEC……Enteroinvasive *E. coli*

EPEC……...Enteropathogenic *E. coli*

EPHI……Ethiopian public Health Institution

ESBL……...Extended-Spectrum β-Lactamase

ETEC……...Enterotoxigenic *E. coli*

EUCAST……..European Committee on Antimicrobial Susceptibility Testing

FMoH……...Federal Ministry of Health

MALDI-TOF MS……..Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

MATE…….. Multidrug And Toxic compound Extrusion

MFS……..Major Facilitator Superfamily

MNEC……..Meningitis-associated *E. coli*

NTC……..No Template Control

PATRIC……Pathosystems Resource Integration Center

PCR……...Polymerase Chain Reaction

PMQR ……..Plasmid mediated quinolone resistance

qPCR…….quantitative Polymerase Chain Reaction

RND……..Resistance-Nodulation-cell Division

SEPEC……...Sepsis-associated *E. coli*

SMR……...Small Multidrug Resistance

ST……...Sequence Type

UPEC……..UroPathogenic *E. coli*

VBNC…….Viable But Non-Culturable

WGS……...Whole Genome Sequencing

WHO……...World health organization

Abstract

Background: Persistence and dissemination of pathogenic bacteria and antibiotic resistance genes (ARGs) in aquatic environments remain a serious public health issue. The aquatic environment may serve as a key transmission pathway for pathogenic bacteria and ARGs to and from humans. Continual faecal discharge into the environments, together with high levels of chemical and pharmaceutical pollutants are creating conditions favourable for the persistence and spread of antibiotic resistant pathogens. The aim of the study was to investigate the distribution of ARGs, and functional and molecular characteristics of potentially pathogenic bacteria in the Akaki river that flows through Addis Ababa, Ethiopia, and its toxicogenomic effect in model organisms.

Methods: Water and sediment samples were collected from five sites along the Akaki river system for isolation of potential bacterial pathogens and detection of ARGs. The diversity and abundance of 84 ARGs and 116 clinically important bacteria were evaluated using DNA qPCR analysis. Potential bacterial pathogens were isolated using selective agar techniques and identified by MALDI-TOF. Isolates were analyzed for their resistance to different classes of antibiotics and further characterized by PCR and whole-genome sequencing. The toxicogenomic effect of the Akaki river water was also assessed by gene expression analysis of selected genes in exposed *Caenorhabditis elegans* and *Pseudomonas aeruginosa* PAO1.

Results: Genes associated with fluoroquinolone, aminoglycoside, β-lactamase, macrolide, multidrug resistance efflux pumps, tetracycline efflux pumps, and vancomycin resistance were detected in the water and sediment samples. The majority of ARGs were identified from sites in close proximity to anthropogenic activities such as hospitals, irrigation systems, and industries. Aminoglycoside acetyltransferase (*AAC(6)-Ib-cr*), aminoglycoside adenylyl-transferase (*aadA1*), β-lactamase (bla_{OXA-10}), quinolone resistance S (*qnrS*), macrolide efflux protein A (*mefA*) and tetracycline resistance (*tetA*) genes were detected at all sampling sites. Surprisingly, much fewer ARGs were detected in the associated sediments, and the sediment collected from the hospital site had the highest diversity and level of resistance genes. Genes such as bla_{OXA-10}, bla_{OXA-2}, *aadA*, *ermC* and *oprM* were persistent in all sediment samples. There was significant variation in the abundance of ARGs between study sites, with those

near the hospitals having the highest abundance in both waters and sediments. *Escherichia coli* strains isolated from the Akaki river were most frequently resistant to erythromycin (97%), doxycycline (78.5%), tetracycline (75.6%), and amoxicillin (75.6%). The overall prevalence of resistance to cephalosporin and carbapenem was 7.9% and 5%, respectively and 80.9% of *E. coli* isolates were MDR. Most *E. coli* isolates (70.8%) harbored enteroaggregative heat-stable toxin 1 (*EAST1*) gene, whereas the heat-labile toxin (*LT*) was detected in only 34 (20.2%) of the isolates. The prevalence of Shiga toxin-producing *E. coli* was 7.7%. The *E. coli* isolates belonged to 20 different sequence types, with ST10, ST69 and ST361 being predominant. The β-lactamase genes were the commonly detected resistance genes in *E. coli*. The most prominent plasmid sequence replicons detected were from the *IncF* family (65%), *Col* (10%), and *IncX3* (7.5%). The *Aeromonas* spp. isolated from the Akaki river were resistant to a wide range of antibiotics with the highest being resistant to amoxicillin (144, 100%), ampicillin (142, 99%), amoxicillin/clavulanate (117, 81%), imipenem (75, 52%), ertapenem (132, 92%) and doxycycline (134, 93%). The heat-labile cytotonic enterotoxin (*alt*) was the predominant toxin gene detected, followed by a heat-stable cytotonic enterotoxin (*ast*). Almost all *Aeromonas* isolates in the current study were new sequence types. *P. aeruginosa* isolates detected in the Akaki river were resistant to different classes of antibiotics and resistance is mediated by diverse group of ARGs with the majority were multi-drug efflux systems. Akaki river water induced a significant change in gene expression in exposed *C. elegans* and *P. aeruginosa* PAO1. Although the levels of almost all analyzed metals were below the permissible limit, the compiled effect resulted in transcriptional changes at the molecular level. A significant spatial variation was observed between the sampling points in the expression of certain genes.

Conclusion: The findings suggest that the water phase, rather than the sediments in the Akaki river, are a potential conduit for the spread of ARGs and thus antibiotic resistant bacteria. The occurrence of antibiotic resistance in *E. coli*, *Aeromonas* spp. and *P. aeruginosa* isolated from Akaki river provides evidence for the need to develop prevention and control strategies to limit the spread of antibiotic-resistant bacteria in the aquatic environment. The presence of virulent *E. coli* and *Aeromonas* spp. in the water could be posing a serious health risk to the

public. The Akaki river water has selection pressure in bacteria and elicits a toxic effect in *C. elegans* at the molecular level result in a higher risk of infectious disease.

Keywords: Aquatic environment, enteric bacterial pathogens, antibiotic resistance *C. elegans, P. aeruginosa* PAO1, toxicogenomic

CHAPTER 1: INTRODUCTION

1.1 Background

Environmental pollution is a major public and animal health problem in both developed and developing countries. There are different forms of pollution that affect the environment in different ways and at different levels of severity, one of which is water pollution (Landrigan *et al.*, 2018). Water is highly vulnerable to pollution and is frequently contaminated with biological and chemical pollutants that affect human and animal health. Due to increasing population growth and industrialization, water pollution is a major global challenge. Water contamination with bacteria, viruses, parasites, and fungi has been steadily increasing globally (Kistemann *et al.*, 2002). Access to safe water for all by 2030 is one of the UN sustainable development goals (UN, 2015). However, pollution-related morbidity and mortality are steadily increasing. Although environmental pollution affects all nations and communities, the burden is significantly higher in developing countries. According to the 2018 Lancet report, 92% of pollution-related deaths occur in low and middle-income countries and the predominant forms of pollution are contamination of drinking water and indoor air pollution (Landrigan et al., 2018). The World health organization (WHO) has reported 12.6 million annual deaths due to environmental pollution, including water and air pollutions (WHO, 2018). Environmental pollution affects human health and imposes a significant burden on the global economy (Landrigan and Fuller, 2014). While the contribution of African countries to the total global environmental pollution is minimal (Maplecroft, 2015), they are more vulnerable and share a more significant global burden than the more wealthy nations (Sarkodie, 2018, Maplecroft, 2015). The Sub-Saharan population is significantly affected by environment pollution due to poor waste management, and the lack of a regulatory monitoring framework (Orisakwe *et al.*, 2019). Sub-Saharan African countries adopt water quality standards from developed countries; however, the necessary implementation and monitoring requirements are not possible (Chikanda, 2009). Increased population growth, urbanization, changing lifestyles, industrialization, and agricultural pollution will be continued as a disproportion of demand and supply of water (Dos Santos *et al.*, 2017). Surface water is more prone to pollution because it serves as a recipient of point and nonpoint source of pollutants. Anthropogenic interventions, including waste disposals from industries, irrigation along the

river (Sarkar and Islam, 2019) and household waste (Ojeda-Benitez *et al.*, 2013) are major sources of surface water pollution.

Ethiopia is referred to as a water tower of Africa; however, surface water pollution is high primarily in major cities such as Addis Ababa and regional capitals. Similar to other developing countries, the water shortage and water quality in Ethiopia are a great challenge faced by the country in the 21st century (Awoke *et al.*, 2016, van den Berg *et al.*, 2019, Beyene *et al.*, 2009). Rapid population growth, poor sewage infrastructure, urbanization and industrialization are among the major contributors to poor water quality in Ethiopia (EPHI, 2017a). The majority of the research into water quality in Ethiopia has focused on drinking water and in particular bottled water, however, little attention has been given to surface water. Surface water such as rivers and lakes have great public health importance because of urbanization and the need for domestic purposes. According to the 2016 Ethiopian demographic and health survey (EDHS) report, 65% at the national level and 57% of rural households have access to improved sources of drinking water (EDHS, 2016). River pollution has been one of the main aquatic environment issues of Addis Ababa. Hospitals, industries, research and diagnostic laboratories, agricultural runoff water, waste from poultry and households directly dispose of their waste to the Akaki rivers or its tributaries without prior treatment (Basha, 2007).

Rivers in Addis Ababa are generally used as a waste disposal site despite the presence of the city's environmental protection policy. Most raw vegetables in the city market are irrigated by the Akaki river and this results in great public health concern. In addition, farmers using Akaki river water for irrigation reported a variety of morbidities including skin disease, wound infection, allergies, and gastrointestinal tract disorders. Vegetables irrigated by the Akaki river water contaminated by toxic substances had a very high risk to human health, such as minor inflammation, cancer, liver damage, mental disabilities, and reproductive diseases (Aschale *et al.*, 2019, Weldegebriel *et al.*, 2012). The study conducted by the Ethiopian public health institution revealed that residents along the Akaki river and farmers that use Akaki river water for irrigation are at higher risk of exposure to pathogens and toxic chemicals (EPHI, 2017a). The downstream residents outside Addis Ababa city use the river water for domestic purposes.

Pesticides used in agricultural activities along the river and metals from industries end up in the Akaki river. The Federal Ministry of Health reported that the level of indicator organisms exceeds the WHO guideline threshold for safe irrigation water quality (FMoH, 2017). Pathogens transmitted through polluted water cause intestinal infections and common water-borne diseases in Addis Ababa such as acute watery diarrhea, typhoid, dysentery, and cholera (Weldesilassie *et al.,* 2017). In addition to the microbial load, the level of chemical pollutants is higher than the WHO and Ethiopian drinking water guideline permissible limit (Melaku *et al.*, 2004). A similar study by Aschale *et al* (2015) also revealed that the level of most heavy metals in the Akaki river was higher than the guideline permissible limit. For instance, the levels of Cr (maximum guideline level 100 µg/L), Mn (maximum guideline level 200 µg/L), and Sr (maximum guideline level 20 µg/L) were above the permissible limits (Aschale *et al*., 2015) of FAO for irrigation water quality guideline (Ayers and Westcot, 1994). The current physicochemical analysis for water quality monitoring whose values are compared against cut-offs in guidelines for which risk levels are fixed in guidelines (Sutadian *et al.*, 2015, Venkatesharaju *et al.*, 2010). This approach will miss the synergetic and antagonistic effects of pollutants at the genetic level. Therefore, advanced risk assessment methods such as gene expression using model organisms such as *Caenorhabditis elegans* and *Daphnia magna* are required to determine aquatic chemical pollutants and their biological effect in animals.

Waterborne diseases are major public health and environmental concerns worldwide and have a substantial contribution to worldwide morbidity and mortality (Nwabor *et al.,* 2016). Consumption of raw fresh vegetable products irrigated with contaminated waters is a major risk factor contributing to gastrointestinal illness in the population, due to the increased contamination with pathogenic bacteria (Sapers G *et al.,* 2014). The effect of waterborne diseases varies in severity from mild gastroenteritis to severe, and occasionally fatal diarrhea, dysentery, typhoid fever, and hepatitis (Abraham *et al.*, 2007), and diarrheal diseases are the most common water-borne diseases. Although it is a global public health problem, the impacts are more severe in developing countries due to limited water treatment facilities. Globally in 2015, diarrhea was the leading cause of death, with an estimated annual death of 1.31 million (Troeger *et al.,* 2017). Contamination of surface water bodies by water-borne pathogens and associated diseases are a major water quality issue worldwide (Jia *et al.,* 2018).

The prevalence of food-borne pathogens varies by region and can be relatively high in developing countries due to poor sanitation infrastructure. In Africa, diarrheal disease is a major cause of morbidity and mortality among infants and young children. Factors such as unimproved water supplies, unhygienic disposal of human waste, poor environmental hygiene and sanitation, and insufficient education (Girma *et al.*, 2018), eating raw beef and raw vegetables (Girmay *et al.*, 2020, ECA, 2016) are major contributors to the disease burden. Most common diarrheal diseases are transmitted through the consumption of contaminated water and food (Sumner *et al.*, 2011, Kotloff, 2017). Diarrheal diseases are a major public health concern in Ethiopia and result in annual death of 47.1 per 100,000 in all age groups (Troeger et al., 2017).

Enteric bacterial pathogens are commonly responsible for water-borne diseases and the pathogens of interest include Enterohemorrhagic *Escherichia coli* (*E. coli* O157:H7), *Aeromonas* spp., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp, *Staphylococcus aureus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Vibrio* species (Kotloff, 2017). The occurrence of Enteropathogenic *E. coli* in surface water increases the risk of waterborne disease. The risk is increased if the water is used for drinking, irrigation of raw eat vegetables, and recreational purposes. Although most *E. coli* strains are commensal gut bacteria of humans and animals, some strains harbor virulence genes associated with various clinical outcomes such as diarrhea, hemorrhagic colitis, urinary tract infections, sepsis, or meningitis. The clinical outcomes caused by *E. coli* infection differ on their host preference, colonization site, and virulence determinants. For instance, *E. coli* O157:H7 is an enterohemorrhagic *E. coli* (EHEC) strain that is recognized as a cause of severe and fatal illness. It is a Shiga toxin-producing *E. coli.* Shiga toxin-producing *E. coli* strains are important human pathogens, causing hemorrhagic colitis and hemolytic-uremic syndrome (Croxen and Finlay, 2010). EHEC infections are associated with the consumption of contaminated water, vegetables, and dairy products (Mead and Griffin, 1998). Potential diarrheal-associated virulence genes in *E. coli* strains isolated from river water have been reported (Widmer *et al.*, 2013, Munshi *et al.*, 2012a).

Aeromonas are emerging opportunistic pathogens and responsible for different clinical outcomes such as cellulitis, gastroenteritis, wound infections, urinary tract infections, and life-threatening conditions such as meningitis and septicemia (Lamy *et al.*, 2009). Human infection caused by *Aeromonas* most commonly occurs in community settings, although infection can also occur in healthcare facilities. Gastrointestinal illness associated with *Aeromonas* infections includes acute watery diarrhea, dysenteric diarrhea, chronic diarrhea, and traveler's diarrhea (Janda and Duffey, 1988). Complications such as small bowel obstruction (Block *et al.*, 1994), acute renal failure (Filler *et al.*, 2000), and hemolytic-uremic syndrome (Fang *et al.*, 1999) can also be caused by enterotoxin producing *Aeromonas* strains. In recent years, reports have shown that *Aeromonas* is an important bacterial wound pathogen related to exposure to environmental or hospital water polluted by *Aeromonas*. *Aeromonas* transmission is through oral consumption of contaminated food or direct contact with contaminated water, dirt, seafood, or food items indirectly on contact with contaminated irrigation water. Not all *Aeromonas* species cause disease in humans. *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas veronii* are the most commonly implicated in human intestinal infections (Janda, 1991). Other pathogens such as *V. cholerae*, *Salmonella*, *Shigella*, and *P. aeruginosa* are frequently reported from surface water and increased risk of public health.

Water-borne diseases are most often self-limited with some supportive cares. However, some individuals may require antibiotic treatment depending on the severity of the disease and types of pathogens (Chalmers, 2014). The increasing spread of antibiotic resistance among enteropathogens significantly affects the treatment outcome of water-borne diseases. Decades of extensive use of antibiotics to treat human and animal infections and as food supplements in livestock (Kemper, 2008, Bacanlı and Başaran, 2019) has led to the rapid emergence of antibiotic-resistant bacteria (Leung *et al.*, 2011). Antibiotic-resistant bacteria are a major global public health problem and have resulted in reduced treatment options for certain infections (Bush *et al.*, 2011). Morbidity and mortality due to infection caused by antibiotic-resistant microbes are among the healthcare crises of the 21st century (Zealand, 2014).

Globally, drug resistance causes 700,000 deaths each year, and the current resistance rate is steadily increasing. Antimicrobial resistance could result in over 10 million annual deaths worldwide by 2050 (Kraker *et al.*, 2016). According to the European Centre for Disease Prevention and Control (ECDC), approximately 33,000 people die annually from infections caused by antibiotic-resistant bacteria in the European Economic Area. The report shows that 58.3% of *E. coli* are resistant to at least one antibiotic (Cassini *et al.*, 2019). In addition, *E. coli* shows increased resistance to different antibiotics; for instance, resistance to quinolones has risen from 19% in 2012 to 26% in 2017 in South Africa (AfricaCDC, 2018). Although complete data is not available, *E. coli* resistance to amoxicillin, trimethoprim and gentamicin is significantly increasing. Similar trends have been observed in other bacterial pathogens (Tadesse *et al.*, 2017). In Sub-Saharan Africa, antibiotic resistance is a major public health concern due to the lack of diagnostic facilities and poor antimicrobial resistance monitoring systems (Kariuki and Dougan, 2014).

Antibiotic-resistance among enteropathogens is becoming a critical concern, especially in developing countries. To control the spread of antibiotic-resistant microbes, research has been predominantly focused on isolates from clinical settings with little concern for the environmental transmission of antibiotic resistance (Martinez, 2009). However, environments outside the healthcare facilities play a significant role in the spread of resistant bacteria (Kraemer *et al.*, 2019, Novovic *et al.*, 2015, Knapp *et al.*, 2017). Antimicrobial resistance cannot be addressed by simply studying the problem in healthcare facilities, and it necessitates combating antibiotic resistance from a one health perspective across the human, veterinary, and environment is crucial to controlling the evolution, persistence, and spread of resistant pathogens (Wang *et al.*, 2018). Reports have shown the evolution and persistence of antibiotic resistance genes in bacteria isolated from various aquatic environments. Factors such as continual discharge of antibiotics to the aquatic environment, heavy metals, organic pollutants, and horizontal gene transfer play a significant role in resistance in the aquatic environment (Widmer *et al*., 2013). The majority of the antibiotics are excreted in their active form and in the environment that can provide selection pressure that can increase the persistence of resistant bacteria in aquatic environments (Munir *et al.*, 2011). Effluent from

healthcare facilities is also a potential source of antimicrobial resistant bacteria in the aquatic environment (Kraemer et al., 2019). In addition, sediments in the aquatic system are considered as an important reservoir for antibiotic resistance genes and facilitate the dissemination of antibiotic-resistant microbes in the environment (Zhu *et al.*, 2017). Although most of the antibiotics have a short half-life in the environment, continual discharge results in pseudo-persistent sub-inhibitory concentrations that promote continual selection of resistant bacteria (Gullberg *et al.*, 2011, Kümmerer, 2009). Antibiotics and heavy metals are major driving forces for the increasing occurrence of antibiotics resistance genes in the environment. Reports have shown that bacteria isolated in surface water polluted by high levels of heavy metals are resistant to both heavy metals and antibiotics (Roberto *et al.*, 2019, Xu *et al.*, 2019) due to that the resistance genes against heavy metals and antibiotics are often colocalized on the same mobile genetic element (Chattopadhyay Madhab and Hans-Peter, 2011). The negative impact of human activities along aquatic environments is a concern for the persistent spread of antibiotic-resistant pathogens (Jia et al., 2018, Jiang *et al.*, 2018). Bacteria can acquire antibiotic resistance through mutations and horizontal gene transfer (Finley *et al.*, 2013) and this contributes to the abundance and diversity of resistance genes in aquatic environments (Smillie *et al.*, 2011). For instance, *E. coli* strains isolated from the aquatic environment, showed significant resistance to currently used antibiotics and the resistance is mediated by diverse plasmid and chromosomal genes (Dhawde *et al.*, 2018, Singh *et al.*, 2018, Diwan *et al.*, 2018, Alwash and Al-Rafyai, 2019). Rivers, especially in urban regions, serve as a reservoir for drug-resistant *E.coli*. For instance, the study done on Chaophraya River, Thailand, has revealed high resistance rates of *E. coli* to fluoroquinolones, amoxicillin, tetracycline, and other antibiotics, and resistance was dominantly attributed to the anthropogenic source of the urbanized watershed (Honda *et al.*, 2016).

As in many regions, the spread of antibiotic-resistant bacteria in clinical settings is well documented in Ethiopia (Moges *et al.*, 2014). Factors such as misuse of antibiotics by health care providers and drug consumption cause the rapid spread of resistant bacteria in Ethiopia. A review of antimicrobial stewardship intervention and clinical outcomes in referral hospitals of Ethiopia has shown that 50% of the prescribed antibiotics are not needed (Gebretekle *et al.*,

2020). A cross-sectional study in Addis Ababa has shown that prescriptions of high doses of broad-spectrum antibiotics, prolonged treatment, and increased prevalence of empirical treatment facilitate the emergence of resistant microbes in health care facilities (Worku and Tewahido, 2018). It has been shown that broad-spectrum antibiotic therapy favours the development of resistant bacteria (Cižman and Plankar Srovin, 2018). A report by the Ethiopian public health institution has also revealed widespread development of antibiotic resistance in many pathogens such as *E. coli* (EPHI, 2017b). A study conducted in major hospitals of Addis Ababa has revealed that the majority of Gram-negative bacteria from surgical site infections were multiple antibiotic and pan-drug resistant (Dessie *et al.*, 2016).

Intensive research has been done on major pathogens, including *E. coli*, in Ethiopian healthcare facilities (Dadi *et al.*, 2020, Tuem *et al.*, 2018, GebreSilasie *et al.*, 2018, Desta *et al.*, 2016); however, the community-acquired resistance, as well as the contribution of environmental factors, is not well characterized in Ethiopia. Rivers in Ethiopia are major waste recipients in major cities. The gut of human and animal is the major sources of antibiotic resistant pathogens and therefore increasing urbanization and population growth contribute to the dissemination of antibiotic resistance in the wastewater recipient rivers. Akaki rivers in Addis Ababa are severely affected by increased population. Industries such as pharmaceutical, breweries, tanneries, distilleries, wineries, and national alcohol liquor factories in Addis Ababa are situated along the course of Akaki river and most discharge their waste directly to the river or its tributaries without prior treatment (Yohannes and Elias, 2017, Aschale *et al.*, 2015). According to the Ethiopian federal environmental protection authority, hospitals in Addis Ababa generated 430.7 tons of contagious waste such as blood and other body fluids, bacterial cultures, wound dressings, and needles. Effluent from healthcare facilities and research laboratories ends up in the Akaki river or its tributaries (FEPA, 2005). Since anthropogenic activities play a significant role in the dissemination and abundance of antibiotic resistance genes (ARGs) and resistant bacteria in rivers in major cities (Chen *et al.*, 2019), discharges from these factories could create selective pressure for evolution of resistance among the microbial communities in the Akaki river.

Assessing the role of the aquatic environment for the evolution and spread of antibiotic resistance and the potential hotspot for the spread of virulent bacterial strains is crucial to control drug resistance and transmission of potentially pathogenic strains. In recent years, increasing levels of ARGs have been reported in aquatic environments (Zhu *et al.*, 2013, Zhou *et al.*, 2017b). However, the majority of these studies have used culture-based methods that usually involve isolating target bacteria on culture media followed by evaluating resistance or virulence genes. Evaluating antibiotic resistance in culturable bacteria from the environment will only provide partial information and will miss detecting non-cultivatable or non-growing bacteria that may potentially carry resistance genes. Therefore, investigating ARGs in bacterial communities in a multi-variate approach provides a comprehensive and quantitative insight on antibiotic resistance in the environment. Quantitative PCR and metagenomics can be used to track the presence of diverse resistance genes within bacterial communities. However, metagenomics fails to detect a low abundance of pathogenic bacteria and ARGs in environmental samples (Suttner *et al.*, 2020). Therefore, in the current study, high-throughput DNA qPCR arrays were used to determine a more comprehensive picture of clinically relevant antibiotic resistance genes and bacteria from river water and sediments together with culture-based methods for functional and molecular characterization of potential pathogens. To the best of our knowledge, this is the first study to investigate potential bacterial pathogens and ARGs in bacterial communities in the Akaki rivers of Ethiopia.

1.2 Literature Review

1.2.1 Overview of Water-Borne Bacterial Pathogens

Water-borne diseases pose an effect on human health and a substantial burden on the global economy. Water-borne diseases cause annual deaths of approximately 2.2 million people globally. It has also a significant impact on the global economy due to increased healthcare costs and loss of work time. It is estimated that water-borne disease causes an economic loss of 12 billion US $ each year for hospitalization and associated costs (Alhamlan *et al.*, 2015). Although water-borne diseases can be caused by bacteria, viruses or parasites, the majority of outbreaks are linked with bacteria. Water contaminated with medically relevant bacteria can cause a variety of clinical outcomes, including gastrointestinal illness, respiratory illness, hepatitis, dermatitis and associated adverse health effects such as neurological disorders and reproductive problems. The presence and increased bacterial load of human origin increase the risk of disease in susceptible individuals. Therefore, screening for these microbes and their diversity is one of the most important strategy of water quality monitoring (Rodrigues *et al.*, 2011). A major mode of water contamination with pathogenic microbes is normally via the release of untreated fecal matter into aquatic environments. The risk of water-borne disease occurs when there is direct contact with contaminated water or indirectly through contaminated food. Contamination of surface waters with bacterial pathogens is a major water quality issue due to the increased risk of disease spread. Aquatic environments act as a hotspot where bacteria from different sources mix with organic and metal pollutants that provide selection pressure and horizontal gene transfer, resulting in the exchange of genetic elements including antibiotic resistance and virulence genes (Finley *et al.*, 2013). Increased levels of fecal microbes in aquatic environment have been shown to increase the risk of water-borne illnesses (Fleisher *et al.*, 1998). Pathogenic bacteria have been reported in many rivers, especially in megacities. To reduce the risk of water-borne disease transmission, the bacterial pathogens represented by the fecal coliforms counts should not exceed 1000/100 mL for safe use of surface water for irrigation (EPHI, 2017a). Water bodies receive a diverse group of pathogenic bacteria from point and non-point sources of pollution. Pathogens from a point source enter the river at different locations such as discharge of domestic sewage, industrial effluent, and mine drainage. The major sources of pollution for rivers are non-point sources

such as fertilizers and insecticides from agricultural land, which comprise up to 80% of the pollution entering major river systems and thus are of major concern with respect to the spread of clinically relevant bacteria in aquatic systems. Other sources, such as poorly sited septic systems, can also introduce diverse group of bacterial pathogens into the river system (Stewart *et al.*, 2008).

1.2.2 Transmission of water-borne pathogens

Water-borne diseases can be transmitted through various routes. Drinking contaminated water and consumption of raw vegetables grown by pathogen contaminated water are the major routes of water-borne disease transmission. The wide range of bacterial pathogens found in environmental waters can subsequently mix with the irrigation water to be used for agriculture and thereby reach products that will be consumed by the community. Direct transmission to the population can also occur if the water is used for domestic purposes such as for drinking and washing. Pathogens in the contaminated water transmitted to vegetables and then to humans are responsible for frequent water-borne disease outbreaks (Pachepsky *et al.*, 2012). For instance, bacillary dysentery is responsible for approximately 165 million cases of bacterial diarrheal diseases annually and almost all of the cases are from developing countries with poor water supply (Sharma *et al.*, 2009). For pathogens transmitted by the fecal-oral route, water is one of the major vehicles for transmission. Seasonal variation is a critical factor influencing waterborne disease trends in the developing world. Scholars have reported the correlation between the prevalence of diarrheal diseases and climate in particular, rainfall. This relationship is complex and therefore, can differ from region to region and thus different transmission routes may be favored. In Africa it is widely reported that transmission of water-borne pathogens peaks during the rainy season likely due to an acceleration of increasing contamination to water bodies (Findley *et al.*, 2005). However, in another study, the peak in diarrheal diseases was during the dry season when water scarcity affected hygienic conditions and favored transmission through fecal-oral route (Mølbak *et al.*, 2000). Diverse bacterial pathogens such as *Escherichia coli, V. cholerae*, *Campylobacter* spp, *Salmonella* spp., *Shigella* spp, *Aeromonas* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., *Legionella* spp., and

Helicobacter pylori have been reported as major etiological agents in most of the water-borne outbreaks worldwide (Benedict et al., 2017).

1.2.3 *Escherichia coli*

One of the major causes of water-borne enteric disease is *E. coli*. Although *E. coli* are generally regarded as commensals bacteria (Kaper *et al.*, 2004), certain strains produce potent toxins that cause infections in humans and animals. *E. coli* in environmental water creates a potential risk for infections in humans and animal especially if the water is used for drinking and other domestic purposes, and for irrigation of raw eat vegetables. Pathogenic *E. coli* strains can be classified as enteric/intestinal pathogenic *E. coli* (InPEC), or extra-intestinal pathogenic *E. coli* (ExPEC) based on their virulence properties and location of infection. The intestinal infections include diarrhea or hemorrhagic colitis, while extra-intestinal infections include urinary tract infections, sepsis, and meningitis. Based on the virulence factors, enteric pathogenic *E. coli* can be further divided into six classes. These are enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and diffusely adherent *E. coli* (DAEC) (Croxen and Finlay, 2010), whereas the extra-intestinal pathogenic include uropathogenic *E. coli* (UPEC), which is the main cause of urinary tract infection, meningitis-associated *E. coli* (MNEC), sepsis-associated *E. coli* (SEPEC) and avian pathogenic *E. coli* (APEC), which is associated with respiratory infections (Kaper *et al*., 2004). Among the intestinal *E. coli* strains, ETEC, EHEC, and EIEC are the most clinically important that can be transmitted by consumption of unsafe water. ETEC serotypes are associated with infantile gastroenteritis and are commonly reported in developing countries where there is inadequate clean water supply. It is the leading bacterial cause of diarrhea in developing countries as well as the most causes of travelers' diarrhea especially people traveling from developed countries to developing regions. It is estimated that ETEC infections cause 650 million cases of diarrhea and 380, 000 associated deaths globally in children. It causes disease by colonizing the small intestine using fimbriae antigens and then production of enterotoxins such as heat-labile and/or heat-stable enterotoxins and leads to diarrhea (Sizemore *et al.*, 2004). EHEC is an enteropathogen that colonizes the colon and causes bloody diarrhea and hemolytic uremic syndrome. Even though

its transmission is mainly through the consumption of raw food, consumption of drinking water contaminated with sewage or human feces is also a possible way of transmission. Outbreaks caused by this strain is mainly associated with consumption of vegetables and fruits. Few reports indicate the presence of asymptomatic carriers may potentially transmit infections to susceptible individuals through the fecal-oral route. This strain encodes several virulence determinants including Shiga toxin and locus of enterocyte effacement (Karch, 2001).

EIEC is one of the pathotypes of diarrheagenic *E. coli* whose pathogenesis in humans resembles that of shigellosis and causes gastroenteritis, dysentery, and enterocolitis. Although this serotype is caused by consumption of contaminated water and food, outbreaks are mainly associated with the consumption of unpasteurized milk and hamburger meat (Cabral, 2010). Several virulence factors such as invasion-associated locus, secreted autotransporter toxin gene (*sen*), and Shigella IgA-like protease homolog gene (*sigA*) involve in the pathogenesis of this serotype (Farajzadeh-Sheikh *et al.*, 2020). The extra-intestinal pathogenic *E.coli* strains carry different combinations of virulence genes from those of InPEC strains and thus cause different clinical outcomes (Ahmed *et al.*, 2011). Scholars have reported that the majority of environmental pathogenic *E. coli* strains in surface waters are ExPEC. For instance, Gomi *et al* (2015) found that almost all *E. coli* isolates in the river water were extra-intestinal pathogens, only 1.5% were intestinal (Gomi *et al.*, 2015).

Presence of high colony forming unit (CFU) of *E. coli* in water is an indication of recent fecal contamination, and therefore presence of enteropathogenes. *E. coli* can serve as an indicator bacteria for water pollution by feacal contaminants. *E. coli* densities of greater than a geometric mean of 200CFU/100 mL indicate that the water is not safe for swimming and bathing (Canada, 1992). High-risk *E. coli* strains have been reported from river water in different countries. A report by Widmer *et al* (2013) in Southeast Asian cities revealed that from 157 river water samples a total of 564 *E. coli* isolates were detected of which 3.9% were observed to have virulence genes associated with diarrhea and Shiga toxin-producing *E. coli* isolates are the most common strain (Widmer *et al.*, 2013). Numerous virulence determinants

including toxins, adhesins, secretion systems, and invasins are involved in *E. coli* pathogenesis. Various virulence genes in *E. coli* strains that were isolated from river water have been reported. Obi *et al* (2004) found that cytotoxic necrotizing factors (c*nf1*and *cnf2*) coding for necrotoxigenic *E. coli*; bundle-forming pilus (*bfpA*) and enteropathogenic attachment and effacement (*eaeA*) coding for enteropathogenic *E. coli* (EPEC), occurred in 35% and 34% of *E. coli* isolates respectively (Obi *et al.*, 2004). Similarly Munshi *et al* (2012) reported that among the tested *E. coli* isolates, 60% harbored the *stx* gene encoding Shiga toxin (Stx) and 40% carried *elt* gene encoding heat-labile enterotoxin (LT). However, none of the isolates contained the *est* gene encoding heat-stable enterotoxin (ST) (Munshi *et al.*, 2012b).

Aquatic environments including rivers especially those that receive human and animal waste are major conduits for the persistence and spread of antibiotic-resistant *E. coli*. For instance, the study done at the Tama River basin, Japan showed *E. coli* strains were resistant to trimethoprim-sulfamethoxazole, ampicillin, and tetracycline. Antibiotic-resistant *E. coli* in the aquatic environment is dominantly associated with anthropogenic source of urbanized watershed areas. *E. coli* isolates collected at effluent points and downstream had significantly smaller mean zones of inhibition to several antibiotics tested compared to isolates collected upstream, exhibited low human activities, of the effluent point (Kobori *et al.*, 2004).

1.2.4 *Salmonella* Species

Contamination of river water with *Salmonella* has been reported globally. Discharge from fields associated with animal husbandry, household waste and untreated sewage disposal contribute to the persistence of *Salmonella* in surface waters, including rivers (Seo *et al.*, 2006). Many serotypes of *Salmonella* are known as gastrointestinal pathogens, and some are capable of causing illness in humans and animals. Contaminated foods and water are the two main sources of *Salmonella* infection, and outbreaks are frequently associated with poor sanitary conditions. Contamination of river water with *Salmonella* species is a great concern since they can survive for long periods in surface waters. Diverse serotypes of *Salmonella* isolates were identified from rivers and wastewaters (Baudart *et al.*, 2000). Globally *Salmonella enterica* subsp. *enterica* serovar *Enteritidi*s is commonly isolated serovar from the

human disease although locally predominant serovars also observed in different locations (Cabral, 2010). Vegetables and fruits grown by contaminated water can carry *Salmonella* and transmit it to the human consumer. A prevalence rate of 19% *Salmonella* was reported from rivers in Kenya (Ew *et al.*, 2015). Of the *Salmonella* species, river water serves as a pool for *Salmonella enterica*. For instance, the report by Mary *et al* (2016) showed that 61% *Salmonella* isolates from the river were *S. enterica* (Callahan *et al.*, 2019). The other report from South Africa has also shown that *Salmonella* species were detected from all sample sites of a river and 93% harbored the *spi*C gene encoding for Salmonella pathogenicity island 2 protein C, 84% harbored the *misL* gene encoding for an autotransporter protein and 87% harbored the *pip*D gene encoding for cysteine protease. The majority of isolates were resistant to sulfamethoxazole, nalidixic acid and streptomycin, but susceptible to quinolones and third generation cephalosporines (Odjadjare and Olaniran, 2015). Similarly, in India, of the total *Salmonella* isolates from surface water, 42.8% were found to be multi-drug resistant (Maloo *et al.*, 2014).

1.2.5 *Vibrio* Species

Other common bacterial pathogens associated with diarrhea due to poor water quality are *Vibrio* species. *Vibrio* spp. are Gram-negative bacteria, facultatively anaerobic, rods, motile or nonmotile and mesophilic or psychrophilic. The genus *Vibrio* is abundant, and a natural inhabitant of water bodies. There are around 100 species within the genus, but only 12 are pathogenic to humans of which the most clinically relevant are *Vibrio cholerae, Vibrio vulnificus* and *Vibrio parahaemolyticus*. *V. parahaemolyticus* is associated with acute food-borne gastroenteritis whereas *V. vulnificus* is an important cause of septicemia and wound infection. Based on the lipopolysaccharide on its surface, *V. cholerae* is divided into several serovarieties. However, only serovar O1 and O139 are associated with the disease cholera. *V. cholerae* O1 strain has been responsible for all major cholera epidemics (Ramamurthy *et al.*, 1993). Pathogenic *V. cholerae* isolates contain three main virulence determinants. These are potent enterotoxin cholera toxin, the colonization factor toxin-coregulated pilus, and the main regulatory protein ToxR which helps cholera spread and pathogenesis. Water bodies are main reservoirs of *V. cholerae*. Therefore, monitoring of *V. cholerae* O1/O139 distribution in the

aquatic environment is crucial for cholera prevention and control programs. Like other water bodies, river water commonly contaminated by *Vibrio* spp. (Seman *et al.*, 2012). Li *et al* (2016) reported *V. cholerae* O1 and O139 in Pearl River Estuary, China. All *V. cholerae* O1/O139 isolates were *ctxAB* negative and 37% carried *tcpA* gene encoding the structural subunit of the toxin-coregulated pilus (Li *et al.*, 2016). Similarly Kokashvili *et al* (2015) reported *V. cholerae, V. parahaemolyticus, V. vulnificus* and other species, and there was seasonal variation on the frequency of clinically relevant *Vibrio* isolates in the aquatic environment (Kokashvili *et al.*, 2015). Environmental *V. cholerae* isolates were found to be susceptible to chloramphenicol, rifampicin, tetracycline, variable to ampicillin and resistant to kanamycin and streptomycin (Seman *et al.*, 2012).

1.2.6 *Campylobacter* Species

The genus *Campylobacter* is Gram-negative bacteria comprises different species and some of them cause infection in human. Aquatic environments are the main sources of *Campylobacter* infections in humans. The human pathogens, *Campylobacter jejuni* and *Campylobacter coli*, are known to be common agents of enteritis in humans and are generally regarded as foodborne pathogens. Infection with *Campylobacter* usually leads to diarrhea manifested as inflammatory diarrhea and abdominal cramps in adults. People can be infected by *Campylobacter* by eating undercooked poultry products, seafood, and meat, contact with animals and drinking untreated water. *Campylobacter* species have been frequently reported from surface water. A study in France by Denis *et al* (2011), for instance, showed that 50% of the river samples were contaminated by *Campylobacter* species, and *C. jejuni* was the most frequently detected species (74.1%), followed by *C. coli* (17.8%) and *Campylobacter lari* (8.1%) (Denis *et al*., 2011). The highest prevalence of *Campylobacter* species was reported in the river than streams, wells, and ponds (Karikari *et al.*, 2016). Higher *Campylobacter* contamination of surface waters has been also reported in Poland (Popowski *et al.*, 1997), India (Baserisalehi *et al.*, 2005) and Nigeria (Ugboma *et al.*, 2012). *Campylobacter* isolates reported by Karkari *et al* (2016) were found to be resistant to different antibiotics tested at a rate of 100% to β-lactams, 98% to erythromycin, 48-69% to the quinolones, 45-55% to

aminoglycosides, 71% to trimethoprim-sulfamethoxazole, 76% to tetracycline and 90% to chloramphenicol (Karikari et al., 2016).

1.2.7 *Shigella* Species

Shigellosis caused by *Shigella* spp. is one of the most common infectious diseases in developing countries and among travelers to endemic areas. The genus *Shigella* consists of four species, *Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei*, and at least 47 serotypes (Peirano *et al.*, 2006). Direct person-to-person, eating contaminated food, and drinking contaminated water are the main modes of transmission of *Shigella*. Several virulence factors such as large invasion plasmid (ipaBCD and ipaH) and genes encoding Shiga toxin (*stx1* and *stx2*) are involving in the pathogenesis of *Shigella* (Newland and Neill, 1988). *Shigella* species can be detected in surface water or sewage. Sharma *et al* (2009) reported *S. flexneri*, *S. sonnei* and *S. dysenteriae* from river Narmada, India and the majority of the isolates were *S. flexneri*. All isolated *Shigella* species were positive for *ipaH* gene whereas the invasion-associated gene *ipaBCD* was present only in *S. flexneri* and the gene, *stx1* was only in *S. dysenteriae* (Sharma *et al.*, 2009). *S. boydii* was found to be the most predominant *Shigella* species of the river catchments in South Africa followed by *S. dysenteriae*, *S. flexneri* and *S. sonnei* (Kinge and Mbewe, 2010). It has been reported that a significant number of environmental *Shigella* isolates are resistant to commonly used antibiotics to treat shigellosis. For instance, the majority of *S. dysenteriae* isolates from the river, Narmada, were resistant to amoxicillin (71.4%), gentamycin (57%), cefotaxime (57%) and amikacin (42.8%). All isolated *S. dysenteriae* were susceptible to chloramphenicol and trimethoprim. On the other hand, *S. sonnei*, 70% were resistant to amoxicillin and 60% to ampicillin, but all the strains were found to be susceptible to cefotaxime, ceftazidime, amikacin, chloramphenicol, ciprofloxacin and norfloxacin. Out of the total *S. flexneri* isolates, 52.5% were resistant to ampicillin, 43% resistant to amoxicillin, 34.8% resistant to ceftazidime, 30% resistant to nalidixic acid, 26% resistant to tetracycline, 17.4% resistant to chloramphenicol, while all the isolates were found to be susceptible to amikacin, cefotaxime, ciprofloxacin, and norfloxacin (Sharma *et al*., 2009).

1.2.8 *Aeromonas* Species

In recent years, *Aeromonas* has become increasingly recognized as enteropathogen involved in many food-borne disease outbreaks (Batra *et al.*, 2016). The genus *Aeromonas* are ubiquitous in the aquatic environment and are easily transmitted to humans and causing a wide range of clinical outcomes, especially gastroenteritis. *Aeromonas* in the aquatic environment does not correlate with currently used indicator bacteria for water quality assessment. The rapid increase of antibiotic resistance in *Aeromonas* species is another concerning feature of this group of bacteria. Not only clinical isolates but also environmental isolates are resistant to clinically relevant antibiotics especially against β-lactam antibiotics (Maravić *et al.*, 2013). More severe gastroenteritis caused by *Aeromonas* species resembles the symptoms of shigellosis. The two species, *Aeromonas veronii* and *Aeromonas caviae*, are most frequently associated with traveler's diarrhea (Block *et al*., 1994). The pathogenicity of *Aeromonas* species is complex and involves a series of virulence factors, toxins, and adhesins. Pathogenic *Aeromonas* strains have a wide range of toxin virulence genes. The toxins are divided into two major groups, cytotoxic enterotoxin, and cytotonic enterotoxin. The cytotonic enterotoxins are bacterial toxins that alter the function of cells without killing whereas cytotoxic enterotoxins kill the host cells. Frequently reported toxin genes from *Aeromonas* strains isolated from diarrheal patients are cytotoxic enterotoxin (*act*), cytotonic enterotoxin, heat-labile cytotonic enterotoxin (*alt*), heat-stable cytotonic toxins (*ast*), hemolysin (*hlyA*) and aerolysin (*aerA*). The lateral flagella (dysenteric infections) and the type III secretion system (*TTSS*) have also been reported from both clinical and environmental isolates. Few reports have also shown Shiga toxin producing *Aeromonas* isolates from diarrheal patients (Chen *et al.*, 2014). A study conducted in Egypt on the *Aeromonas* strains isolated from food samples was found to be highly virulent and a significant number of isolates carried *alt*, *aerA*, *act*, and *ast* toxin genes. Significant numbers of these isolates were also multi-drug resistant (Hammad *et al.*, 2018). A similar study in Israel among patients with diarrhea showed that the majority of patients from which *Aeromonas* were isolated were negative for other diarrhea-causing agents. This indicates that *Aeromonas* may be responsible for the clinical outcome. In this study, most isolates harbored several virulence genes (*act, hlyA, aerA, alt, TTSS,* and others) and resistant to β-lactam antibiotics (Senderovich *et al.*, 2012).

1.2.9 Pseudomonas aeruginosa

Pseudomonas aeruginosa is an important opportunistic human pathogen and due to its high metabolic versatility, it adapts to a variety of environmental conditions including the aquatic environments (Hall *et al.*, 1998). It can grow and multiply in a wide range of aquatic systems including rivers, lakes, oceans, and battled water (Kimata *et al.*, 2004). The presence of *P. aeruginosa* in moist environments as found in hospital sinks and air-conditioning units is a major problem (Ayliffe *et al.*, 1974). River water is a source of *P. aeruginosa* infection especially if the water used for recreational and domestic purposes (Medema *et al.*, 1997). Frequent outbreaks of *P. aeruginosa* infections have been reported globally from exposure to surface water (Mena and Gerba, 2009). Infection with *P. aeruginosa* is an opportunistic and is thus more prevalent among those immunocompromised such as burn victims, cystic fibrosis, acute leukemia, organ transplants, and intravenous-drug users (Bodey *et al.*, 1983). Natural resistance to a diverse group of antibiotics is one of the features of *P. aeruginosa*, also to a tendency to develop acquired resistance to antibiotics (Suzuki *et al.*, 2013). In recent years multi-drug resistant *P. aeruginosa* causes serious problems in the management of infections. Not only the clinical isolates but also environmental *P. aeruginosa* isolates were found to be multi-drug resistant due to selection pressure by antibiotics and other chemical pollutants (Magalhães *et al.*, 2016). The *P. aeruginosa* isolates in surface water of Dhaka city, Bangladesh showed high resistance to different antibiotics (Nasreen *et al.*, 2015). Increased abundance of *P. aeruginosa* in urban rivers impacted by domestic sewage (Cui *et al.*, 2019), and high-risk clones of *Pseudomonas* strains have been reported from different environments. For instance, ST277 and São Paulo metallo-β-lactamase (SPM-1) producing strain was identified from an urban river in Brazil (Turano *et al.*, 2016). In Ethiopia, *P. aeruginosa* is one of the pathogens commonly associated with hospital acquired infections (Hailu *et al.*, 2016), however, no reports have been found showing its distribution in the environment outside the healthcare facilities.

1.2.10 Other Waterborne Bacterial Pathogens

There are other groups of bacteria with the potential to be transmitted via polluted water, including *Mycobacterium avium* complex and *Helicobacter pylori*. These groups do not

correlate with commonly used indicator bacteria for water quality assessment. *Mycobacterium avium* complex organisms can grow in a wide range of temperature and Ph in the environment. Due to their ability to resist disinfectants, chlorine, and another stressor, they are ubiquitous in surface waters. This in part due to their ability to form biofilms in the aquatic environment. Their transmission includes the consumption of potable water. *H. pylori* are the major causative agent for gastritis and has been associated with the pathogenesis of peptic and duodenal ulcer diseases. Ingestion of contaminated food and water is the main source of the transmission of *H. pylori* (HealthCanada, 2006).

2.2.11 Detection Methods for Water-borne Pathogens

Currently used indicator bacteria for water quality assessment does not absolutely indicate the presence or absence of pathogenic bacteria, therefore a direct analysis of specific pathogens is required (Anderson *et al*., 2005). There are currently established phenotypic and molecular techniques to detected bacterial pathogens in the water. Although the culture-dependent methods are commonly used to detect pathogens from water, their results are not always reliable because of low sensitivity and presence of viable but non-culturable (VBNC) bacteria in the aquatic environment. Pathogens present in small numbers in water result in false negatives in the culture-based method. The presence of inhibitors especially in polluted water is another challenge to grow bacteria in a culture-based method. However, due to the simplicity and cost, culture-based methods are being used to characterize pathogens phenotypically and genotypically. In addition, the culture method is time and labor-intensive (Ramírez-Castillo *et al.*, 2015).

Current advanced molecular methods detect and quantify diverse pathogens in water from total extracted DNA. These include, polymerase chain reaction (PCR) based methods, qPCR arrays, and DNA sequencing. These platforms have their own strength and drawbacks. While DNA sequencing provides the most reliable information, it is costly and time-consuming, and not available in resource-limited settings. PCR is commonly used molecular technique to detect waterborne pathogens by amplifying a specific target DNA sequence. It can simultaneously detect several target pathogens in a sample in a single reaction (multiplex

PCR). Since PCR is very sensitive, proper primer design and optimal reaction conditions are required. PCR has its drawbacks, one of which is its inability to discriminate between viable and non-viable bacteria since both contain DNA. The other challenge of PCR and other molecular techniques to detect pathogens is the presence of inhibitors in the water such as humic acids and metals to which PCR is sensitive (Ramírez-Castillo *et al.*, 2015).

DNA qPCR arrays, also known as oligonucleotide microarrays, is a powerful genomic technology method that is a collection of assays for profiling or identification of bacterial species, antibiotic resistance, and virulence genes in clinical and environmental samples. Since the method can detect thousands of target bacteria, virulence, and resistance genes simultaneously, it is cost-effective. High density immobilized nucleic acids are coated onto a glass slide and this allows the rapid detection of multiple genes of multiple organisms, resistance genes or virulence factors simultaneously in the sample. Microbial profiling helps to determine the microbial composition in a sample. In addition to the identification of microbes, profiling also can help to determine the abundance of each microbe by using a control sample. In the case of identification, the array helps to identify which bacterial pathogens or resistance, or virulence genes are present in a sample without quantification. The principle of DNA qPCR array is coupled with PCR amplification of target genes of target microbes or genetic elements. Short DNA oligonucleotide probes are designed to be complementary to a gene in a target organism or genetic element. The PCR products are detected by target-specific fluorescent probe. For identification, the assay includes 16S rRNA gene of bacterial species. Antibiotic resistance genes and virulence factors databases were used to design assays for antibiotic resistance and virulence factors, respectively (Ramírez-Castillo *et al.*, 2015).

DNA sequencing is a powerful technique for determining the bases in the genome of bacteria or other organisms. Whole-genome sequencing provides more detailed and reliable information about an organism than other standard laboratory techniques. This technique can be used for the identification of specific strains, genotyping, serotyping, virulence genes,

antibiotic resistance mechanism, and other important information (Ramírez-Castillo *et al*., 2015).

1.2.12 Dissemination of Antibiotic Resistance in Aquatic Environment

The presence of several driving factors makes aquatic environments conduits of resistant pathogens and ARGs. Increased antibiotic exposure results in greater selective pressure for evolution to drug resistance via a variety of mechanisms. There are two major ways of ARGs dissemination in the environment, exogenous antibiotic-resistant bacteria having ARGs released into the environment and selection for resistance mutations due to environmental stress because of pollutants in the environment. In the case of exogenous antibiotic resistance, like other pollutants, resistance genes and/or resistant bacteria are released into the river system from point and non-point sources such as wastewater treatment plants and runoff from irrigation sources (Berglund, 2015, Baker-Austin *et al.*, 2006).

The pollutants including antibiotic residues and metals co-select for ARGs in indigenous bacteria or facilitate resistance gene transfer between bacteria through mobile genetic elements. Metal-mediated antibiotic resistance in the environment is mainly grouped into co-resistance or cross-resistance. In cross-resistance, resistance to one agent can confer resistance to both metal and antibiotics. For instance, *tetL* protein in bacteria transport both tetracycline and cobalt, resistance development against cobalt also leads to tetracycline resistance (Figure1a). The second mechanism is a selection of resistance by co-resistance in which resistance to both heavy metals and antibiotics are present on the same mobile genetic element such as plasmid, transposon and integron, and resistance to one agent leads to resistance to the other agent. For instance, pHCM1 (Salmonella typhi CT18 resistance plasmid) plasmid contains both streptomycin resistance (*strB*) and mercury resistance (*merD*) genes so that resistance to streptomycin, also confers resistance to mercury (Figure 1b). There is also a third mechanism conferring resistance in bacteria, co-regulatory resistance. In this case, different regulatory systems are transcriptionally linked to each other, and exposure to one toxicant results in resistance to another toxicant. For instance, *mex* and *czc* operons cause increased expression of metal efflux and certain antibiotic resistance such as imipenem through an

unknown pathway (Figure 1c). Since metals are not biodegradable, they can exert-long term resistance selection pressure on bacteria. (Baker-Austin *et al*., 2006).

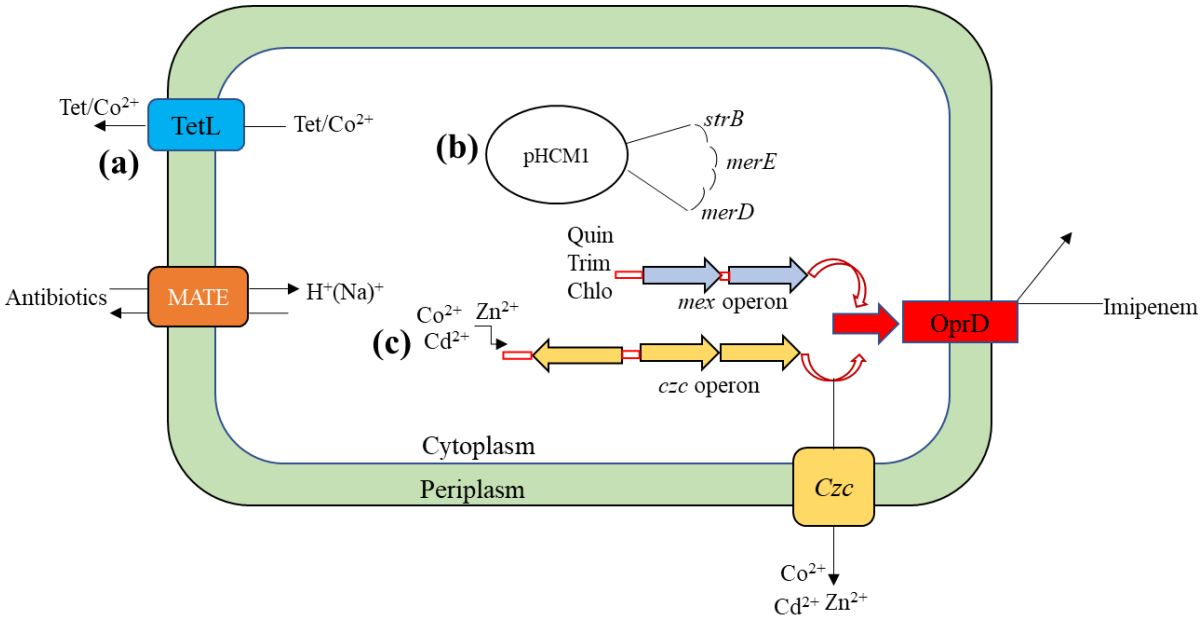

Figure 1.1: Molecular mechanisms of how metal and antibiotic co-select for resistance. (a) Cross resistance in which one cellular process confers resistance to both Co^{2+} and tetracycline (Tet) resistance. In this case TetL protein mediates transport of both cobalt and tetracycline, and thus any change on the TetL leads to resistance to cobalt and tetracycline. (b) Co-resistance in which the resistance determinants to both heavy metal and antibiotics are co-localize on the same genetic element. Resistance to Streptomycin B (Mediated by *strB* gene) also confers resistance to Mercury (Mediated by *merD* gene). (c) Co-regulatory resistance in which different regulatory systems are transcriptionally linked and exposure to one agent causes resistance to another through an unknown pathway (Baker-Austin *et al*., 2006). Abbreviations: Chlor, chloramphenicol; Quin, quinolone; Tet, tetracycline; TetL, tetracycline efflux protein; Trim, trimethoprim; strB, gene encoding streptomycin-modifying enzyme; MATE, multidrug and toxic compound extrusion; merD, gene encoding mer operon regulatory protein; merE, gene encoding mercury-efflux protein.

Antibiotic resistance in bacteria can be determined by both chromosome and extra chromosome genetic elements. Plasmid-borne resistance genes are one of the common mechanisms conferring resistance transfer between bacteria in the aquatic environment due to the presence of a diverse group of bacterial species, elevated carbon concentration, and therefore, increased bacterial activity (Costanzo *et al.*, 2005). Carbon concentration in the environment facilitates the transfer of ARGs among the bacterial community. For instance, increased concentration of CO_2 facilitates the transfer of ampicillin resistance genes carrying plasmid, pUC19, into *E. coli.* This is mediated by enhancing the uptake of DNA by pilus, increasing pore sizes, and providing more membrane channels (Liao *et al.*, 2019a). Antibiotic resistance genes could also originate in the antibiotic-producing organisms and afterward transfer these to pathogenic bacteria. For example, tetracycline resistance genes *otrA* and *otrB* have been found in the tetracycline-producing organism *Streptomyces rimosus* and in pathogenic *Microbacterium* (Pang *et al.*, 1994). Although antibiotics are known to persist in aquatic environments, antibiotic resistance genes may persist longer, even after antibiotics are removed or the host bacteria have died (Salyers and Amábile-Cuevas, 1997).

The ability of bacteria to form biofilm in an aquatic environment is one of the factors for the evolution of drug-resistant bacterial strains. Biofilms can be comprised of a single bacterial species such as *Vibrio cholerae* (Teschler *et al.*, 2015) or more commonly they are a complex and diverse population of microorganisms together (bacteria, fungi, and protozoa) embedded in the extracellular matrix of polysaccharides. In aquatic habitats, biofilms develop not only in sediment and sand but also on floating aggregates. Natural environments such as lakes, streams, and rivers have become targets for antibiotic resistance testing in addition to hospitals and other health sectors (Singer *et al.*, 2016). Antibiotic-resistant bacteria from the aquatic environment is a major contributor to global antibiotic resistance spread and the rate of distribution varies with geographical locations (Watkinson, 2008). The report by Khan *et al* (2019) in aquatic environments, Sweden for instance found a high prevalence of antibiotic resistance among *Enterobacteriaceae* (Khan *et al.*, 2019). It has been reported that the concentration of antibiotic resistance genes in the river is significantly correlated with anthropogenic activities. For instance, the study by Pruden and coworkers has shown that the

concentration of *sul1* gene was significantly associated with animal feeding operations (Pruden *et al.*, 2012).

1.2.13 Biological Water Quality

Quality water is one of the UN sustainability development goals of agenda 2030 “safe and affordable drinking water to all by 2030” (UN, 2015). Although sufficient water is available elsewhere, due to different factors such as poor infrastructure, poor management policy and lack of political commitment safe water is not accessible to all and thus causes millions of deaths each year. Ethiopia adopted drinking water quality policy from developed nations and implemented nationally, however, water accessibility and quality is still low. A report by Central Statistical Agency of Ethiopia (CSAE) showed that only 14% of Ethiopian population gets water from low-risk sources (CSAE, 2017). Surface water quality in Ethiopia is poor and in the majority of Ethiopian rivers the level of indicators bacteria (*E. coli* and total coliforms) exceeds WHO standard and are not safe for recreational and swimming purposes (Kebede *et al.*, 2020).

The quality of surface water in rivers and lakes depends on several interrelated factors that work together to reduce water quality. Of the aquatic environments, rivers seem to have received the most attention because of their rapid transport of antibiotics, ARGs, and resistant bacteria (Pruden *et al*., 2012). Increasing industrialization and urbanization have led to several problems of river water quality management. Quality water is very essential to the health, and economic wellbeing of humans and animals (Raghav and Shrivastava, 2016). There are two major components used to monitor water quality assessment, physico-chemistry and biological. Although water can be contaminated by physical, chemical, and biological pollutants, exposure to biological pollutants especially pathogens associated with water-borne diseases needs to be given higher priority in regulatory programs for domestic water supplies (Sivaranjani *et al.*, 2015).

1.2.14 Biological Water Quality Assessment

There are different analytical methods to assess water quality including enumeration of indicator bacteria, bioaccumulation, and toxicity testing. Isolation and enumeration of indicator bacteria widely used standard method especially in resource limited areas. Indicator bacteria are species whose presence indicates that fecal pollution has occurred. They are accompanied by pathogens, but do not necessarily cause disease by themselves. The reasons for using indicator organisms rather than pathogens are mostly pathogens are more difficult to grow and identify than indicator organisms, and some pathogens require special growth media and there are many different types of pathogens that it is difficult to test for all. Pathogens are less likely to be detected in water since they are present in smaller numbers than indicator bacteria. Different bacteria can be used as an indicator bacterium but should have the following features. First the bacteria must be fecal origin and consistently present in the fresh fecal waste. Second the bacteria must occur in greater numbers than pathogen and be more resistant to environmental stresses and persist for a longer time than the pathogen. Third simple, reliable, and inexpensive methods should exist for the isolation, enumeration, and identification of the indicator bacteria. Three types of coliform bacteria are commonly used to test water quality, total coliforms & *E. coli*, fecal coliforms, and fecal streptococci. Total coliforms are a family of *Enterobacteriaceae* and includes the aerobic and facultative anaerobic, Gram-negative, rod-shaped, non-spore-forming bacteria that ferment lactose with gas production within 48 hours incubation at 35^{o}C. Total coliforms consist of *E. coli*, *Enterobacter*, *Klebsiella* and *Citrobacter*. Human and animal feces discharged into the aquatic environment contain high numbers of coliforms. Total coliform is commonly useful for determining the quality of drinking water, shellfish harvesting waters, and recreational waters (APHA, 1998). Fecal coliforms are coliform bacteria that can grow at 44.5-45.5°C and ferment lactose and produce acid and gas within 48 h incubation. Almost all strains of *E. coli* meet this definition except *E. coli* O157:H7 which does not grow and produce acid and gas at 44-45.5°C. Other coliforms that meet this group include strains of *K. pneumoniae, Citrobacter freundii*, and some *Enterobacter* species (*Enterobacter aerogenes* & *Enterobacter cloacae*). The occurrence of fecal coliforms in water indicates the presence of fecal material from warm-blooded animals (Anderson *et al.*, 2005). Fecal streptococci comprise *Enterococcus faecalis*,

Streptococcus bovis, Streptococcus equinus and *Streptococcus avium*. Since they commonly colonize the gastrointestinal tract of both humans and warm-blooded animals, they are used to detect fecal contamination in water. Members of the fecal streptococci survive longer than other bacterial indicators but do not replicate in the environment. Fecal streptococci are useful for implying the presence of viruses in water. Enterococci are also used as indicator bacteria to assesses water quality for drinking, recreational purposes, and water re-use applications. They provide strong indication of fecal contamination as they are environmental stress resistant than *E. coli* (Anderson et al., 2005).

1.2.15 Model Organism for Toxicogenomic Risk Assessment

The physicochemical and bacteriological approaches are commonly used standard methods to monitor water quality. Advanced analytical technologies including gas chromatography-mass spectrometry and high-performance liquid chromatography able to detect numerous pollutants in water. However, the current technologies fail to detect all pollutants and their interactions which are critical to assess water toxicity. Therefore, whole sample analysis using model organisms as a toxicogenomic risk assessment method at genetic level is crucial by exposing the test water sample. Toxicogenomic methods using model organisms have been used to determine chemical toxicity of water at the molecular level. Organisms respond to environmental pollutants by altering the expression of different groups of genes. Measuring the gene expressions can provide detailed insight into the molecular toxicity of pollutants (Xiong *et al.*, 2017). This helps to determine the effect of pollutants in larger animals including humans. *Caenorhabditis elegans* (*C. elegans*) and *Daphnia magna* (*D. magna*) are the two commonly used model organisms to perform toxicity testing of water. *C. elegans* is a free-living nematode under the family *Rhabditidae* and feeds mainly on bacteria. The nematode is maintained easily in the laboratory where it is grown on agar plates seeded with *Escherichia coli* mutant strain OP50 (*E. coli* OP50). The nematode has been successfully used as a model organism to address basic biological questions, including development, aging, and neurobiology. In addition, in recent years the nematode has been used to study host innate immunity, microbial pathogenesis, and drug discovery. The advantages of using *C. elegans* in toxicity assessment is that they are small in size (1mm) and transparent. The nematode has

rapid generation time (from egg to gravid adult in 2 days, 300 eggs laid in 3 days) and all the worms in a population can be genetically identical which can avoid the cofounding effect because of the genetic variability on the physiological responses (Boyd *et al.*, 2010). *C. elegans* is the best model organism to study *in vivo* system since it is less complex than human but share high homology. *C. elegans* have ~60%–80% of human genes. The nematodes used to study high variability of biological processes such as gene regulation, sex determination, cell signaling, metabolism, ageing and apoptosis (Kaletta and Hengartner, 2006). Bacteria such as *P. aeruginosa* PAO1 can be also used to assess the pollution status of water at the genetic level that can predict also how the aquatic environment induces antibiotic resistance evolution.

1.3 Statement of the problem

Water-borne diseases are a serious public health issue and are caused by a group of pathogens including many bacteria, viruses, protozoa, and helminths. Water-borne diseases are responsible for thousands of deaths each year especially in tropical regions with poor sanitation. They are mainly caused by enteric pathogens and although most human intestinal pathogens cannot survive for long periods outside the human or animal body, they can remain viable in aquatic environments long enough to reinfect humans. Despite the advanced technologies for health care, improved management, and increased use of therapeutics in the past decades, diarrheal diseases remain among the top five causes of child death. Diarrheal disease is commonly caused by ingestion of contaminated foods and water (Soboksa *et al.*, 2020).

Getting to clean water for agriculture and irrigation of vegetables is a major challenge. Since farmers in megacities have no other options, they use highly polluted urban river sources. This results in the contamination of crops with pathogens and causes public health issues where raw vegetables are eaten. In resource limited nations, use of untreated wastewater and manure to produce vegetables is a major contributing factor to the contamination that causes frequent water-borne disease outbreaks. Pathogens from different potential sources enter the river system. The sewage treatment technology is not able to eliminate all pathogens. Many pathogens end up in the effluent which is then discharged into receiving river and implicated

in human and animal diseases linked with the use of contaminated water for domestic purposes and agriculture (Cho *et al.*, 2010).

As stated previously indicator organisms are commonly used for water quality assessment. Presence of indicators above the permissible limit representing the presence of pathogenic bacteria. However, debate exists regarding indicator organisms and their ability to represent the potential presence or absence of pathogenic bacteria (Dickerson *et al.*, 2007). Metabolic phenotype-based detection of an organism does not consider the genetic elements involved in pathogenesis. Therefore, detection of potential pathogens and their virulence genes in such organisms is needed to accurately assess the health risks associated with aquatic environments (Lauber *et al.*, 2003).

The emergence and spread of antibiotic-resistant bacteria is the public health challenge of the century and it is rising to dangerously high levels globally (Port *et al.*, 2014). The emergence of multi-antibiotic resistant "Superbugs" such as those strains carrying the $bla_{\mathrm{NDM-1}}$ gene is a cause of particular concern. Extensive use of antimicrobial treatments in humans and animals together with the presence of chemical pollutants increase resistant bacterial populations in aquatic systems (Munir *et al*., 2011). Although epidemiology and mechanism of resistance are well documented, most of them have focused on isolates from clinical patients in health care facilities. However, antibiotic-resistant bacteria are widespread in the environment, including water and sediment (Port *et al*., 2014). Because of the high per capita antibiotic use and host density, healthcare facilities are believed to be the major focal points for resistance evolution. However, continual antibiotic discharge into the environment from animal agriculture facilities also creates selective pressure for antibiotic resistance (Andersson and Hughes, 2012). The presence of resistant bacteria associated with river water has been a crucial public health concern. Although antibiotics concentration in the environment is lower than that obtained in vivo, it is high enough to drive antibiotic resistance and it has been reported that long term bacterial exposure to low concentration of antibiotics in streams lead to the development of antibiotic resistant bacteria (Bryskier, 2005). The presence of increased levels of heavy metals in aquatic environments is another contributing factor to the emergence

of drug-resistant bacteria. Heavy metal-resistant bacteria are most likely resistant to antibiotics as well. Heavy metals such as Cu, Hg, Cd, Co, and Zn in various ion forms have been associated with antibiotic resistance (Seiler and Berendonk, 2012).

Horizontal gene transfer is a mechanism of resistance development in the aquatic environment. Bacteria including non-pathogenic species found in the aquatic environment can serve as a source from which pathogens can acquire antibiotic resistance genes, and in turn, they can become resistant by acquiring resistance genes from human-derived pathogens. The current sewage treatment processes are not able to reduce or eliminate resistant bacteria (Levy, 1997). Spontaneous mutations are also an important mechanism of novel resistance in the bacterial community in the aquatic environment. For instance, mutations on the quinolone resistance determining region in bacteria isolated from the aquatic environment are commonly associated with the presence of antibiotics such as ciprofloxacin and metal pollutants. Bacterial biofilm formation in aquatic environments is also a major contributing factor for the evolution and persistence of antibiotic-resistant strains. In biofilms, bacterial are 10 to 1,000 times more resistant to specific antimicrobial agents compared to their planktonic counterparts. This induced resistance is caused by different factors such as poor antibiotic penetration into the polysaccharide matrix, presence of either non-growing cells or cells that triggered stress responses under unfavorable environmental conditions within the biofilm matrix (Gilbert *et al.*, 2002). Determining the epidemiology and spatial variations of antibiotic-resistant bacteria and ARGs in aquatic environments is profoundly important to human and animal health. However, the extent to which aquatic environments contribute toward the persistence and spread of antibiotic-resistant bacteria and ARGs in the aquatic environment are poorly understood in Ethiopia.

Rivers in Addis Ababa are severely polluted by industrial and municipal solid and liquid wastes. There are many industrial establishments along the Akaki river and most of them are discharge their effluents directly into this river or its tributaries without any prior treatment. The polluted river water is widely used by downstream residents for domestic purposes and to grow raw eat vegetables which are sold and consumed by inhabitants of Addis Ababa.

Water from the rivers is being used for various purposes; domestic, irrigation, industrial consumption, washing of materials, bathing, cattle consumption, and waste disposal. Farmers in Addis Ababa use surface irrigation along the Akaki river to grow vegetable production which accounts for about 60% of the total market supply for the city. There is a great public health concern of food poisoning since 60% of the city's food consumption is supplied by urban farmers, who irrigate their crops using wastewater in the Akaki river (UAO, 2002).

1.4 Significance of the study

Given the frequent history of acute watery diarrhea outbreaks in Addis Ababa (WHO, 2016), it is interesting to assess the presence of potential pathogens may be present in the Akaki river that could potentially cause for epidemic disease associated with these environmental isolates. Many developing countries have little or no data about antibiotics and antibiotic resistance in an aquatic environment. Similarly, information on the prevalence rate of potentially pathogenic bacteria, their drug resistance patterns, and primary resistance mechanisms in the rivers are unavailable not only for the Akaki river but also in other Ethiopian rivers crossing urban settlements. Therefore, this is an important study that contributes to the understanding of these variables associated with potential bacterial pathogens, persistence, and spread of antibiotic resistance genes and drug resistance levels. This knowledge will be beneficial for defining control and preventative strategies for infectious diseases. The current physicochemical water toxicity assessment method is missing the synergistic and antagonistic effects of pollutants at the genetic level. Therefore, advanced assessment techniques are required to determine aquatic chemical pollutants and their biological effect in animals. Toxicogenomic assays can determine the biological effects of pollutants through the identification of transcriptional changes at the molecular level. In the current study, we used transcriptomics as an environmental risk assessment tool to better identify the target mechanisms of toxicity. Since chemical pollutants in the river could potentially contribute to the evolution and persistence of antibiotic resistance, we used *P. aeruginosa* PAO1 bacterial model to determined expressional levels of stress-associated genes, virulence genes and antibiotics/heavy metal resistance genes.

1.5 Study Hypothesis

- Akaki river is a conduit for the persistence and spread of antibiotic resistance genes, and the water poses a high risk to public health.
- Bacterial isolates from the Akaki river are virulent and resistant to commonly used antimicrobials.
- Akaki river water has a significant adverse biological effect on an animal model *Caenorhabditis elegans* and a bacterial model *P. aeruginosa* PAO1

1.6 OBJECTIVES OF THE STUDY

General Objective

- To determine the functional and molecular characterization of potential bacterial pathogens in the Akaki river sand its toxicogenomics effects on model organisms, Ethiopia

Specific Objectives

- To assess diversity and abundance of antibiotic resistance genes and clinically relevant bacteria in Akaki river, Addis Ababa, Ethiopia
- To determine the phenotypic antimicrobial profile pattern of potential pathogenic bacteria in the Akaki river
- To determine the mechanism of antibiotic resistance and virulence factors in bacterial isolates from the Akaki river
- To determine expressional levels of genes associated with antibiotic/metal resistance, virulence, and oxidative stress in *P. aeruginosa* PAO1 grown in Akaki river water
- To investigate the toxicogenomic effect of Akaki river water in the animal model organism *C. elegans*

CHAPTER 2: MATERIALS AND METHODS

2.1 Study Area

The study was conducted in Addis Ababa, the capital city of Ethiopia, supporting a population of approximately 5 million, and as the headquarter of the African Union hosts many visitors from diverse countries. Two major rivers are flowing through the center of Addis Ababa, the Little Akaki and the Big Akaki rivers (Figure 2.1). The Big Akaki river originates in the Northeastern, while the Little Akaki river originates in the Northwestern part of Addis Ababa. The Big Akaki river passes through residential and commercial areas of the city whereas Little Akaki passes through an industrial zone. The two rivers flow into the Aba-Samuel reservoir and form one of the biggest tributaries of the Awash River. Several socio-economic activities such as health care facilities, markets, industries, diagnostic, and research laboratories as well as agriculture and farming activities are situated along the rivers. The majority of health care facilities are located along the Big Akaki river. In general, the Akaki rivers are grouped into three segments, upper catchment, middle catchment, and lower catchment. The upper catchment comprises streams that drain from different parts of Geferesa, Mount Entoto, and Legedadi dams joining together to form both Little Akaki and Big Akaki rivers. The middle catchment is the full course of the rivers within the city, Addis Ababa, before leaving the sub-city Akaki Kality. The lower catchment is the course of the rivers from Akaki Kality sub-city down to Aba-Samuel reservoir.

2.2 Study design and Sampling Points

A series of cross sectional study design was employed to investigate the functional and molecular characterization of potential bacterial pathogens in Akaki river and its toxicogenomic effects. Water samples were collecteds from five sampling points, Gefersa (GE), Mekanissa (MK), Batu (BA), Zewditu (ZE), and Aba-Samuel (AB) along the course of both rivers for bacteriological and antibiotic resistance genes (ARGs) analysis. In addition to water samples, sediment samples were also collected from the same site for AGRs and clinically relevant bacteria analysis. For toxicogenomic analysis water samples were obtained only from the Little Akaki River (GE, Alert (AL), MK, BA, and AB) (Figure 2.1). The sites were chosen based on anthropogenic activities such as industries, irrigation, healthcare

facilities, and other possible sources of pollutants. GE is the upstream and drinking water reservoir, MK is residential & irrigation area, BA is an industry-dominated area and irrigation zone, ZE is located in residential and healthcare facilities, AL is located in residential, and some healthcare facilities and AB is the downstream reservoir. The sampling area covers a total of 54 km, from upstream to downstream of the river.

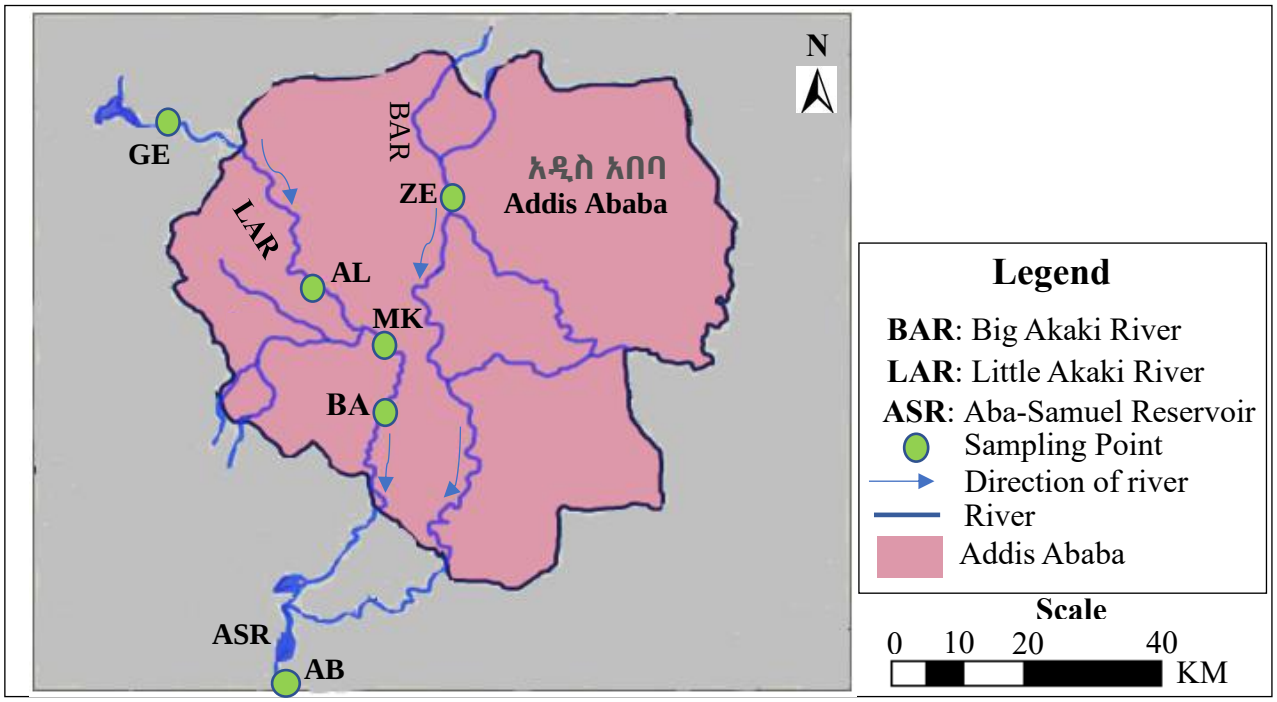

Figure 2.1: Map of Little Akaki and Big Akaki rivers flowing through Addis Ababa, Ethiopia. The sampling points include Gefersa (GE), Mekanissa (MK), Alert (AL), Batu (BA) from Little Akaki river and Zewditu (ZE) from Big Akaki river. Both rivers join in the Aba-Samuel (AB) downstream reservoir. GE is upstream of the Little Akaki river (9°03'43.2"N 38°38'34.9"E), MK is located in irrigation and residential area (8°58'25.7"N38°43'59.6"E), AL is located in residential and health care facilities (8°59'16.8"N 38°42'34.4"E), BA is located in an industry dominated area and irrigation zone (8°55'52.0"N 38°45'26.3"E), ZE is located along residential and few healthcare facilities (9°01'02.9"N 38°45'20.2"E) and AB is downstream reservoir (8°47'15.7"N 38°42'25.7"E). The estimated distances between sampling sites are 15.08 (GE-AL), 4.07km (AL-MK), 9.17 km (MK-BA), 25.35 km (BA-AB) and 52.16 km (ZE-AB).

2.3 Sample Collection

2.3.1 Sample Collection for DNA and Bacterial Isolation

Water samples were collected from five sites of the rivers (GE, MK, BA, ZE and AB) based on anthropogenic activities such as irrigation, population density, industry, tributaries, health care facilities and origin of the river for DNA qPCR arrays, isolation, and characterization of potential pathogenic bacteria. At each site 1 Litter water sample for DNA isolation and 250 mL, water sample for bacterial isolation were collected from 15-20 cm below the surface of the water in sterile bottles in the flow of the stream. Sediment samples were also collected for DNA qPCR arrays from the same five sampling sites and 500 mg of wet weight was used for DNA isolation. The samples were transported in an ice-box to AHRI for immediate processing.

2.3.3 Sample Collection for Toxicogenomic Analysis

For toxicogenomic analysis using *Caenorhabditis elegans* and *Pseudomonas aeruginosa* PAO1, water samples were collected from four sites of the Little Akaki river (GE, AL, MK, and BA) and a downstream reservoir (AB). From each sampling point, 1L water sample was collected in pre-cleaned polyethylene bottles by immersing into the upper surface of the river, 15-20 cm deep and transported in ice box to AHRI for immediate processing. The water was heated to 95^{o}C for 30 minutes in a water bath to eliminate microbial activity. It was then stored at 4^{o}C and shipped at room temperature to Orebro University, Sweden for gene expression studies.

2.4 Sample Processing and Analysis

2.4.1 Detection and quantification of ARGs and pathogenic bacteria

Water samples were prefiltered through a 25 µm isopore polycarbonate filter to remove debris and then 50 mL water filtered again through a 0.22 µm isopore polycarbonate filter (Merck Millipore, Ireland) using a vacuum filtration apparatus. The filters were transferred into PowerWater DNA Bead Tube and the filters were stored at -20℃ until DNA extraction. Total DNA was extracted from the membrane filters using Qiagen DNeasy PowerWater Kit (Qiagen, USA) and from sediment samples using FastDNA SPIN Kit (MP Biomedicals) according to the manufacturers' instructions (100µL eluted in EB). The concentration and purity of the DNA were measured by NanoDrop Spectrophotometer (DeNovix, USA) and stored at -20ºC until shipped to Orebro University, Sweden on dry ice.

Microbial DNA qPCR Arrays (QIAGEN, Sweden) were used to determine the diversity and abundance of ARGs and clinically relevant bacteria in the Akaki river (Annex I). Antibiotics Resistance Genes DNA qPCR Array (Qiagen, Sweden. BAID-1901ZRA) and the water and sepsis DNA qPCR Arrays (Qiagen, Sweden. BAID-1405Z and BAID-1903Z, respectively) for bacterial detection were used according to the manufacturer's instructions. The kits are designed to detect species-specific bacterial and fungal ribosomal rRNA genes or antibiotic resistance and virulence factor genes. The assay background and possible contamination were assessed using a No Template Control (NTC). NTC is used to establish the lower cycle threshold value for positive calls ($\Delta CT \geq 6$, and the upper CT value for negative calls ($\Delta CT < 3$) (Khan *et al.*, 2019). The PCR reactions were conducted in a final volume of 25 µl containing microbial qPCR Master mix and a template (10 ng per reaction). The qPCR thermocycling conditions for SYBR Green consisted of an initial PCR activation step for 10 min at 95°C followed by 40 cycles of 95ºC for 15 s denaturation and 60°C for 2 min annealing and extension in a CFX96^{TM} Real-Time system (Bio-Rad Laboratories, Canada) according to the manufacturer's instruction.

2.4.2 Bacterial Isolation and Identification

The membrane filter technique was employed for isolation of *E. coli*, *Vibrio cholerae*, *Salmonella*, *Aeromonas* species, *P. aeruginosa*, *Klebsiella* species and *Shigella* species following the standard protocols. The water samples were prefiltered through 25μm isopore polycarbonate filters to remove debris and serially diluted (10 fold serial dilution with sterile distilled water) and filtered again through 0.45 μm isopore polycarbonate according to the standard protocol and placed on different selective and differential culture media, including Chromocult Coliform Agar, Thiosulfate Citrate Bile-salt Sucrose agar, Pseudomonas agar, MacConkey agar and Salmonella-Shigella agar, and incubated for 24 h at 37°C (Figure 2.2). Enumeration of *E. coli* was carried out using membrane filtration method (WHO, 1998) using Chromocult® Coliform Agar. After 24 h incubation at 37^{o}C, the number of colonies per 100 mL of a sample was evaluated from plate count (Annex II). Isolates were sub-cultured for identification and molecular analysis. Identification was performed by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany). MALDI-TOF-MS identifies bacteria by analyzing the profiles of bacterial macromolecule that are obtained from whole bacteria and compares them against the MALDI database of known microbes. The method follows a proteomic approach that allows rapid and accurate identification of bacteria. For identification, overnight grown bacterial colony applied on disposable MALDI target plate and mixed with a matrix containing α-Cyano-4-hydroxycinnamic acid (CHCA) dissolved in acetonitrile (50%) and 2.5% trifluoroacetic acid (Bruker Daltonik GmbH, Bremen, Germany) according to manufacturer's protocol. The plates were shipped to Orebro University, Sweden for identification (Annex III).

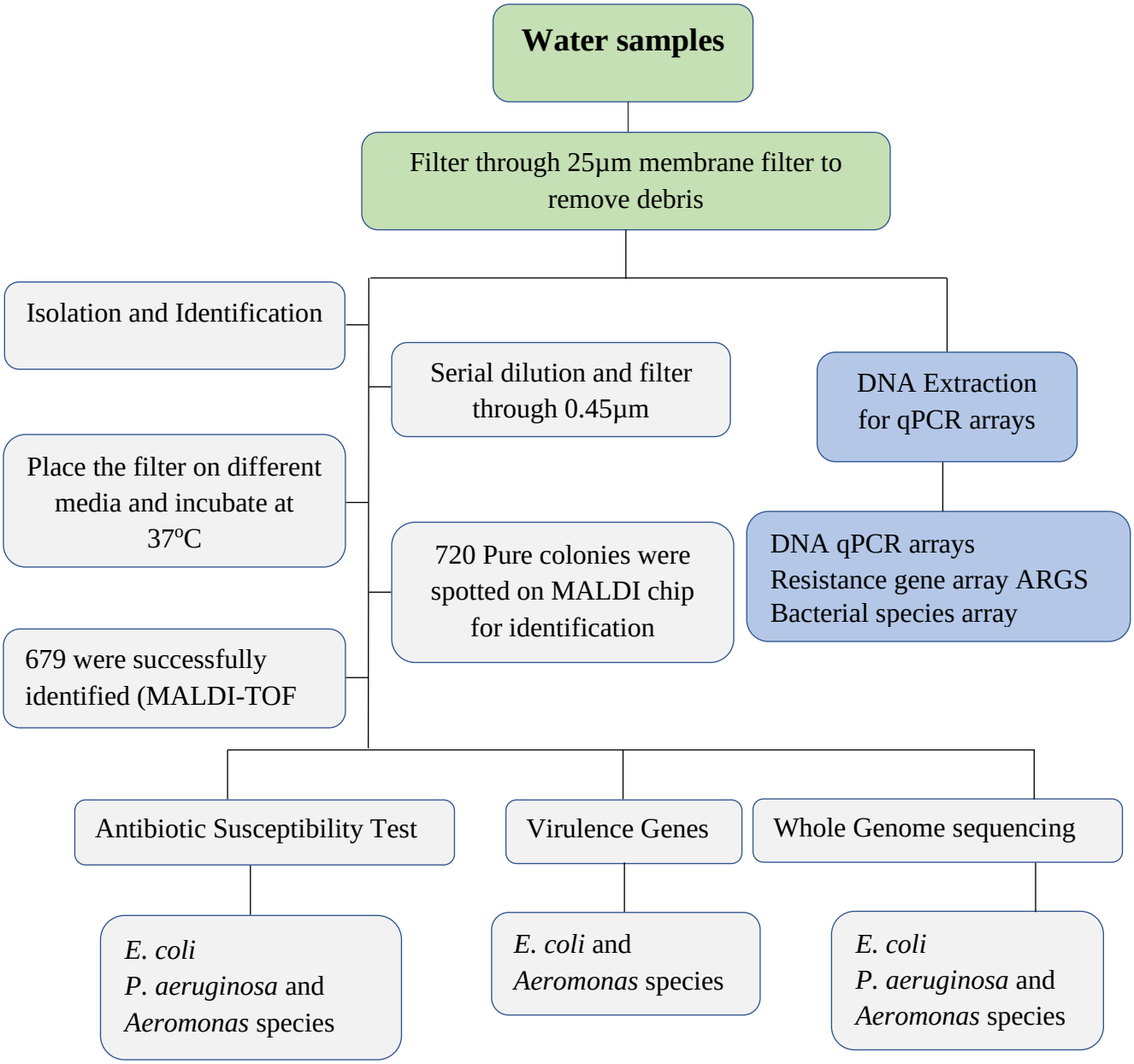

Figure 2.2: A flow diagram of the laboratory procedure for isolation, identification, and characterization of potential antibiotic resistant pathogenic bacteria in Akaki river.

2.4.3 Antimicrobial susceptibility testing

E. coli, *Aeromonas* spp., and *P. aeruginosa* were tested for their resistance to antibiotics belonging to different classes as listed in Annex IV. The antibiotics tested were tetracycline (TET, 30 µg), doxycycline (DOX, 30 µg), kanamycin (KAN, 30 µg), gentamicin (GEM, 10 µg), cefoxitin (FOX, 30 µg), cefotetan (CTT, 30 µg), cefotaxime (CTX, 30 µg), cefixime (CFM, 30 µg), ceftazidime (CAZ, 5 µg), ceftriaxone (CRO, 30 µg), cefepime (FEP, 30 µg), amoxicillin (AML, 25 µg), amoxicillin-clavulanate (AMC, 44/24 µg), ampicillin (AMP, 10 µg), norfloxacin (NOR, 5 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NAL, 30 µg), imipenem (IMP, 10 µg), ertapenem (ETP, 10 µg), meropenem (MEM, 10 µg), azithromycin (AZM, 15 µg), erythromycin (ERY, 15 µg), sulfamethoxazole/trimethoprim (SXT, 23.75/1.25 µg) and chloramphenicol (CHL, 30 µg). Antibiograms were done on Mueller Hinton agar plates with the disk-diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST-2019) standard procedures to determine the zone of inhibition. Interpretation of results was done using the EUCAST-2019 standards (EUCAST, 2019). All strains showing ‘‘resistant’’ or ‘‘intermediate’’ behavior was included under the category ‘‘resistant’’ and the others were classified as ‘sensitive’.

2.4.4 Detection of virulence genes in *E. coli* and *Aeromonas* species

Genomic DNA from microbial isolates was isolated using GenElute Bacterial Genomic DNA extraction kit following the manufacturer’s instruction (Sigma-Aldrich, Germany) from an overnight grown culture in Tryptic soy broth (TSB). The concentration and quality of purified DNA were measured by NanoDrop Spectrophotometer (DeNovix, USA). All *E. coli* isolates (n=168) were analyzed using QIAGEN multiplex-PCR (m-PCR) kit (Qiagen, Germany) to determine the presence of enteroaggregative *E. coli* heat-stable enterotoxin 1 (*EAST1*), heat-stable enterotoxin (*STa*), heat-labile enterotoxins (*LT*), Shiga toxins *(Stx1*, *Stx2*) and adhesin genes (*F4*, *F6* and *F18*). These genes are involved in the pathogenesis of *E. coli* and are associated with diarrhea. Those genes which had similar product sizes were confirmed using a conventional PCR separately. The presence of six virulence-associated genes (cytotoxic enterotoxin (*act*), heat-labile cytotonic enterotoxin (*alt*), heat-stable cytotonic enterotoxin (*ast*), hemolysin (*hlyA*) and shiga-toxins (*stx-1* and *stx-2*) were determined in *Aeromonas*

species by conventional PCR. The nucleotide sequences of the primers to amplify the target genes are listed in Annex V Table-1 and Table 2 for *E. coli* and *Aeromonas* species, respectively. The reaction mixture for m-PCR consisted of 25 µl 2x QIAGEN Multiplex PCR Master Mix, 5 µl 10x primer mix (0.2 µM), template (600 ng per reaction) and nuclease free water with a reaction volume of 50 µL. The reaction mixture for conventional PCR consisted of 2.5 µL 10X Taq buffer, 0.5 µL dntps, 1 µL primer mix (both forward and reverse), template (80 ng/µl), 0.125 µL Taq DNA polymerase, and nuclease-free water for a total reaction volume of 25 µL. The PCR cycles for m-PCR include first, thermal cycles were applied for 15 minutes at 94°C, and then 40 cycles were applied individually as follows: 30 seconds at 94°C (denaturation), 90 seconds at 60°C (annealing), 90 seconds at 72°C (extension), and final extension for 10 minutes at 72°C. The PCR cycles for conventional PCR include initial denaturation for 30 s at 95°C and then 35 cycles were applied individually as follows, 60 seconds at 72°C (extension), and final extension for 10 minutes at 72°C. To determine the presence and absence of the target genes, PCR products were visualized using 2% agarose gel electrophoresis (Annex V).

2.4.5 Whole Genome Sequencing and Genome-Based Analysis

A total of 66 representative *E. coli* (n=27), *P. aeruginosa* (n=18), and *Aeromonas* spp. (n=21) isolates were selected based on their phenotypic antibiotic susceptibility results and the sampling points and subjected to whole-genome sequencing (WGS) for subsequent analysis. The bacterial isolates were shipped to Orebro University, Sweden on dry ice. DNA was isolated from revived overnight cultures using the Phenol: Chloroform: Isoamyl alcohol (PCI) (25:24:1, Phenol pH=8) method (Lemarchand *et al.*, 2005) (Annex VI). The quality of DNA was checked by gel electrophoresis on 0.8% agarose gel and quantified by NanoDrop 2000 Spectrophotometer prior to storage at -20°C. Genomic DNA was shipped to Eurofins Genomics, Germany for sequencing. WGS was performed using an Illumina HiSeq 2500 to generate 250-bp paired-end reads. Sequence read quality was checked by FastQC version 0.11.9. The raw reads of sequence data were trimmed using trimmomatic-0.39 to remove adapters and de novo assembly was performed by SPAdes genome assembler version 3.14.1 using default settings. Insilco Clermont *Escherichia coli* phylo-typing tool (*arpA, chuA, yjaA*

and *TspE4.C2*) (http://clermontyping.iame-research.cente) was used for phylotyping of *E. coli* strains. Multilocus sequence typing (MLST) was performed using MLST 2.0 server of the Center for Genomic Epidemiology (CGE) (http://genomicepidemiology.org/). For *E. coli*, *adk, fumC, gyrB, icd, mdh, purA* and *recA* and for *P. aeruginosa acsA, aroE, guaA, mutL, nuoD, ppsA* and *trpE* alleles were used for typing (Larsen *et al.*, 2012). *Aeromonas* sequence type was performed by *Aeromonas* PubMLST database using six alleles (*gltA*, *groL*, *gyrB*, *metG*, *ppsA* and *recA*) (https://pubmlst.org/organisms/aeromonas-spp/). Serotype (*E. coli* and *P. aeruginosa*) and fimH type (*E. coli*) were determined using SerotypeFinder 2.0 and FimTyper 1.0 respectively (CGE). The presence of antibiotic resistance genes, virulence determinants, and plasmid incompatibility groups were analyzed using the bacterial analysis pipeline at the CGE server by uploading assembled genomes data to the respective bioinformatics tool. In addition to the CGE, Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/home) was used to determine the efflux pumps conferring resistance to different clinically relevant antibiotics. The general genome feature of *E. coli*, *P. aeruginosa* and *Aeromonas* isolates was determined by Pathosystems Resource Integration Center (PATRIC) (https://www.patricbrc.org). Sequence identity of certain genes was determined by EMBOSS Clustal Omega Multiple Sequence Alignment bioinformatics tools (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.5 Toxicogenomic Analysis

2.5.1 *C. elegans* Exposure and gene expression

2.5.1.1 Preparation of *C. elegans* and Exposure

Caenorhabditis elegans N2 were maintained on nematode growth medium plates seeded with *Escherichia coli* OP50 at 20°C according to standard protocol (Stiernagle, 2006). Adult *C. elegans* hermaphrodites were collected in centrifuge tubes and lysed using a bleaching solution (5N NaOH, 1% HOCl) to release the eggs. The eggs were then washed three times with M9 buffer (KH2PO4, Na2HPO4, NaCl, and 1M MgSO4) before being resuspended in 10 ml of M9 buffer and kept in a tube rotator overnight for hatching. Approximately 1000 synchronized L1 worms were exposed to the environmental water samples and a K-medium control (51 mM NaCl, 32 mM KCl, in Milli-Q water) in a 24 well plate and incubated at 20°C for 48 h until they reached young adult. Five replicates were tested for each water sample and control.

2.5.1.2 RNA Extraction and RT-PCR

After 48-hrs exposure, the nematodes were harvested by centrifugation at 2500 rpm and suspended in 700µl of Trizol reagent (Sigma) and stored at -80 ºC overnight. RNA was extracted by Direct-zol™ RNA MidiPrep Kit (Zymo Research, USA) according to the manufacturer's instruction. The quality and concentration of RNA were measured by NanoDrop Spectrophotometer (DeNovix, USA) at 260 nm. cDNA synthesis was done by qScript cDNA synthesis kit (Quanta Biosciences, USA) using 1µg of RNA. The reaction mix consisted of 10 µl qPCR mix, 5 µl nuclease-free water, 3 µl primer, and 2 µl template. The level gene expression was measured by SYBR Green qPCR kit (Kappa Biosciences, USA) following the manufacturer's instruction. The thermocycling conditions for the qPCR consisted of a denaturation step for 5 min at 95ºC followed by 40 cycles of 95ºC for 2 s and 60ºC for 30 s in CFX96TM Real-Time system (Bio-Rad Laboratories, Canada). In total 30 genes were tested, including those associated with heat shock and general response (*hsp-70*, *hsp-16.2*, *hsp-16.48*, *hsf-1*, *sip-1* and *hsp-16.1*), oxidative stress (*cyp-35A2*, *cat-2*, *sod-1* and *skn-1*), innate immunity (*tir-1*, *tol-1*, *abf-2*, *clec-60* and *lys-7*), metal response (*mtl-1*, *mtl-2*,

pgp-5, *aip-1*, *cdr-1*, *ftn-1*, *hmt-1*, *numr-1*, *cdf-2* and *hif-1*) and development (*vit-4, vit-5, vit-6, daf-12* and *daf-16*) (Kumar *et al.*, 2015). The *Fes* gene was used as a reference gene and internal control and exposure to K-medium prepared with Milli-Q water was a negative control throughout the experiment (Annex VII).

2.5.2 *P. aeruginosa* PAO1 exposure to Akaki river water and gene expression

2.5.2.1 Growth conditions and exposure

Pseudomonas aeruginosa PAO1 was used in the current study to determine the effect of Akaki river water on the expression of antibiotics/metal resistance, virulence, and stress response genes. The growth medium was either nutrient broth or nutrient agar in all experiments. The nutrient broth was prepared with the water samples collected from the Akaki river and aa MQ water control. After preparation, media was filtered through 0.22 µm filter membrane, and sterility was checked before the actual experiment. To determine the late exponential phase of bacterial growth for RNA extraction, PAO1 was incubated at 30°C for 12 h in nutrient broth medium, and growth was monitored by measuring the optical density (OD) at 600 nm every 30 min until 2 h incubation and then every hour (OD600) using Ultrospec 10 spectrophotometer (Amersham, London, UK). The growth curve was constructed by GraphPad Prism 8 for windows.

2.5.2.2 Bacterial RNA Extraction and RT-PCR

The late log growth phase of PAO1 was found to be 7-8 h (OD600 ~1) and 7.5 h was used as a late log growth for the actual experiment. After incubation for 7.5 h at 30°C in nutrient medium prepared by experimental and MQ water, 1ml was collected in a clean Eppendorf tube for RNA extraction. Isolation of RNA was performed by NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. To remove contaminant genomic DNA, extracted RNA samples were treated with DNAase (ArcticZymes, Tromso, Norway) before cDNA synthesis. The quality and concentration of RNA were measured by NanoDrop Spectrophotometer (DeNovix, USA) at 260 nm. The integrity of RNA was checked by running gel electrophoresis. cDNA synthesis was done by qScript cDNA synthesis kit (Quanta Biosciences, USA) using 1µg of RNA. The level of gene

expression was measured by SYBR Green qPCR kit (Kappa Biosciences, USA). The reaction mix consisted of 10 µl qPCR mix, 5 µl nuclease-free water, 3 µl primer, and 2 µl template. The thermocycling conditions for the qPCR consisted of a denaturation step for 5 min at 95ºC, followed by 40 cycles of 95ºC for 2 s and 60ºC for 30 s in CFX96TM Real-Time system (Bio-Rad Laboratories, Canada). A total of 29 genes were tested, including those associated with Antibiotics/Metal resistance genes (*mexA, oprM, cat, mexT, mexE,* mexX, *czcA* and *AmpC),* virulence-associated genes *(rhL, ptxR, exoS, ppyR, mvfR, pilA, cupA, pcrV and lasB)* and stress response genes *(dnaK, sigX, rpoS, recA, radA, oxyR, lexA, algU, groEL, groES, rpoH and pvdS*) (Khan *et al*, unpublished data). To select a reference gene, three candidate genes, *rpsL*, *rpoD* and *cubA* were evaluated for their expression before the actual experiment and *rpsL* expression was found to be the most consistent in all conditions and therefore, used as a reference gene and internal control. Growth in a control, nutrient broth prepared with Milli-Q water, was a negative control throughout the experiment (Annex VIII).

2.6 Statistical Analysis

Identification of ARGs and pathogenic bacteria was analysed by the ΔCT ($\Delta CT = CT_{\text{Test sample}} - CT_{\text{No template control}}$) method using data analysis template excel software provided by the manufacturer (Qiagen). For profiling, the ΔΔCT method was used to calculate the fold change of each gene and bacterial species. A heat map was constructed by GraphPad Prism version 8.3 for Windows (GraphPad Software, La Jolla California, USA). Principal coordinate analysis (PCA) was performed to evaluate differences in ARGs and bacterial community profiles among sampling sites based on the Bray–Curtis distance of ARGs and bacterial relative abundance. A Pearson correlation matrix was used to determine the correlation among ARGs and bacterial community and $P<0.05$ was considered significant (XLSTAT, 2019). The correlation plot between ARG profiles and bacterial communities was determined by Redundancy analysis using Canoco 5.0 software package (Braak and Smilauer, 2012). Venn diagram was constructed using MyDraw software for Windows (https://www.mydraw.com/) for the five sampling sites to show the number and diversity of ARGs detected.

The antibiotic susceptibility data were analyzed using SPSS version 20 (IBM, USA). Descriptive statistics were summarized using frequencies and cross-tabulations and tables and graphs were used for data presentation. Co-occurrence of virulence determinants in *E. coli* isolates from the Akaki river was determined by the correlation matrix. Statistical association of susceptibility patterns and occurrence of virulence genes was determined by X^2 test or Fisher's exact test as appropriate. Graphs were generated using GraphPad prism 8.01for windows (GraphPad Software, La Jolla California USA). An unpaired t-test was used to determine the statistical differences of *E. coli* counts between sampling sites. Phylogenetic comparison of resistance genes was performed by EMBOSS Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

For gene expression assays, the raw CT value was normalized using the respective reference gene, and the fold change was calculated against the control K-medium for *C. elegans* and nutrient broth prepared with Milli-Q water for PAO1 data. Data from qRT-PCR ($2^{(-\Delta\Delta CT)}$) were exported to GraphPad Prism. One-way ANOVA (Tukey's multiple comparisons test) was used to compare the fold change in the relative expression of genes in *C. elegans*. One-way ANOVA (Dunnett's test) was used to compare the fold change in the relative expression of genes in *P. aeruginosa* PAO1. A P value ≤ 0.05 was considered statistically significant.

2.7 Ethics Statement

The research was approved by the Addis Ababa University institutional research board, College of Health Sciences (IRB-CHS, Ref. No AAUMF 03-008), national IRB (Ref. No 310/83/2018) and Armauer Hansen Research Institute/All Africa Leprosy TB Rehabilitation and Training centre (AAERC) research ethics committee (Ref. No PO26/17). Permission for water sample collection was obtained from the Addis Ababa Water and Sewerage Authority. Ethiopian Biodiversity Institute authorized the exportation of DNA and Bacterial isolates to Orebro University, Sweden.

CHAPTER 3: RESULTS

3.1 Antibiotic resistance genes in bacterial communities in Akaki River, Addis Ababa, Ethiopia

The current study aimed to establish the extent and diversity of antibiotic resistance determinants and the associated clinically relevant bacterial population at various locations along the Akaki river in Addis Ababa, Ethiopia. A total of 84 clinically relevant antibiotic resistance gene subtypes associated with resistance to β-lactams, aminoglycosides, fluoroquinolones, macrolides, and vancomycin, as well as multidrug resistance and tetracycline efflux pumps, were analysed from surface waters and sediments collected from 5 different sites of the Akaki river system. From the total targeted antibiotic resistance genes, fewer were detected in GE (8.3%) and AB (21.4%) whereas the majority of ARGs were detected in MK (60.7%), BA (64.3%), and ZE (65.5%) sites. All water samples contained β-lactam, fluoroquinolone, aminoglycoside, macrolide, and tetracycline resistance genes at the class level. However, spatial variation was observed for the diversity of ARGs between sampling locations (Figure 3.1a). Fewer resistance genes were detected in the Gefersa reservoir, farthest site upstream where the Little Akaki river originates, which is the source of drinking water (GE), and the downstream sampling point, the Aba-Samuel reservoir (AB) where both Little and Big Akaki rivers form a large tributary to Awash River. None of the multidrug resistance efflux pumps, class A, B, and C β-lactam, vancomycin, and erythromycin resistance gene were detected in the upstream of the rivers. Only $bla_{\text{OXA-10}}$, *AAC(6)-Ib-cr*, *aadA1*, *mefA*, t*etA* and *qnrS* were persistent in all sampling sites, but were in lower amounts upstream and downstream sites (Figure 3.2). Unique resistance genes were detected in several sampling sites. For instance, vancomycin resistance gene (*vanB)*, bla_{KPC}, $bla_{\text{per-2group}}$, bla_{OXA23} and *qepA* were detected only in ZE. Whereas three β-lactamase genes (bla_{NDM}, $bla_{\text{CFE-1}}$ and $bla_{\text{OXA-23}}$) were detected only in BA just down-stream of an industrial area. Two carbapenemase genes ($bla_{\text{IMP-2}}$ and $bla_{\text{IMP-5}}$) were detected only downstream of the river. Like the β-lactamase genes, the middle catchment of the river contained the majority of aminoglycosides resistance genes. Not only the diversity of ARGs but also their abundance demonstrated spatial variation (Figure 3.3). In general, the number of resistance genes detected (Figure 3.1a) and their abundance (Figure 3.3) upstream (GE) and downstream (AB)

of the river were much lower than the other three sites. For instance, only 18% of fluoroquinolone and 3.6% of β-lactam resistance genes were detected upstream of the Little Akaki River.

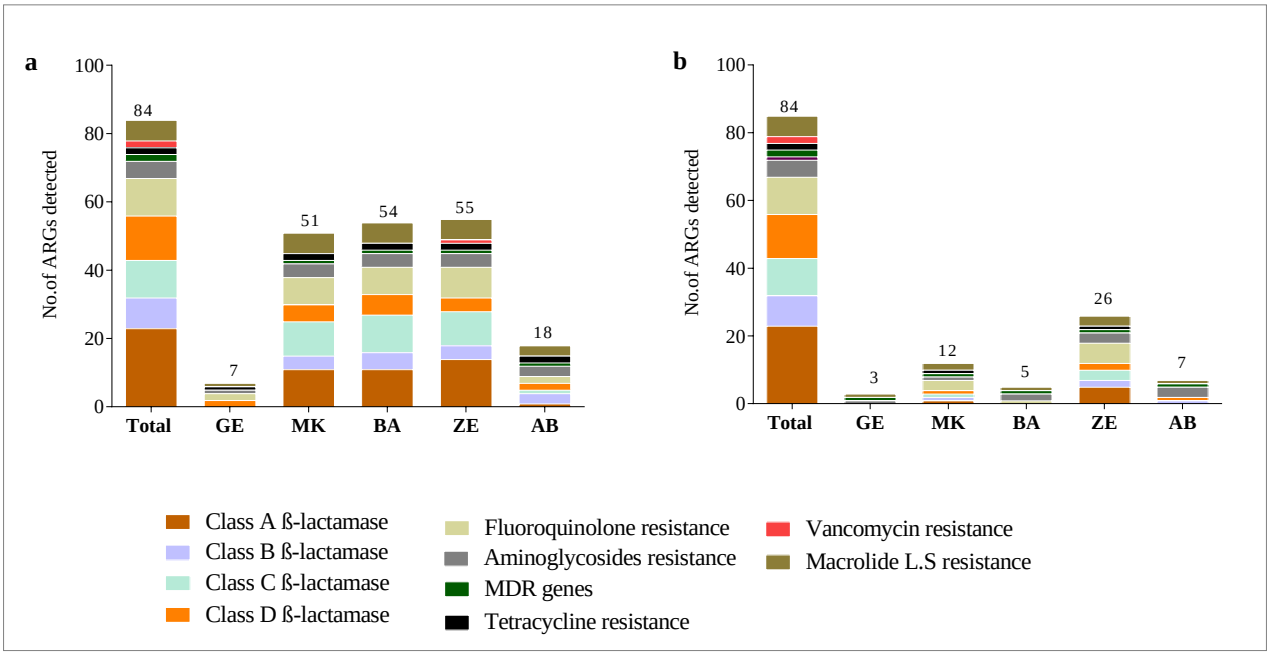

Figure 3.1: The antibiotic resistance genes detected from (a) Akaki river water and (b) sediment in Addis Ababa, Ethiopia. Water and sediment samples were obtained from five sampling points along Little Akaki (GE, MK, and BA), Big Akaki (ZE) and a downstream reservoir (AB). MDR indicates multi-drug resistance efflux pump genes and Macrolide L. S. resistance indicates macrolide, lincosamide and streptogramin resistance. Total indicates the total numbers of antibiotic resistance genes tested in the qPCR array.

The study investigated 55 β-lactamase genes belonging to four sub-classes (A, B, C and D) and found that their distribution and diversity showed spatial variation among sampling sites. The majority (69%) of the tested β-lactam resistant genes were detected in the rivers. The proportion of β-lactam resistance gene at sampling points was 1.9%, 28.8%, 31.7%, 30.8% and 6.7% for GE, MK, BA, ZE, and AB, respectively. The β-lactamase gene *bla*$_{\text{OXA-10}}$ was persistently detected in all sampling sites (Table 3.1). The Gefersa reservoir, upstream of the Little Akaki river contained only two β-lactam resistant genes (*bla*$_{\text{OXA-58}}$ and *bla*$_{\text{OXA10}}$), both

from class D. Majority of β-lactamase genes were detected in the three sites (MK, BA and ZE) nearest the industrial, healthcare facilities and agricultural activities. In some sites, unique β-lactamase genes were detected. For instance, *bla*$_{NDM}$, *bla*$_{CFE-1}$ and *bla*$_{OXA-23}$ were detected only in BA sampling site in which the majority of industries are established, whereas *bla*$_{KPC}$, *bla*$_{Per-2\ group}$ and *bla*$_{SHV238G240K}$ were detected in a site near the hospitals (Figure 3.2). None of class A, B, and C β-lactam resistance genes was observed upstream of the river. Two carbapenemase genes (*bla*$_{IMP-2}$ and *bla*$_{IMP-5}$) were detected only downstream of the river. All tested class C β-lactamase genes were detected in the river, and except *bla*$_{FOX}$, all were near the industrial, hospitals, and agricultural regions of the rivers, MK, BA, and ZE. The *bla*$_{BES-1}$, *bla*$_{BIC-1}$, *bla*$_{CTX-M-8}$, *bla*$_{IMI\&NMC-A}$, *bla*$_{SFC-1}$, *bla*$_{SHV(156D)}$, *bla*$_{SME}$, *bla*$_{IMP-1}$, *bla*$_{IMP-12}$, *bla*$_{VIM-7}$, *bla*$_{OXA-18}$, *bla*$_{OXA-45}$, *bla*$_{OXA-50}$, *bla*$_{OXA-51}$, *bla*$_{OXA-54}$, *bla*$_{OXA-55}$ and *bla*$_{OXA-60}$ not detected in any of the sampling sites (Figure 3.3a).

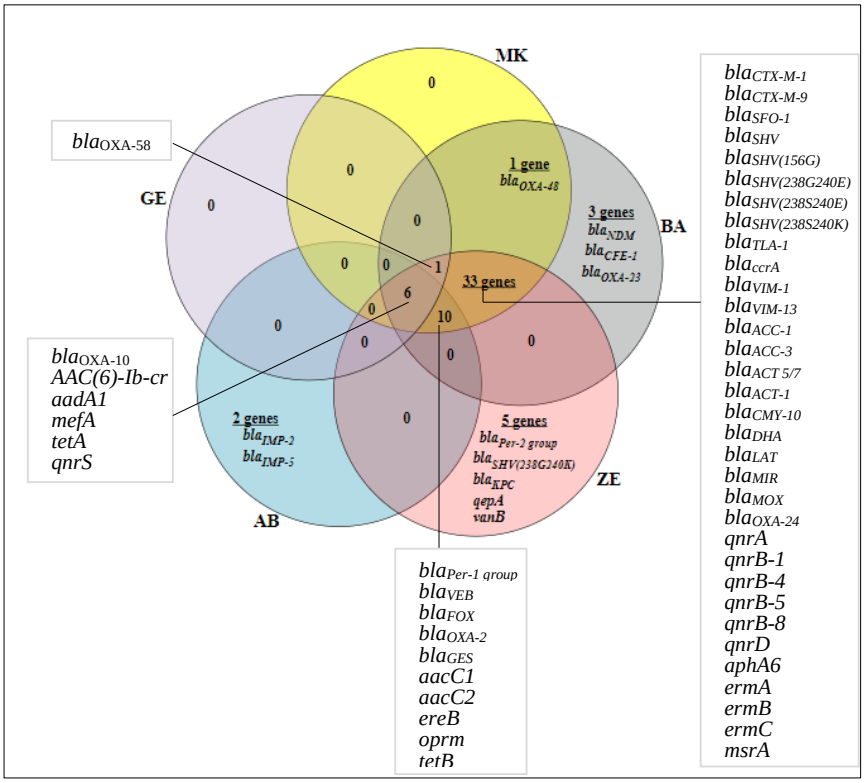

Figure 3.2: Venn diagram showing the number and diversity of ARGs detected from Akaki river water. Samples were collected from five sampling points (GE, MK, BA, ZE and AB).

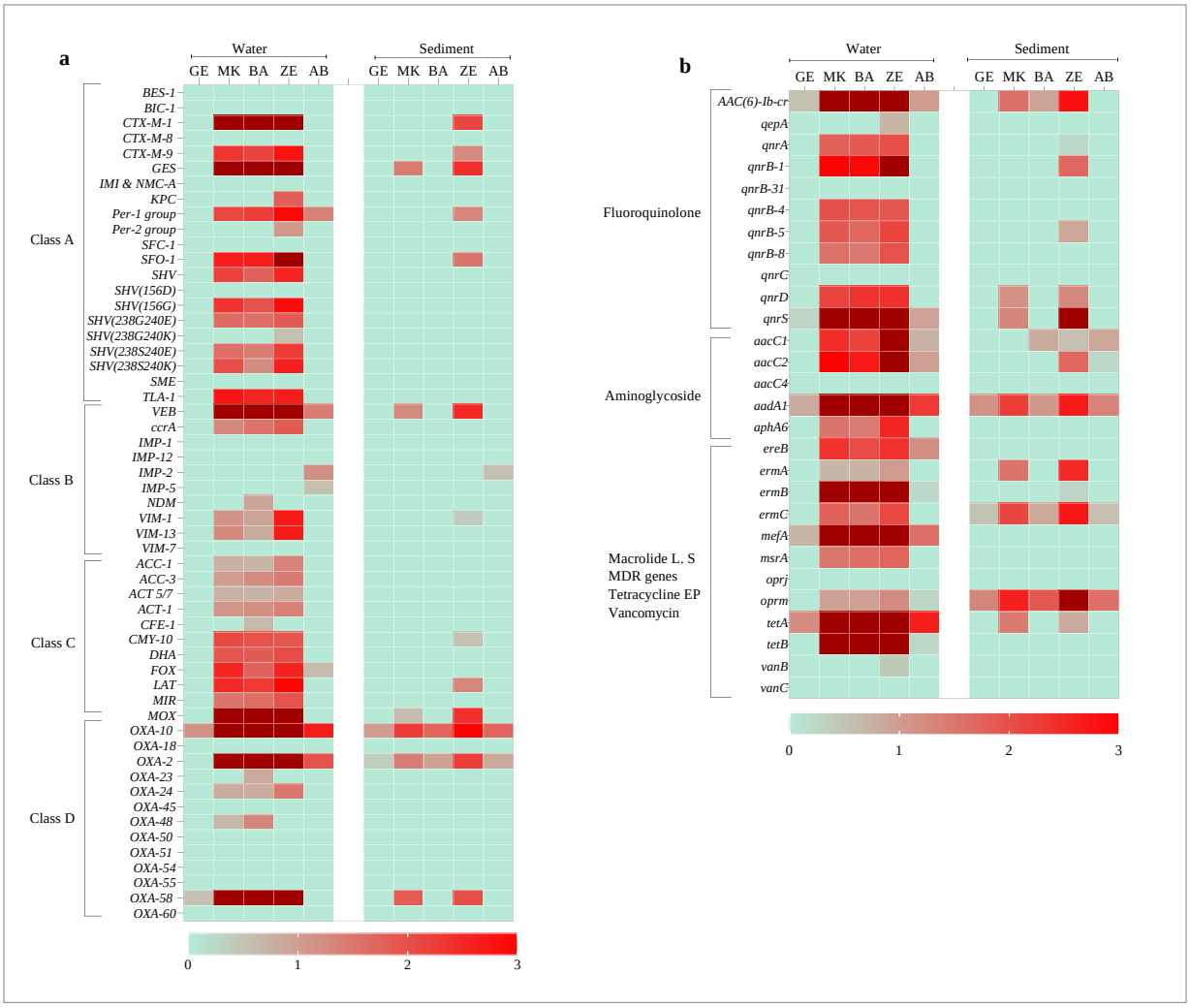

Figure 3.3: Relative abundance of (a) β-lactamase genes and (b) aminoglycoside, fluoroquinolone, macrolide, MDR efflux pump, tetracycline efflux pump and vancomycin resistance genes in bacterial communities of Akaki river water and sediment samples. Genes are grouped by antibiotics class are demarcated by black line on the left side. Green indicates absence and red indicates presence of genes. The gradient of the red reflects the different abundance of resistance gene based on fold change. The dark red indicates high abundance (high fold change) of a particular gene.

Table 3 1: Diversity and distribution of β-lactamase genes identified from water and sediment at different locations in the Akaki river.

Antibiotic resistance genes	Water*					Sediment*				
	GE†	MK†	BA†	ZE†	AB†	GE†	MK†	BA†	ZE†	AB†
bla$_{\text{BES-1}}$										
bla$_{\text{BIC-1}}$										
bla$_{\text{CTX-M-1 Group}}$										
bla$_{\text{CTX-M-8 Group}}$										
bla$_{\text{CTX-M-9 Group}}$										
bla$_{\text{GES}}$										
bla$_{\text{IMI \& NMC-A}}$										
bla$_{\text{KPC}}$										
bla$_{\text{Per-1 group}}$										
bla$_{\text{Per-2 group}}$										
bla$_{\text{SFC-1}}$										
bla$_{\text{SFO-1}}$										
bla$_{\text{SHV}}$										
bla$_{\text{SHV(156D)}}$										
bla$_{\text{SHV(156G)}}$										
bla$_{\text{SHV(238G240E)}}$										
bla$_{\text{SHV(238G240K)}}$										
bla$_{\text{SHV(238S240E)}}$										
bla$_{\text{SHV(238S240K)}}$										
bla$_{\text{SME}}$										
bla$_{\text{TLA-1}}$										
bla$_{\text{VEB}}$										
bl$_{a\text{ccrA}}$										
bla$_{\text{IMP-1 group}}$										
bla$_{\text{IMP-12 group}}$										
bla$_{\text{IMP-2 group}}$										
bla$_{\text{IMP-5 group}}$										
bla$_{\text{NDM}}$										
bla$_{\text{VIM-1 group}}$										
bla$_{\text{VIM-13}}$										
bla$_{\text{VIM-7}}$										
bla$_{\text{ACC-1 group}}$										
bla$_{\text{ACC-3}}$										
bla$_{\text{ACT 5/7 group}}$										
bla$_{\text{ACT-1 group}}$										
bla$_{\text{CFE-1}}$										
bla$_{\text{CMY-10 Group}}$										
bla$_{\text{DHA}}$										
bla$_{\text{FOX}}$										
bla$_{\text{LAT}}$										
bla$_{\text{MIR}}$										
bla$_{\text{MOX}}$										

bla$_{\text{OXA-10}}$										
bla$_{\text{OXA-18}}$										
bla$_{\text{OXA-2 Group}}$										
bla$_{\text{OXA-23 Group}}$										
bla$_{\text{OXA-24 Group}}$										
bla$_{\text{OXA-45}}$										
bla$_{\text{OXA-48 Group}}$										
bla$_{\text{OXA-50 Group}}$										
bla$_{\text{OXA-51 Group}}$										
bla$_{\text{OXA-54}}$										
bla$_{\text{OXA-55}}$										
bla$_{\text{OXA-58}}$										
bla$_{\text{OXA-60}}$										

* Shaded boxes indicate presence and white boxes absence of resistance gene

† GE, Gefersa; MK, Mekanissa; BA, Batu; ZE, Zewditu; AB, Aba-Samuel

The presence and abundance of five major aminoglycoside-resistant genes (*aacC1*, *aacC2*, *aacC4*, *aadA1*, and *aphA6*) were determined, of which *aadA1* was consistently observed at all sampling sites (Figure 3.3b, Table 3.2). Four (*aacC1*, *aacC2*, *aadA1* and a*phA6*) were detected in the three sites (MK, BA, and ZE). Eleven fluoroquinolone-resistant genes (*AAC(6)-Ib-cr*, q*epA*, *qnrA*, *qnrB-1*, *qnrB-31*, *qnrB-4*, *qnrB-5*, *qnrB-8*, *qnrC*, *qnrD* and *qnrS*) were evaluated, of which *AAC (6)-Ib-cr* and *qnrS*, were persistently detected in all sampling sites. The middle catchment of the river was where the majority of fluoroquinolone resistance genes were detected. The upstream and downstream of the river contained only two fluoroquinolone-resistant genes (*qnrS* and *AAC*(*6*)-*Ib-cr*). Macrolides-associated resistant genes (*ermA, ermB*, *ermC*, *mefA* and *msrA*) were also assessed and the results revealed that all sampling sites were found to be positive for *mefA*. The three anthropogenically impacted sampling sites (MK, BA, and ZE) contained all tested macrolides resistance genes. Out of the analysed two multidrug resistance efflux pump genes (*oprj* and *oprM*), only *oprm* was detected in four sites (MK, BA, ZE, and AB). We also determined two tetracycline resistance genes (*tetA* and *tetB*) and *teA* was persistently detected in all sampling sites (Figure 3.3b; Table 3.2). The gene *tetB* was detected in four sites (MK, BA, ZE and AB). One vancomycin gene (*vanB*) was detected in the hospital site and none of the sampling sites contained *vanC*.

Table 3 2: Antibiotic resistance genes for fluoroquinolones, aminoglycosides and other clinically relevant antibiotics detected at different locations in water and sediment of the Akaki river.

ARGs	Water*					Sediment*				
	GE†	MK†	BA†	ZE†	AB†	GE†	MK†	BA†	ZE†	AB†
Fluoroquinolone										
AAC(6)-Ib-cr	■	■	■	■	■		■	■	■	
qepA				■						
qnrA		■	■	■						
qnrB-1 group		■	■	■					■	
qnrB-31 group										
qnrB-4 group		■	■	■						
qnrB-5 group		■	■	■					■	
qnrB-8 group		■	■	■						
qnrC										
qnrD		■	■	■			■		■	
qnrS	■	■	■	■	■		■		■	
Aminoglycosides										
aacC1		■	■	■	■			■	■	■
aacC2		■	■	■	■				■	
aacC4										
aadA1	■	■	■	■	■	■	■	■	■	■
aphA6		■	■	■						
Erythromycin, macrolide, MDR§, EP§, Tetracycline EP and vancomycin										
ereB		■	■	■	■		■			
ermA		■	■	■						
ermB		■	■	■			■		■	
ermC		■	■	■			■	■	■	■
mefA	■	■	■	■	■					
msrA		■	■	■						
oprj										
oprM		■	■	■	■	■	■	■	■	■
tetA	■	■	■	■	■		■		■	
tetB		■	■	■	■		■		■	
vanB				■						
vanC										

* Shaded boxes indicate presence and white boxes absence of resistance gene

† GE, Gefersa; MK, Mekanissa; BA, Batu; ZE, Zewditu; AB, Aba-Samuel

§ MDR: multi drug resistance, EP: efflux pumps

Sediment samples were also collected from all 5 sites in the river and assessed for the presence and relative abundance of the antibiotic resistance genes in a similar manner as with the water samples. Fewer ARGs were detected in Akaki river sediment samples and there were no unique genes detected in the sediment samples that were not detected in the water samples (Figure 3.1b). Two β-lactam (*bla*$_{\text{OXA-10}}$ and *bla*$_{\text{OXA-2}}$), 1 aminoglycoside (*aadA1*), 1 macrolide (*ermC*) and 1 multi-drug efflux pump (*oprM*) resistance genes were persistent in all sampling sites (Table 3.1 and 3.2). A relatively higher number of ARGs were detected in the sediment samples collected from ZE sampling site compared to other sites. For instance, only two β-lactamase genes were detected from BA sampling sites whereas 13 β-lactamase genes were detected from ZE sites. Not only the β-lactamase gene but also other resistance classes showed spatial variations between sampling sites. Unique genes detected at ZE includes *bla*$_{\text{CTX-M1}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{Per-1 group}}$, *bla*$_{\text{SFO-1}}$, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{CMY-10}}$, *bla*$_{\text{LAT}}$, *qnrb-1*, *qnrb-5* and *aaC2*. Like the water samples, *bla*$_{\text{IMP-2}}$ was detected only downstream of the river (AB). Out of the 11 fluroquinolones resistance genes tested, 6 were detected in the samples of which 3 were only in ZE sampling point. No Fluoroquinolone resistance gene was detected from upstream and downstream reservoirs of the river. Three out of 5 aminoglycoside resistance genes were detected in ZE site and *aadA1*was persistent in all sampling points.

The relative abundance of ARGs detected in both waters and sediments of the Akaki river system is presented in figure 3.3. The abundance has shown spatial variation among sampling sites, with the highest abundance observed in the three anthropogenic impacted sites (MK, BA, and ZE) and the lowest upstream (GE) and downstream (AB) of the river. The abundance of *tetA* and *bla*$_{\text{OXA-10}}$ genes was highest in all sites compared to other genes. The fold changes in the middle catchment of the river also showed some variations. The most abundant genes in the middle catchment of the river were *bla*$_{\text{CTX-M1}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{VEB}}$, *bla*$_{\text{MOX}}$, *bla*$_{\text{OXA-10}}$, *bla*$_{\text{OXA-58}}$, *AAC (6)-Ib-cr*, *qnrs*, *aadA1*, *ermB*, *mefA*, *tetA* and *tetB*. The abundance of resistance genes in the three sites (MK, BA and ZE) was almost consistent. However, the site near hospitals had a relatively higher abundance of some genes such as *bla*$_{\text{SHV}}$, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{VIM-13}}$, *ACC-1*, *ACC-3*, *bla*$_{\text{DHA}}$, *bla*$_{\text{LAT}}$, *bla*$_{\text{MIR,}}$ and *bla*$_{\text{OXA-24.}}$ On the other hand, the abundance of *bla*$_{\text{OXA-18}}$ was relatively higher in BA site. Similarly, the relative abundance of resistance genes detected in the sediment sample was highest in ZE site. The abundance of all persistently detected

resistance genes in the sediment samples (*bla*$_{OXA-10}$, *bla*$_{OXA-2}$, *aadA1*, *ermC* and *oprM*) was highest in ZE site. In multivariate analysis, it was observed that GE and AB, ZE, and BA were clustered together whereas MK was clustered with none of the other sampling sites (Figure 3.4a).

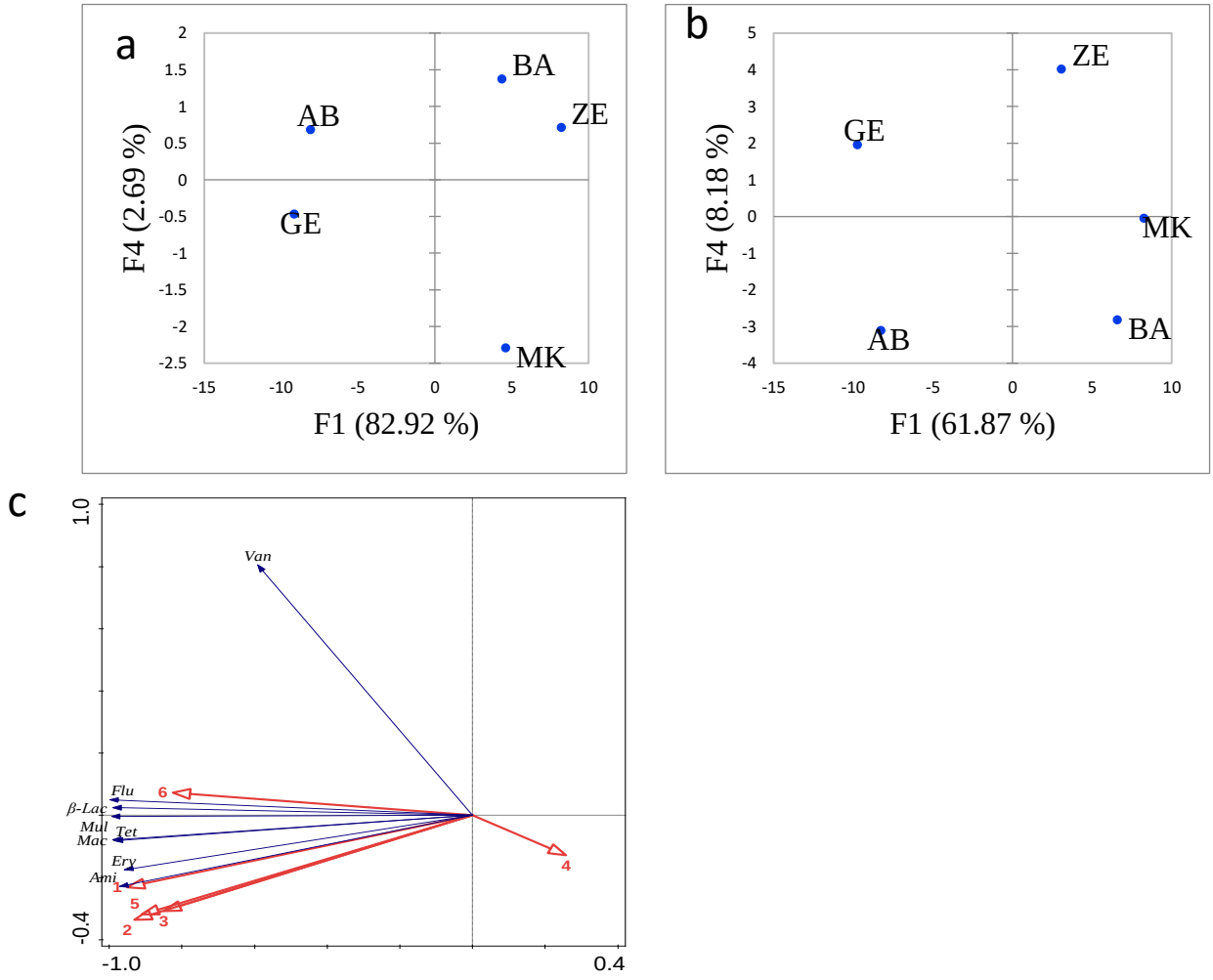

Figure 3.4: Correlation matrix biplots displaying similarities between (a) ARGs and (b) clinically relevant bacteria profiles of GE, MK, BA, ZE and AB based on the Bray–Curtis distance analysis. (c) Correlation between ARGs (Flu (Fluoroquinolone resistance, Ami (Aminoglycoside-resistance), β-lac (β-lactam resistance), Mac (Macrolide), MDE (Multidrug resistance efflux pump), Tet (Tetracycline efflux pump), Van (Vancomycin resistance) and Ery (Erythromycin resistance)) abundance and bacterial community using Redundancy analysis at phyla level. The red arrows represent bacteria at phylum level and blue arrow

shows ARGs classes. The numbers from 1-6 indicates bacteroidetes (1), firmicutes (2), proteobacteria (3), actinobacteria, fusobacteria and verrucomicrobia respectively.

Using microbial DNA qPCR arrays for water and sepsis, we determined the diversity, distribution, and relative abundance of clinically relevant bacteria in the Akaki river. The results revealed that the water samples contained diverse clinically relevant bacteria with some spatial variations between sampling sites. The bacteria identified in the Akaki river were in general grouped into six phyla; actinobacteria, fusobacteria, firmicutes, bacteroidetes, proteobacteria, and verrucomicrobia (Figure 3.5a). The most abundant phylum identified in the river water samples was proteobacteria followed by firmicutes, actinobacteria, bacteroidetes, fusobacteria and verrucomicrobia. The distribution at the phyla level showed a spatial variation between sampling sites. Three phyla (actinobacteria, firmicutes and, proteobacteria) were persistently detected in all sampling sites. All the six phyla were detected in the middle catchment of the rivers (MK, BA, and ZE). However, fusobacteria, verrucomicrobia and bacteroidetes were not identified in the upstream and downstream of the rivers (GE and AB).

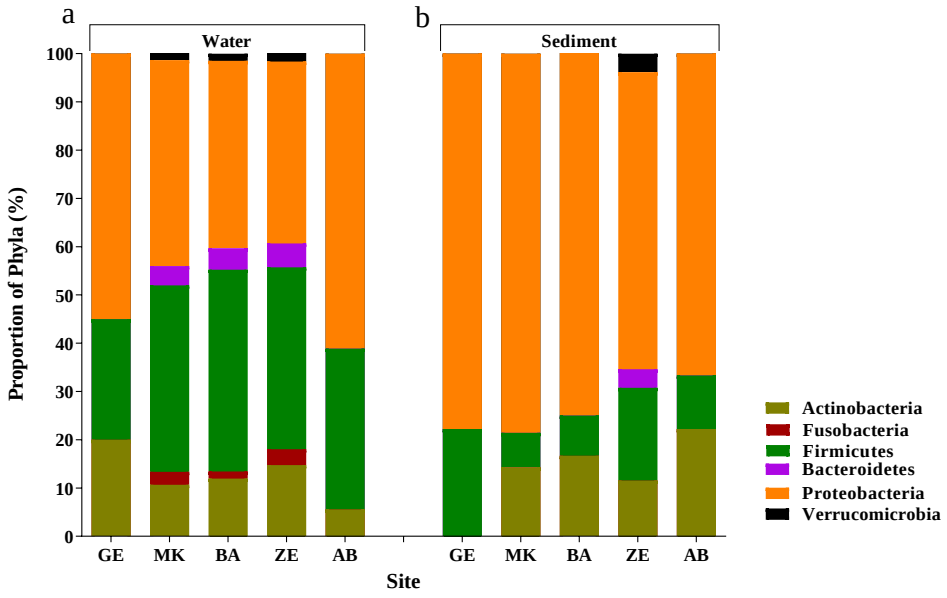

Figure 3.5: Distribution of clinically relevant bacteria at the phyla level detected by qPCR from Akaki river (a) water and (b) sediment samples.

The major bacterial species persistently detected in all sampling sites were *Escherichia coli*, *Aeromonas* species, *Clostridium sordellii*, and *Klebsiella oxytoca*. Diarrhea causing bacteria such as *Shigella dysenteriae, Salmonella enterica, Yersinia enterocolitica, Vibrio cholerae* and others were more frequently detected in the middle catchment of the river (Figure 3.6). The bacterial richness and diversity were highest at the three sites (MK, BA and ZE). Fewer bacterial species were identified upstream and downstream of the river. For instance, only 16 potential pathogenic bacteria were detected in the upstream of the river (GE). In multivariate analysis MK and BA, GE and AB were clustered together (Figure 3.4b). Site-specific bacteria were also detected in the middle catchment of the river. For instance, *V. cholerae* was identified only in BA sampling site whereas only a sample collected from ZE site contained *Vibrio vulnificus*. The majority of tested bacteria were not detected in the sediment samples (Figure. 3.5b). Two phyla (Firmicutes and Proteobacteria) were persistently detected in all sampling sites. At species level, *Aeromonas* species, *Brevundimonas diminuta* and *Brevundimonas vesicularis* were persistently identified in all sampling sites. The sediment sample collected from the hospital site (ZE) contained relatively higher number of bacteria (Figure 3.6). Similarly, to the water samples, the bacterial diversity in the upstream and downstream reservoirs was low.

The correlation between the mean fold change of ARGs and bacteria at phyla level was determined and found to be significantly correlated (Figure 3.4c). A correlation between the abundance of bacteroidetes and firmicutes to β-lactamases and erythromycin resistance genes, fusobacteria, proteobacteria, firmicutes, and bacteroidetes to aminoglycosides, macrolides, and multidrug resistance efflux pump, fusobacterial, Bacteroidetes, and firmicutes to fluoroquinolone and tetracycline efflux pump, was observed. In addition to the phylum level, redundancy analysis with Pearson correlation coefficient was performed to determine the significant relationship between the fold change of each bacterial species and ARGs. The bacterial richness was positively correlated with the abundance and diversity of antibiotic resistance genes. In the three sites (MK, BA, and ZE), a positive correlation with the abundance of the majority of antibiotic resistance genes was observed. The spatial difference of bacterial diversity and their abundance in each site also resulted in presence of unique antibiotic resistance. In the redundancy analysis, the fold change of some bacteria was

significantly correlated with the fold change of ARGs. For instance, the majority of ARGs were positively correlated with *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, *Clostridium perfringens*, *Enterococcus faecium*, *Lactobacillus gasseri*, *Aeromonas enteropelogenes* and *Micrococcus luteus*. The upstream and the downstream sites had fewer bacterial compositions with a low level which resulted in few antibiotic resistance genes compared to other sites.

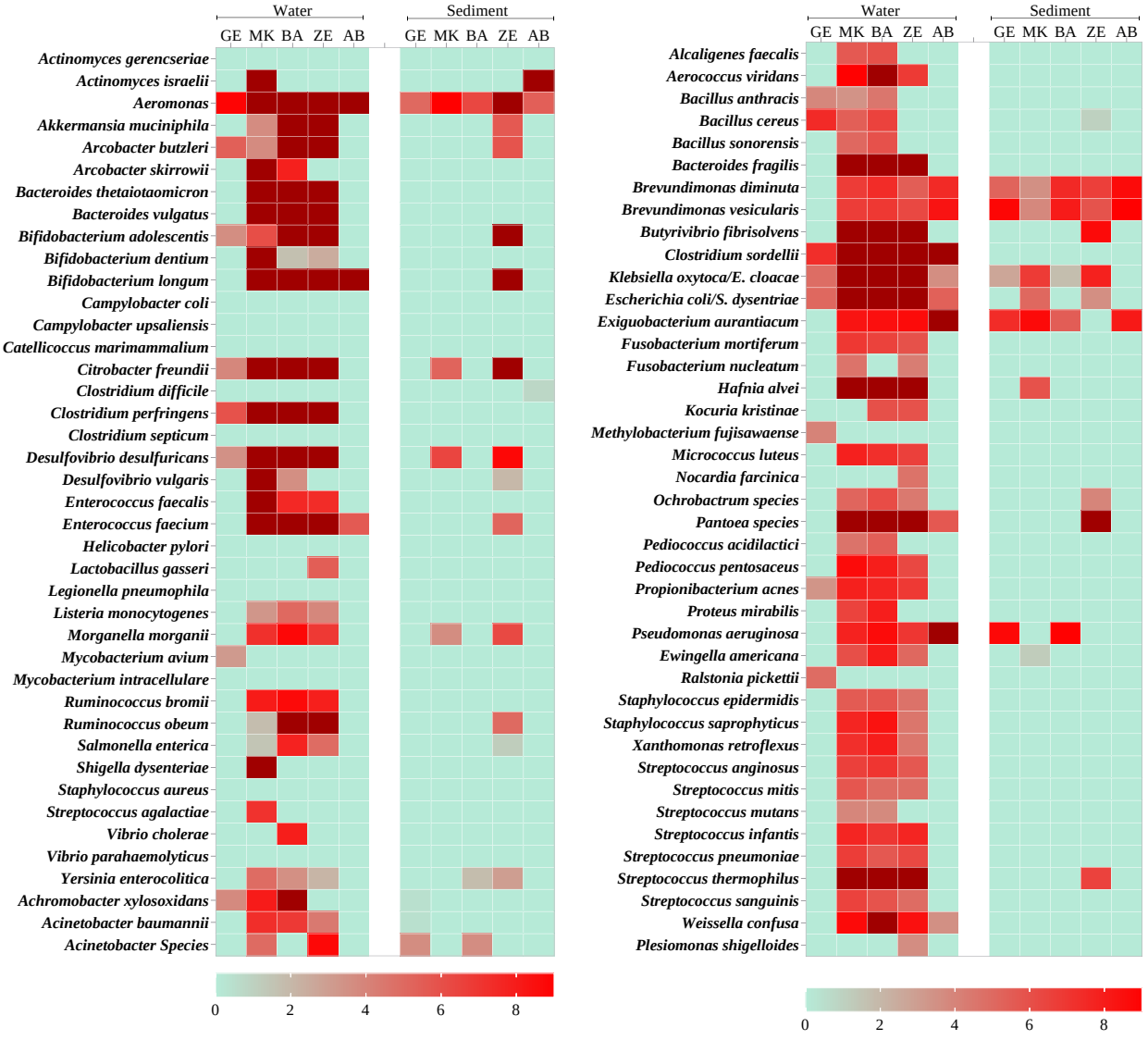

Figure 3.6: Relative abundance of clinically relevant bacteria from Akaki river water and sediment samples. The log fold change of each bacterium was calculated using ΔΔCT method. Green color indicates absence and red color indicates presence of genes. Gradient of the red colors reflects increasing abundance based on the difference in the fold change of detection of bacterial DNA. The dark red indicates a high abundance based on high fold change of a particular bacterial species.

3.2 Functional and molecular features of *Escherichia coli* isolates from Akaki river, Ethiopia

In this study, *E. coli* strains were isolated from the Akaki river to determine the phenotypic antibiotic resistance levels, distribution of virulence genes, and mechanism of resistance. The levels of *E. coli* were also determined in the Akaki rivers to evaluate the bacteriological quality of the water. The level of *E. coli* showed a significant variation between sampling sites. The cfu at site MK, BA and ZE was higher than the upstream (GE) and downstream (AB) sites of the river. The overall mean count of *E. coli* in the present study were 3.7, 8.5, 7.4, 8.2, and 6.2 $\log_{10}$ CFU 100 mL^{-1} in GE, MK, BA, ZE, and AB, respectively (Figure 3.7). The variation was significantly higher in MK (P=0.0006), BA (P=0.002) and ZE (P=0.0008) compared to the upstream reservoir (GE). The results were higher than the WHO recommended standard of quality of water for irrigation (WHO, 2006). A total of 209 *E. coli* suspected strains were isolated from the five sites of the river, of which 168 *E. coli* were confirmed by MALDI TOF-MS. The majority of the isolates were detected in the middle catchment of the rivers (MK, BA, and ZE) and fewer numbers from the upstream and downstream of the river.

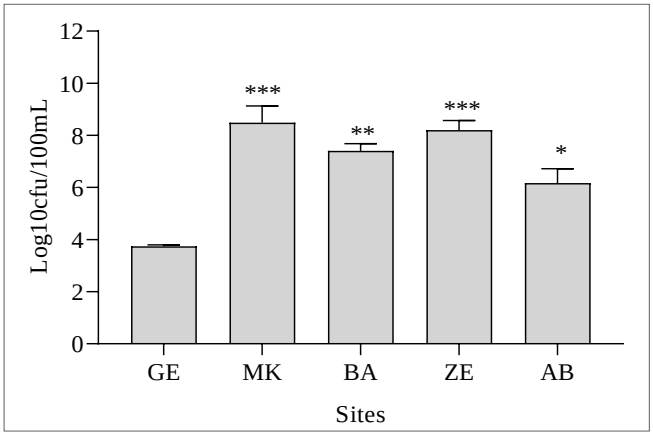

Figure 3.7: Abundance of *E. coli* at the sampling points in the Akaki river. GE, MK, and BA located in the Little Akaki river whereas ZE is in the Big Akaki river. AB is the downstream reservoir. Unpaired *t*-test was applied to determine the statistical difference of cfu between GE and other sampling sites. Where * refers to $p < 0.05$, ** refers to $p < 0.01$, and ***refers to $p < 0.001$. For enumeration two biological replicates were used to each sampling sites.

3.2.1 **Antibiotic Resistance Patterns**

All confirmed *E. coli* isolates (168) were tested for their resistance to 24 different antibiotics. The *E. coli* isolates included in the present study have shown resistance to at least three antibiotics tested. The most frequent resistance was against erythromycin (97%), followed by doxycycline (78.5%), tetracycline (75.6%), and amoxicillin (75.6%) (Figure 3.8). The overall prevalence of cephalosporin resistance was 7.9%, and to 2nd, 3rd, and 4th generation cephalosporins was 1.8%, 10.9%, and 8.3 %, respectively. Resistance to 3rd generation cephalosporin was higher than other cephalosporin sub-groups. The highest resistance was observed to cefotaxime and cefixime in the cephalosporin antibiotics. The prevalence of resistance to carbapenem antibiotics in the current study was low, 2 to 5% of the three carbapenem antibiotics. Resistance to penicillin and tetracycline was higher compared to other classes of antibiotics. Of the total *E. coli* isolates, 36.3% were resistant to amoxicillin/clavulanic acid and a different resistance pattern was observed to other penicillin antibiotics. The frequency of multi-drug resistant (MDR) was evaluated in the *E. coli* isolates. In this study, MDR is defined as an isolate showing resistance to three or more antibiotic classes (EUCAST, 2019). It was observed that 80.95% (n = 136) were resistant to three or more classes of antibiotics, and thus categorized as MDR.

3.2.2 Distribution of Virulence Genes in *E. coli*

All *E. coli* isolates were investigated for their pathogen potential by determining the presence of virulence-associated genes, including five toxins (*EAST1*, *STa*, *LT*, *Stx1* and *Stx2*) and three adherence factors (*F4, F6* and *F18*). Figure 3.9a shows the distribution of virulence genes among *E. coli* isolates from the Akaki river. The majority of *E. coli* isolates (119, 70.8%) harbored enteroaggregative heat-stable toxin 1 (*EAST1*) gene, whereas the heat-labile toxin (*LT*) was detected in 34 (20.2%) *E. coli* isolates. The prevalence of Shiga toxin-producing *E. coli* was 7.7%. Two Shiga toxin-associated genes were determined with a prevalence rate of 6% and 1.8% for *stx-1* and *stx-2,* respectively. The prevalence of adhesin genes, *F4*, *F6*, and *F18* were 28.6%, 12.5% and 58.3%, respectively. The distribution of virulence genes in *E. coli* was correlated with the site of isolation (Figure 13.9b). The majority of isolates from MK and ZE sites contained *EAST1* gene. Of the *EAST1* positive isolates, 38.7% and 26.4 % were

detected in MK and ZE sampling sites, respectively. Fewer strains that were isolated from the upstream reservoir (GE) and lower stream catchment (AB) of the river contained *EAST1*.

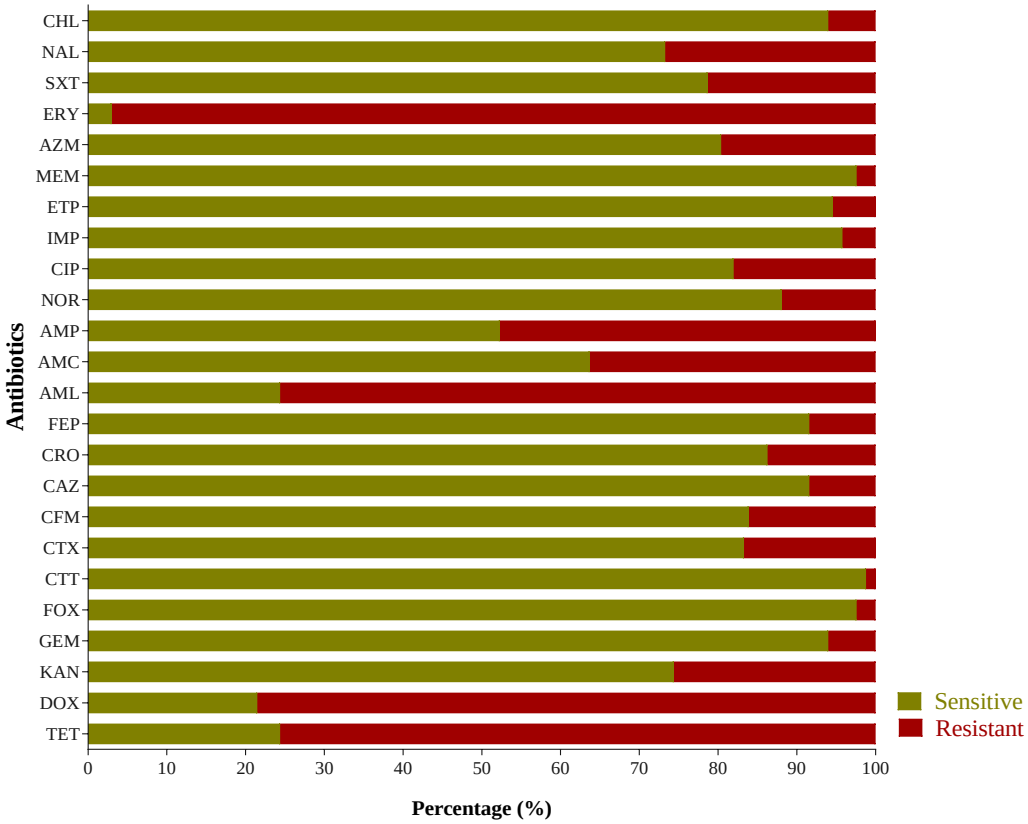

Figure 3.8: An antibiogram of *E. coli* isolates from Akaki river. Interpretation was carried out using the European Committee on Antimicrobial Susceptibility Testing (EUCAST-2019) standard. Strains showing ‘‘resistant’’ or ‘‘intermediate’’ was subsumed under the category ‘‘resistant’’ and the others were classified as ‘sensitive’. Abbreviations: TET, tetracycline; DOX, doxycycline; KAN, kanamycin; GEM, gentamicin; FOX, cefoxitin; CTT, cefotetan; CTX, cefotaxime; CFM, cefixime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AML, amoxicillin; AMC, amoxicillin-clavulanate; AMP, ampicillin; NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; IMP, imipenem; ETP, ertapenem; MEM, meropenem; AZM, azithromycin; ERY, erythromycin; SXT, sulfamethoxazole / trimethoprim and CHL, chloramphenicol.

The gene *stx-2* was detected only in strains that were isolated in MK, mostly associated with residential areas and agriculture. The majority of heat-stable enterotoxin-a (*STa*) positive strains were isolated from ZE site. None of the isolates upstream or in the recipient catchment of the river contained *STa*, *stx-1*, and *stx-2* enterotoxins. Co-occurrence of the 6 major virulence genes using Pearson correlation matrix showed a positive correlation among the majority of the resistance genes, however, a significant positive correlation was observed between *EAST1* and *F6*, *EAST1* and *F18*, *STa* and *F6*, *STa* and *stx-2*, *F4*, and *F6*, *F6* and *F18*. Alternatively, *LT* was significantly negatively correlated with *F4* (Table 3.3).

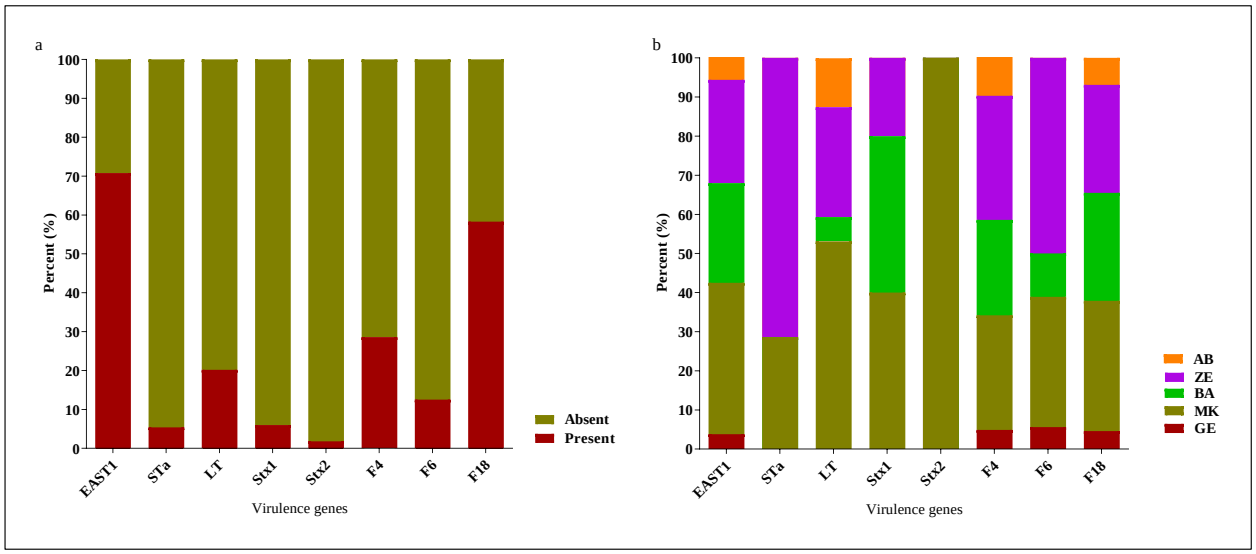

Figure 3.9: The (a) prevalence and (b) spatial distribution of virulence genes in *E. coli* isolated from the different sites of the Akaki rivers.

Correlation of antibiotic resistance patterns and diversity of virulence genes showed a significant association between resistance to cephalosporins and the occurrence of some enterotoxins. For instance, *E. coli* isolates that were resistant to ceftriaxone and ceftazidime were more likely to contain *EAST1*, *STa* and *F18* virulence genes. Similarly, doxycycline and chloramphenicol resistance had a significant association with the presence of some virulence genes (*EAST1* and *STa*). Isolates that were resistant to norfloxacin were less likely to have adhesion genes such as F4 and F6 (Table 3.4).

Table 3 3: Co-occurrence of virulence determinants in E. coli isolates from Akaki river

Gene	***EAST1***	***STa***	***LT***	***stx1***	***stx2***	***F4***	***F6***	***F18***
EAST1	1	0.036	0.128	0.051	0.087	-0.087	0.203**	0.201**
Sta		1	0.012	-0.060	-0.032	0.142	0.390**	0.147
LT			1	-0.127	-0.068	-0.155*	-0.101	0.035
Stx1				1	-0.034	0.119	0.133	0.060
Stx2					1	-0.085	0.221**	0.023
F4						1	0.239**	0.134
F6							1	0.210**
F18								1

* Correlation is significant at 0.05 level

** Correlation is significant at 0.01 level

3.2.3 Sequencing and Phylogeny

A total of 27 *E. coli* isolates were subjected for whole genome sequencing and the results are presented in Table 3.5. The draft genomes ranged in size from 4.5Mbp to 6.1Mpb and the contigs ranged between 42 and 1,507. The mean GC content was found to be 50.9% and the mean coding sequence (CDS) was 4,994. The *E. coli* strains were assigned to six phylogroups (A, B1, B2, C, D and F), 11 isolates were members of phylogroup A (40.8%), 7 were members of phylogroup B1 (25.9), 2 were members of phylogroup B2 (7.4%), 1 was a member of phylogroup C (3.7 %), 5 were members of phylogenetic group D (18.5%), and 1 was a member of phylogroup F (3.7%). Serotypes of the *E. coli* isolates varied widely, and the predominant serotypes were O9 (n =5) and O8 (n=3). The 27 *E. coli* isolates belonged to 20 different sequence types, where ST10, ST69, and ST361 were the predominant sequence types detected (Table 3.6). The fimH type was also determined using fimH typer of the center of genomic epidemiology and found 17 different FimH types (Table 6). The most common fimH type was fimH 54, followed by fimH27and one *E. coli* strain was untypable.

Table 3 4: Association between antibiotic resistance and presence of virulence gene in *E. coli* isolates from Akaki river

AST		EAST1				STa				LT				Stx1				Stx2				F4				F6				F18			
		+	-	X^2	P	+	-	X^2	P	+	-	X^2	P	+	-	X^2	P	+	-	X^2	P	+	-	X^2	P	+	-	X^2	P	+	-	X^2	P
TET	S	27	14	0.65	0.43	2	39		1	7	34	0.33	0.56	3	38		0.70	1	40		0.57	13	28	0.26	0..60	2	39		0.10	20	21	2.0	0.15
	R	92	35			7	120			27	100			7	120			2	120			35	92			19	105			78	49		
GM	S	112	46		0.6	7	151		0.09	31	127		0.43	10	148		0.53	3	155		0.83	47	111		0.165	21	138		0.63	92	66		0.59
	R	7	3			2	8			3	7			0	10			0	10			1	9			1	9			6	4		
FOX	S	115	49		0.33	8	156		0.19	34	130		0.40	10	154		0.78	3	161		0.93	41	116		0.25	20	144		0.41	97	67		0.19
	R	4	0			1	3			0	4			0	4			0	4			0	4			1	3			1	3		
CTT	S	117	49		0.5	8	158		0.1	34	132		0.62	10	156		0.88	3	163		0.96	47	119		0.49	20	146		0.23	97	69		0.66
	R	2	0			1	1			0	2			0	2			0	2			1	1			1	1			1	1		
SXT	S	84	34	0.02	0.5	7	111		0.46	27	91	1.7	0.19	10	108		0.02	2	116		0.65	35	83	0.23	0.63	17	101		0.31	73	45	2.3	0.15
	R	35	15			2	48			7	433			0	50			1	49			13	37			4	46			25	25		
AM	S	66	22	1.6	0.21	4	84		0.44	15	73	1.2	0.28	7	81		0.20	2	86		0.53	23	65	0.53	0.46	11	77	00	1.00	54	34	0.69	0.40
	R	53	27			5	75			19	61			3	77			1	79			25	55			10	70			44	36		
CIP	S	99	38	0.95	0.33	5	132		0.15	29	108	0.31	0.57	10	127		0.13	3	134		0.55	40	97	0.41	0.51	18	119		0.25	79	58	0.05	0.81
	R	19	11			3	27			5	25			0	30			0	30			7	23			2	28			18	12		
IMP	S	113	48		0.34	9	152		0.67	33	128		0.56	10	151		0.64	3	158		0.87	48	113		0.09	21	140		0.38	94	67		0.69
	R	6	1			0	7			1	6			0	7			0	7			0	7			0	7			4	3		
CTX	S	95	45		0.07	8	132		0.53	30	110		0.28	9	131		0.47	3	137		0.57	44	96		0.04	18	122		052	86	54	3.3	0.06
	R	24	4			1	27			4	24			1	27			0	28			4	24			3	25			12	16		
MEP	S	115	49		0.24	9	155		0.8	33	131		0.59	10	154		0.78	3	161		0.93	48	116		0.25	21	143		0.58	96	68		0.55

	R	4	0			0	4			1	3			0	4			0	4			0	4			0	4			2	2		
NA	S	84	39	1.4	0.23	5	118		0.19	25	98	0.02	0.96	8	115		0.46	3	120	1.1	0.39	40	83	3.5	0.61	17	106		0.28	75	48	1.3	0.25
	R	35	10			4	41			9	36			2	43			0	45			8	37			4	441			23	22		
KAN	S	91	34	0.9	0.33	5	120		0.17	24	101	0.32	0.56	9	116		0.22	3	122	1.0	0.40	36	89	0.01	0.91	17	108	0.54		73	52	0.01	0.97
	R	28	15			4	39			10	33			1	42			0	43			12	31			4	39			25	18		
ERT	S	4	1		.54	0	5		0.75	2	3		0.26	0	5		0.73	0	5		0.91	2	3		0.44	1	4		0.49	4	1		0.30
	R	115	48			9	1454			32	131			10	153			3	160			46	117			20	143			94	69		
CRO	S	98	47	5.4	0.02	8	137		0.64	29	116	0.03	0.84	9	136		0.59	3	142		0.64	44	101		0.15	20	125		0.17	90	55	6.0	0.01
	R	21	2			1	22			5	18			1	22			0	23			4	19			1	22			8	15		
CAZ	S	106	48	3.5	0.04	6	148		0.29	32	122		0.43	10	144		0.40	2	152		0.23	44	110		0.60	17	137		0.07	93	61	3..2	0.07
	R	13	1			3	11			2	12			0	14			1	13			4	10			4	10			5	9		
CHL	S	113	45		0.32	6	152		0.01	33	125		0.36	9	149		0.46	3	155		0.83	44	114		0.30	18	140		0.11	93	65	0.30	0.58
	R	6	4			3	7			1	9			1	9			0	10			4	6			3	7			5	5		
DOX	S	26	10	0.04	0.83	1	35		0.38	8	28	0.11	0.73	2	34		0.63	1	35		0.51	13	23	1.2	0.25	6	30	0.72	0.39	21	15		1.00
	R	93	39			8	124			26	106			8	124			2	130			35	97			15	117			77	55		
AZM	S	94	41	0.48	0.48	5	130		0.07	28	107	0.10	0.74	10	125		0.10	3	132		0.51	40	95	0.37	0.53	15	120	1.2	0.27	79	56	0.01	0.92
	R	25	8			4	29			6	27			0	33			0	33			8	25			6	27			19	14		
AML	S	28	13	0.16	0.68	1	40		0.30	4	37		0.52	5	36	3.7	0.05	2	39		0.14	9	32	1.1	0.28	6	35	0.22	0.78	23	18	0.11	0.73
	R	91	36			8	119			30	97			5	122			1	126			39	88			15	112			75	52		
NOR	S	104	44	0.19	0.66	8	140		0.7	30	118		0.62	8	40		0.33	3	145		0.68	46	102		0.03	21	127		005	86	62	0.03	0.87
	R	15	5			1	19			4	16			2	18			0	20			2	18			0	20			12	8		
CFM	S	98	43	0.75	0.38	6	135		0.15	27	114	0.64	0.42	9	132		0.50	3	138		0.58	44	97		0.06	19	122		0.30	87	54	4.0	0.04
	R	21	6			3	24			7	20			1	26			0	27			4	23			2	25			11	16		
ETP	S	113	46		0.51	7	152		0.07	32	127		0.57	10	149		0.56	3	156		0.84	48	111		0.04	21	138		0.37	94	65		0.29
	R	6	3			2	7			2	7			0	9			0	9			0	9			0	9			4	5		
FEP	S	110	48		0.15	9	149		0.56	30	128		0.11	10	158		0.53	3	155		0.83	47	111		0.16	20	138		0.63	95	63		0.06
	R	9	1			0	10			4	6			0	10			0	10			1	9			1	9			3	7		

Table 3 5: General genome features of *E. coli* isolates from Akaki river. The variables are determined by PATRIC bioinformatics tool

Isolates	Contigs	GC (%)	Genome Size (Mbp)	CDS	tRNA	rRNA
ZEC-1	117	50.76	4.6	4,646	65	4
ZEC-2	173	50.5	5.1	5,264	70	4
ZEC-3	75	50.64	4.7	4,697	68	5
ZEC-4	136	50.51	5.4	5,448	72	6
ZEC-5	73	50.84	4.9	4,903	72	5
ZEC-6	131	50.72	5.3	5,346	66	5
ZEC-7	113	50.78	5.0	4,911	66	5
ZEC-8	153	50.61	4.9	5,031	69	6
ZEC-9	111	50.67	5.2	5,266	66	5
ZEC-10	62	50.67	5.0	4,944	69	6
ZEC-11	42	50.56	4.5	4,444	66	6
MEC-12	83	50.85	6.1	6,113	68	4
MEC-13	71	50.64	4.9	4,857	22	3
MEC-14	98	50.68	4.8	4,797	64	4
MEC-15	81	50.93	4.7	4,663	65	4
MEC-16	188	50.73	4.5	4,580	69	6
MEC-17	72	50.50	5.3	5,272	75	4
MEC-18	121	50.79	4.6	4,561	62	6
MEC-19	63	50.66	4.8	4,686	71	4
MEC-20	111	50.65	5.0	4,982	70	4
MEC-21	115	50.74	4.9	4,869	70	4
MEC-22	1,507	50.79	5.4	5,270	94	5
BEC-23	100	50.74	5.2	5,297	73	6
BEC-24	187	50.70	4.8	4,928	69	6
BEC-25	1,470	50.46	4.6	5,366	76	5
BEC-26	81	50.80	4.6	4,493	68	4
AEC-27	183	50.88	5.1	5,206	65	4

Table 3 6:Genetic serotyping and plasmid identification among *E. coli* isolates

Isolates	Site	*FimH*	ST	Serotype	Plasmid Replicons	
					Inc	*Col*
ZEC-1	ZE	34	46	O8:H4	*IncFIB(H89-PhagePlasmid)*	*Col(MP18)*
ZEC-2	ZE	54	10	O43:H19	*IncFIB(AP001918) and IncFII*	
ZEC-3	ZE	137	10	O114:H40	*IncQ1*	
ZEC-4	ZE	27	69	O17/O77:H18	*IncFIA, IncFIB(AP001918), IncFII(29) and IncFII(pRSB107)*	*Col8282*
ZEC-5	ZE	35	448	O8:H8		
ZEC-6	ZE	27	69	O17/O77: H18	*IncFIA, IncFIB(AP001918), IncFII, IncFII, IncQ1 and IncY*	*Col156*
ZEC-7	ZE	27	405	O102:H6	*IncFIA, IncFIB(AP001918), IncFII and IncFII*	
ZEC-8	ZE	233	401	O159:H34	*IncFIB(K)*	
ZEC-9	ZE	27	69	O15:H1	*Col156, IncFIA, IncFIB(AP001918), IncFII and IncFII*	*Col156*
ZEC-10	ZE	32	847	:H2*	*IncFIB(AP001918), IncFII and IncY*	
ZEC-11	ZE	31	154	O134:H38		
MEC-12	MK		746		*IncFII(K)* and IncFII(K)	*Col440I*
MEC-13	MK	142	90	O8:H9	*IncFIA, IncFIB(AP001918), IncFII(pAMA1167-NDM-5), IncQ1 and IncY*	
MEC-14	MK	24	10	O21:H12	*IncFIB(K) and IncFII*	
MEC-15	MK		746	O173:H37		
MEC-16	MK	237	120	O9:H10		
MEC-17	MK	76	998	O50/O2:H6	*IncFIB(AP001918), IncX1 and IncY*	
MEC-18	MK	69	216	O3:H4	*pSL483*	
MEC-19	MK	153	1722	O1:H25		
MEC-20	MK	54	361	O9:H30	*IncFIB(pB171), IncFII, IncI1-I(Gamma) and IncY*	*Col8282*
MEC-21	MK	54	361	O9:H30	*IncFIB(pB171) and IncFII(29)*	
MEC-22	MK	289	1308	O75:H10	*IncC*	
BEC-23	BA	30	131	O25: H4	*IncFIA, IncFII and IncI1-I(Gamma)*	
BEC-24	BA	54	3997	O89:H37	*IncFIA, IncFIB(AP001918), IncFII, IncFII, IncFII and IncI1-I(Gamma)*	*Col156*
BEC-25	BA	61	224!	O9:H30	*IncFIA, IncFIB(AP001918), IncFIB(pHCM2), IncFII(29), IncFII(pAMA1167-NDM-5) and IncX1*	
BEC-26	BA	54	361	O9:H30	*IncFIA(HI1) and IncR*	
AEC-27	AB	54	541	O148:H40	*IncFII(Yp), IncFII(pECLA) and IncX3*	

3.2.4 Antibiotic Resistance Genes, Plasmid Profiles, and Virulence Genes

Antibiotic resistance genes involved in inactivation of antibiotics, efflux pumps and chromosomal mutations conferring resistance to diverse group antibiotics were determined using the center of genomic epidemiology and comprehensive antibiotic resistance database (Table 3.7). Except for two isolates (ZEC-10 and MEC-18), all *E. coli* isolates harbored at least three resistance genes. The diversity of resistance genes in the *E. coli* strains was showed variation between sampling sites. Significant numbers of genetic determinants conferring resistance to the antibiotic were observed in anthropogenically impacted sampling sites compared downstream of the river. The diversity of resistance genes was highest in MK sampling sites followed by ZE. The lower diversity was observed in the downstream of the rivers (AB). Plasmid-mediated rifampin resistance genes were detected only in *E. coli* isolated from anthropogenic non impacted downstream site. In addition to the enzyme-mediated antibiotics resistance genes, we have determined the distribution of efflux pumps and found that all isolates harbored at least 7 antibiotics efflux pumps.

The β-lactamase genes were the most frequently detected resistance genes, followed by aminoglycosides (Table 3.7). The majority of resistance genes were detected from strains that were isolated from MK and ZE, the two anthropogenically impacted sampling sites. Aminoglycoside resistance genes were persistently detected in *E. coli* isolates regardless of sampling sites. Out of the 27 *E. coli* strains, 22 (81.4%) contained at least one aminoglycoside resistance gene. Two aminoglycoside resistance genes, aminoglycoside 3'-phospho-transferasen (*aph(3'')-Ib*) and streptomycin phosphor-transferase (*aph(6)-Id*), were more frequently detected. The gene, *aph (6)-Id* was correlated with strains that were isolated from MK, whereas *aph(3'')-Ib* and *aac(3)-IIa* were correlated with both MK and ZE sites. Few *E. coli* strains harbored aminoglycoside (3") (9) adenylyl-transferase genes (*aadA1*, *aadA2* and *aadA5*). Isolates from BA and ZE sampling sites carried *aadA5* gene. Although *aadA1* was detected from isolates from MK and BA sites, it was more correlated with isolates from MK sampling site (Figure 3.10).

Table 3 7: Antimicrobial resistance genes detected in *E. coli* isolated from Akaki river

Isolate	Site	AMG[a]	βL[b]	Mac[c]	Qui[d]	Others
ZEC-1	ZE	*aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-35}}$ & *blaTEM_190*	*mdf(A)* & *mph(A)*	*qnrS1*	*sul2*, *tet(A)* & *dfrA14*
ZEC-2	ZE		*ampC1*	*mdf(A)*		
ZEC-3	ZE	*aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{TEM-1B,}}$ *bla*$_{\text{ampC}}$ & bla$_{\text{TEM-1}}$	*mdf(A)*		*sul2*, *tet(A)* & *dfrA8*
ZEC-4	ZE	*aadA5* & *mphA*	*bla*$_{\text{TEM-1B}}$ *and bla*$_{\text{ctx\_-m\_3}}$	*mdf(A)*		*tet(B)*
ZEC-5	ZE	*aadA2*		*mdf(A)*	*qepA4*	*sul1*, *tet(A)* & *dfrA12*
ZEC-6	ZE	*aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{TEM-1B}}$	*mdf(A)*		*sul2*, *tet(B)*& *dfrA17*
ZEC-7	ZE	*aadA5*	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$ & *ampC1*	*mdf(A)* & *mph(A)*	*aac(6')-Ib-cr*	*sul1*, *tet(B)* & *dfrA17*
ZEC-8	ZE			*mdf(A)*		
ZEC-9	ZE	*aac(3)-IIa*, *aadA5*, *aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{TEM-1B}}$	*mdf(A)* & *mph(A)*	*qnrS1*	*sul1*, *sul2 tet(A)* & *dfrA17*
ZEC-10	ZE		*bla*$_{\text{TEM-1B}}$ & ampC1	*mdf(A)*		*tet(A)*, *dfrA5* & *fosA7*
ZEC-11	ZE			*mdf(A)*		
MEC-12	MK	*aac(3)-IIa*, *aph(3'')-Ib*, *aph(3')-Ia* & *aph(6)-Id*	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXY 1-7}}$ & *bla*$_{\text{TEM-1B}}$		*qnrB1* & *aac(6')-Ib-cr*	*sul2*, *tet(A)*,*dfrA14* & *catB3*
MEC-13	MK	*aac(3)-IId*, *aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{OXA-1}}$ *and bla*$_{\text{TEM-1B}}$	*mdf(A)* &*mph(A)*	*aac(6')-Ib-cr*	*sul2*, *tet(B)* & *dfrA17*
MEC-14	MK	*aph(6)-Id*	*ampC1*	*mdf(A)*		
MEC-15	MK		*ampC1*& *bla*$_{\text{TEM-156}}$	*mdf(A)*		
MEC-16	MK	*aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{CTX-M-15}}$ & *bla*$_{\text{TEM-1B}}$	*mdf(A)*	*qnrS1*	*sul2* & *tet(A)*
MEC-17	MK	*aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{CTX-M-15}}$ & *bla*$_{\text{TEM-1B}}$	*mdf(A)*		*sul1*, *sul2*, *tet(A)* & *dfrA7*

MEC-18	MK	*ampH*		*mdf(A)*		
MEC-19	MK	*aadA2, aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{CTX-M-15}}$	*mdf(A)*	*qepA4* & *qnrS1*	*sul1, sul2, tet(A), dfrA12* & *dfrA14*
MEC-20	MK	*aadA1*	*bla*$_{\text{OXA-1}}$ & *bla*$_{\text{TEM-1B}}$	*mdf(A)*		*tet(B)* & *catA1*
MEC-21	MK	*aadA1*	*bla*$_{\text{OXA-1}}$ & *bla*$_{\text{TEM-1B}}$	*mdf(A)* & *mph(A)*		*tet(B)* & *catA1*
MEC-22	MK	*aac(3)-IIa, & aph(6)-Id*	*bla*$_{\text{NDM-1}}$, *bla*$_{\text{TEM-141}}$, *bla*$_{\text{TEM-150}}$, *bla*$_{\text{TEM-1A}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{TEM-206}}$, *bla*$_{\text{TEM-214}}$, *bla*$_{\text{TEM-79}}$ & *ampC1*	*mdf(A)*		*sul1* & *catA1*
BEC-23	BA	*aadA5* & *mphA*	*bla*$_{\text{CTX-M-15}}$ & *bla*$_{\text{TEM-1B}}$	*mdf(A)* & *mph(A)*		*sul1, tet(A)* & *dfrA17*
BEC-24	BA	*aadA5*	*bla*$_{\text{CTX-M-3}}$ & *bla*$_{\text{TEM-1B}}$	*erm(B), mdf(A)* & *mph(A)*		*sul1, tet(B)* & *dfrA17*
BEC-25	BA	*aadA1, aadA2* & *ampH*	*bla*$_{\text{CMY-79}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-169}}$ & *bla*$_{\text{TEM-33}}$	*erm(B), mdf(A)* &*mph(A)*	*qepA4*	*sul1, tet(B)* & *dfrA12*
BEC-26	BA	*ampH*	*bla*$_{\text{TEM-1B}}$	*mdf(A)*		
AEC-27	AB	*aph(3'')-Ib* & *aph(6)-Id*	*bla*$_{\text{OXA-1}}$ & *bla*$_{\text{TEM-1B}}$	*mdf(A)*	*qnrS1* & *aac(6')-Ib-cr*	*sul1, sul2 catB3* & *ARR-3*

[a] Aminoglycoside [b] β-Lactamase [c] Macrolide [d] Fluoroquinolones and aminoglycoside

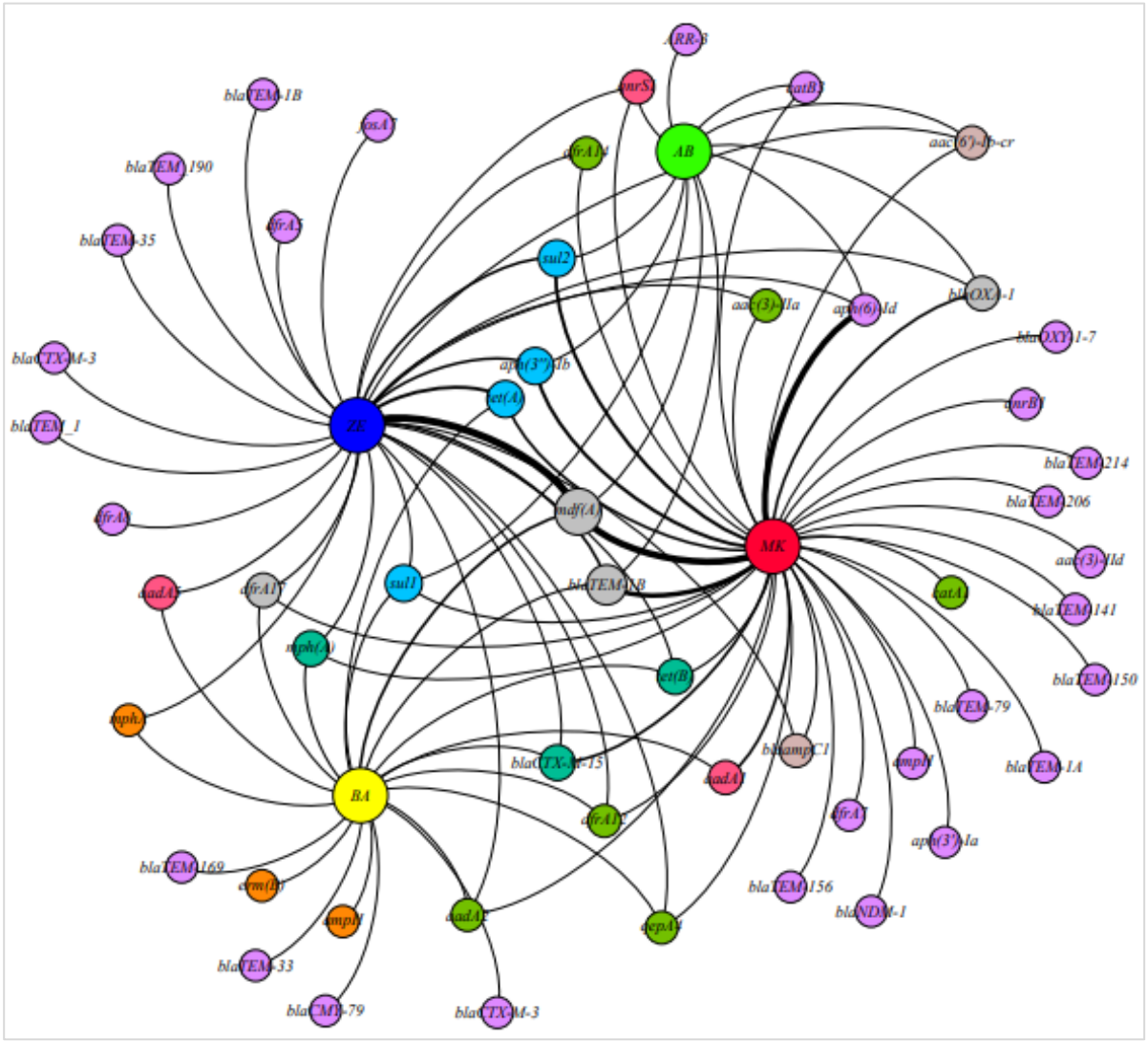

Figure 3.10: Gephi networking analysis revealing occurrence of antibiotic resistance gene subtypes in *E. coli* according to sampling sites. The size of the node is proportional to the number of connections, that is the degree of weight.

The β-lactamase genes were detected in the majority of the sequenced isolates (Table 3.7). The most frequently detected β-lactamase gene was *bla*$_{\text{CTX-M-15}}$, followed by *bla*$_{\text{OXA-1}}$, bla$_{\text{CTX-M-1}}$, and *bla*$_{\textit{TEM-1B}}$ and were significantly correlated with strains isolated from MK site. The diversity of β-lactamase genes showed some spatial variations between sampling sites (Figure 3.10). For instance, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXY-1-7}}$, *bla*$_{\text{TEM-150}}$, and *bla*$_{\text{TEM-1A}}$ were present in *E. coli* strains that were isolated from MK site, while the *bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-35}}$, *bla*$_{\text{TEM-190}}$ and *bla*$_{\text{CTX-M-3}}$ were detected in strains isolated from ZE. Genes for extended-spectrum β-lactamase (ESBL) production were detected in 77.7% (21/27) of *E. coli* strains. TEM-type ESBL was the predominant (19/27), followed by CTX-M type. Of the TEM-type, *bla*$_{\text{TEM-1B}}$ variant was more frequently detected. Considerable diversity of genetic types of CTX-M producers were detected and the most dominant subtype was *bla*$_{\text{CTX-M-15}}$ variant which was detected from 8 *E. coli* strains and their nucleotide sequence was identical (Table 3.8). The nucleotide sequences of the *bla*$_{\text{CTX-M-15}}$ in the present study were identical to those previously reported *bla*$_{\text{CTX-M-15}}$ in *E. coli* isolated from stool and water ((NZ_CM015666.1, MT188708.1, and MT188705.1), *K. pneumoniae* isolated from abdominal drainage (MN688549.1) and *Aeromonas* species isolated from water (MT188707.1). The lower similarity was observed with *bla*$_{\text{CTX-M-15}}$ detected in *K. pneumoniae* that was isolated from veterinary setting (CP039952.1) with 43.35% identity. In addition, the nucleotide sequence of *bla*$_{\text{TEM-1B}}$ genes identified in the current study was identical with previously reported *bla*$_{\text{TEM-1B}}$ genes in clinical and environmental *E. coli* strains.

The majority of *E. coli* isolates contained macrolide (*mdf(A)*), sulphonamide (*sul1*) and tetracycline (*tetA*) resistance gene. One isolate that was identified from the downstream site (AB) carried fosobiocine, *fosA7* gene. The gene confers resistance to chloramphenicol was detected from five isolates, 4 from MK and 1 from AB sites, three of them harbored *catA1* gene and the rest two carried *catB3*. We identified four plasmid-mediated quinolone resistance genetic determinants (*aac(6')-Ib-cr, qnrS1, qepA4* and *qnrB1*) in 10 *E. coli* strains. In addition, mutations in the quinolone resistance determining region (*gyrA, gyrB, parC* and *parE*) evaluated and there were alterations in the gyrA gene sequence in a total of 9 *E. coli* strains (33.3%), and in a majority of them, there were multiple point mutations. Multiple point mutations were detected in the *gyrA* gene at the amino acid 83 sites that confer resistance to

nalidixic acid whereas a single mutation was observed conferring resistance to ciprofloxacin. No *gyrB* mutations were detected in any strain (Figure 3.11). We identified two types of *gyrA* (S83L and D87N), three types of *parC* (S80I, E84V, and S80R), and four types of *parE* (S458T, I529L, S458A, and S458A) mutations. Except for one *E. coli* strain (MKEC-9), all isolates that were resistant to ciprofloxacin and/or nalidixic acid had a mutation in the quinolone resistance determining region. The strain MKEC-9 was phenotypically resistant to both ciprofloxacin and nalidixic acid. Mutations such as on parC: p.E84V and parE:p.I529L were detected in *E. coli* strains that were isolated only from BA sampling sites and parC: P.S80R and parE:p.S458T only in ZE sampling site.

Table 3 8: Percent Identity Matrix performed by Clustal Omega, shows the identity in percentage of the similarity of *bla*$_{\text{CTX-M-15}}$ sequences in *E. coli* isolates from Akaki river and previously reported *bla*$_{\text{CTX-M-15}}$ from selected bacterial strains.

CP039952.1	100.00	43.35	43.35	43.35	43.35	43.35	43.35	43.35	43.35	43.35	43.35	43.35	43.35	43.40
ZEC-1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
MEC-15	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00		
MEC-16	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00			
MEC-17	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00				
MEC-19	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00					
BEC-23	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00						
BEC-25	100.00	100.00	100.00	100.00	100.00	100.00	100.00							
NZ_CM015666.1	100.00	100.00	100.00	100.00	100.00	100.00								
MT188708.1	100.00	100.00	100.00	100.00	100.00									
MT188705.1	100.00	100.00	100.00	100.00										
MN688549.1	100.00	100.00	100.00											
MT188707.1	100.00	100.00												
ZEC-7	100.00													

- The red font color indicates isolates in the current study whereas the others are generated from Gene Bank database

The plasmid profile of all 27 *E. coli* isolates was determined using Plasmid Finder (Center for Genomic Epidemiology). The result showed that a single or combination of plasmids was present in most isolates and the distribution was highly heterogeneous. The most prominent were replicons of the *IncF* family (65%), followed by *Col* (10%) and *IncX3* (7.5%). Other

groups of plasmids were also detected in *E. coli* isolates (Table 3.6). Majority of the isolates harbored *IncFIB* (AP001918) and *IncFII* plasmids that can carry and transfer multidrug resistance and virulence functions. Bacteriocins-associated plasmid was detected from seven isolates, and *Col156* was predominant. Five isolates also harbored Phage-like plasmid, *IncY*.

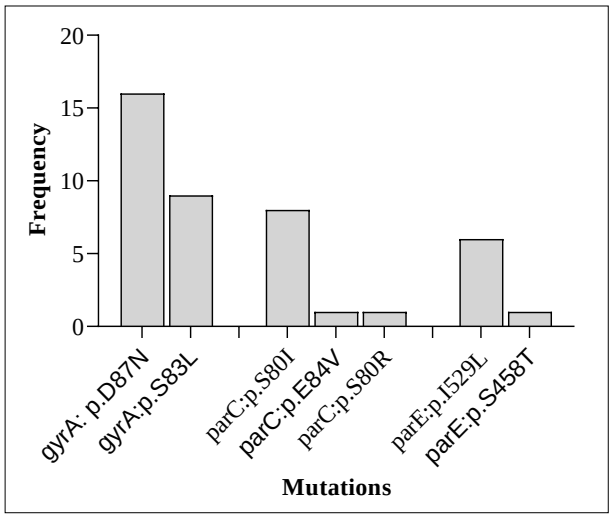

Figure 3.11: Frequency of mutations in quinolone resistance determining regions of *E. coli* isolates from Akaki river. Mutations were detected using the genomic epidemiology databases.

The virulence gene profiles were also assessed in 27 *E. coli* isolates whose genomes were sequenced. The majority of the isolates harbored virulence genes associated with adherence, toxins, protease, evasion, secretion systems and other subgroups (Table 3.9). Toxin-associated virulence genes were detected in 11 of the 27 *E. coli* isolates. The toxin genes identified include the heat-resistant agglutinin (*hra*) from 3 isolates, plasmid-encoded enterotoxin (*senB*) from 2 isolates, secreted autotransporter toxin (*sat*) from two isolates, hemolysin F (*hlyF*) from two isolates, and enterotoxigenic *E. coli* (ETEC) autotransporter A (*eatA*) from one isolate. The predominant evasion-associated gene was increased serum survival (*iss*), which was detected in 14 *E. coli* isolates. The virulence gene, *Iha* an adhesin in enterohemorrhagic *E. coli* O157:H7 was detected in three *E. coli* strains that were isolated from ZE and one from BA site. In most cases, isolates from ZE site contained more virulence determinants when compared to isolates from other sites.

Table 3 9:Distribution of the virulence factors among the *E. coli* isolates in the Akaki river.

Isolates	Virulence genes						
	Adherence	**Toxin**	**Protease**	**Evasion**	**Secretion system**	**Iron uptake**	**Others**
ZEC-1	fimA,yagW/ecpD, csgF,fimD, csgB & fimF			*Iss* and *gad*			*terC*
ZEC-2	*Iha, irp2* and yagX/ecpC	*sat,cea,cba, pic, mchB* & *mchCmchF*	sat	*Iss* and *traT*	*capU* and *aaiC, fliP, gspI* and *gspF*	*sitA, fyuA* and *iutA*	*terC*
ZEC-3	*afaD, irp2,* ydiV, fimH, fimD and csgE		*ompT* and *ompA*		*ppdD* and *flgH*	*fyuA*	*terC*
ZEC-4	*iha,afaD, papC iucC air* fimA fimE and yagZ/ecpA	*AstA* and *veatA*	*ompT*	*Iss, traT, gad, kpsE, kpsMIII_K96, lpfA dsbA* and *traT*	*ppdD* and *spaS*	*chuA, sitA* and *iutA*	*eilA* and *terC*
ZEC-5	*irp2 lpfA,* fimH and yagZ/ecpA			*gad* and ruvB		*fyuA,*	*terC*
ZEC-6	*iha, iucC irp2 Air lpfA* yagZ and fimB	*senB* and gtrB	*ompT*	*iss traT kpsE, gad* and *kpsMIII_K96*		*chuA sitA, fyuA, iutA* and *sitA*	*eilA* and *terC*
ZEC-7	*irp2 Air,* csgF			*traT kpsE, kpsE, kpsMII_K5, gad* and traJ		*chuA* and *fyuA*	*eilA and terC*
ZEC-8	*LpfA,* ydiV ppdD csgG and fimG			dsbA	invC	*etsC*	*terC*
ZEC-9	*papA_feiA_F8, lpfA, papC irp2* and *Air,*	*senB*	*ompT*	*iss traT kpsE vgad*		*chuA, sitA* and *fyuA*	*eilA and terC*
ZEC-10	*papC iroN* and *lpfA*	*hlyF,* and *mchF*	*ompT*	*iss traT gad Cia* and *cvaC*		*sitA* and *etsC*	*terC*
ZEC-11	ydiV and *lpfA*	*ompT*		*iss Gad*	spaS	*sitA*	*terC*
MEC-12				*traT*		*fyuA*	
MEC-13				*Iss* and *Gad*		*sitA*	*terC*
MEC-14				*Iss*			*terC*
MEC-15				*iss*	*capU*		*terC*

MEC-16	*lpfA*			*iss* and *gad*	*capU,*	*sitA*	*terC*
MEC-17	*focG sfaE sfaD, sfaS focC ibeA iroN* and *irp2*	*vat hlyF, cnf1, pic cma cvaC* and *hra*	*ompT*	*Iss* and *kpsE, kpsMII_K1, mchF gad tcpC* and *usp,*		*chuA, sitA, fyuA* and *yfcV*	*ClbB, neuC and terC*
MEC-18				*iss* and *gad,*			*terC*
MEC-19	*LpfA* and *Air*			*kpsE* and *gad,*		*chuA,*	*eilA and terC*
MEC-20		*cia*		*gad*	*capU*	*sitA*	*terC*
MEC-21				*gad*	*capU*	*sitA,*	*terC*
MEC-22	LpfA	hra		gad and *traT*			terC
BEC-23	*iha, papA_F43 iucC* & *irp2*	*sat, hra,* and *cia*	*ompT*	*iss, traT kpsE, kpsMII_K5, gad* & *usp*		*chuA,sitA,fyuA iutA, yfcV*	*terC*
BEC-24	*iucC,*			*traT*		*sitA, iutA,*	*terC*
BEC-25	*irp2* and *lpfA)*			*traT* and *gad,*		*fyuA*	*terC*
BEC-26				*gad*			*terC*
AEC-27				*gad*			*terC*

3.3 Functional and molecular features of *Aeromonas* isolates in Akaki river, Ethiopia

In the present study, antibiotic resistance levels, resistance mechanism and virulence genes associated with diarrhea were investigated in *Aeromonas* species isolated from the Akaki river. A total of 144 *Aeromonas* isolates (*A. caviae* (62), *A. hydrophila* (33) and *A. veronii* (49)) were recovered from the five sampling sites in the current study. The predominant species isolated in all sites was *A. caviae*. They were tested for their resistance patterns against the antibiotics listed in Annex III and reported in Figure 3.12. These antibiotics were chosen to represent most classes of antimicrobials, as well as known activity against wildtype *Aeromonas* as previously reported (Igbinosa and Okoh, 2012). The highest rate of resistance was against doxycycline (134, 93%), amoxicillin (144, 100%), sulfamethoxazole/ trimethoprim (98, 68%%), ampicillin (142, 99%), amoxicillin/clavulanate (117, 81%), tetracycline (115, 80%), ertapenem (132, 92%) and imipenem (75, 52%). Considerable numbers of strains were resistant to cephalosporins, with a prevalence to third-generation cephalosporin, ceftazidime at 30%.

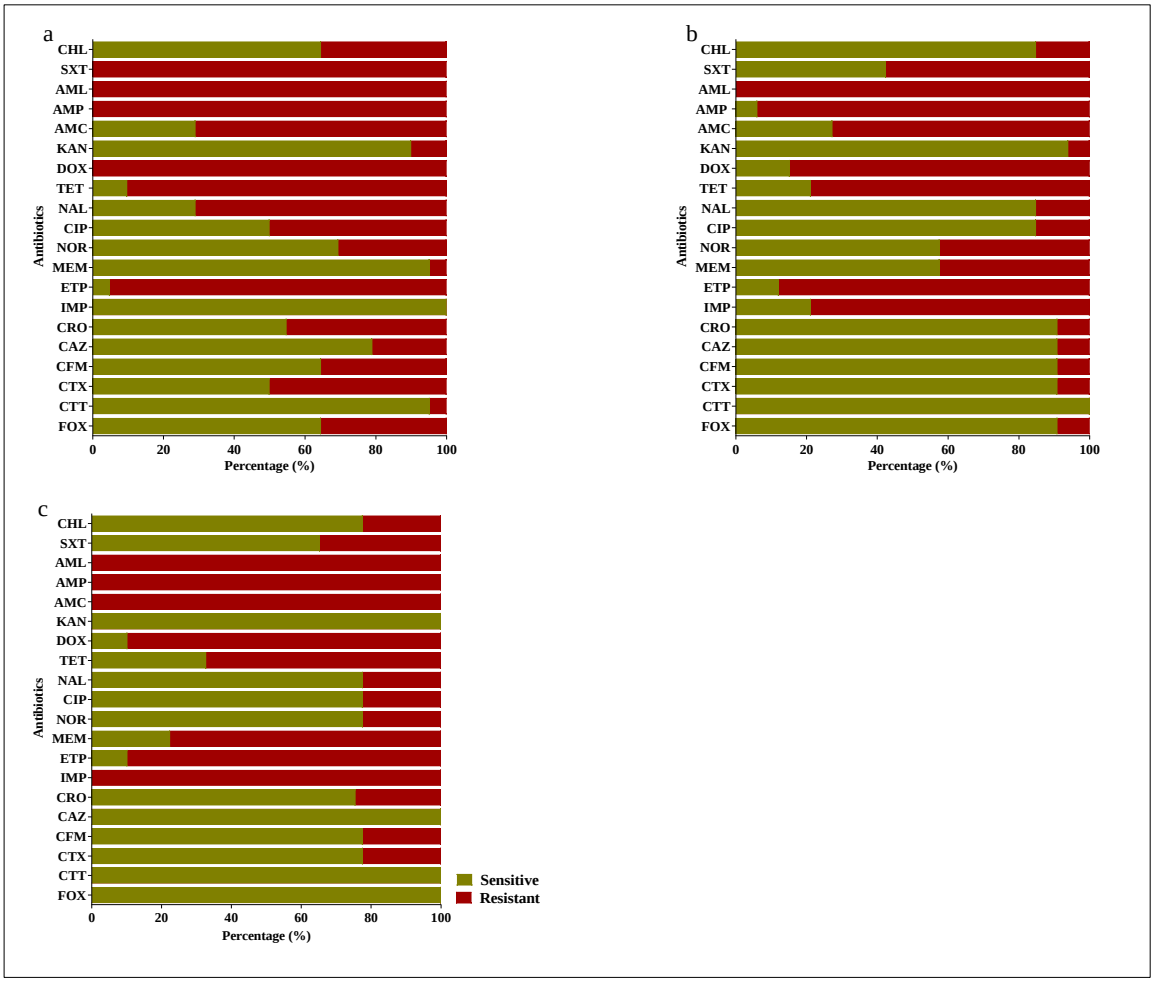

Figure 3.12: Proportion of antibiogram patterns of *A. cavae* (a), *A. hydrophilla* (b) and *A. veronii* (c). Result interpretation was carried out using the European Committee on Antimicrobial Susceptibility Testing (EUCAST-2019) standard. All strains showing ‘‘resistant’’ or ‘‘intermediate’’ behavior was subsumed under the category ‘‘resistant’’ and the others were classified as ‘Sensitive’. Abbreviations: TET, tetracycline; DOX, doxycycline; KAN, kanamycin; FOX, cefoxitin; CTT, cefotetan; CTX, cefotaxime; CFM, cefixime; CAZ, ceftazidime; CRO, ceftriaxone; AMP, ampicillin; AML, amoxicillin; AMC, amoxicillin-clavulanate; NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic Acid; IMP, imipenem; ETP, ertapenem; MEM, meropenem; SXT, sulfamethoxazole / trimethoprim and CHL, chloramphenicol.

Different resistance patterns were observed among the *Aeromonas* species to the antibiotics studied. Most of *A. caviae* isolates were resistant to penicillin with the highest resistance to ampicillin (62,100%) and amoxicillin (62,100%). The percentage of resistance to amoxicillin-clavulanate was 71%. Almost all *A. caviae* strains found to be resistant to ertapenem (59, 95%) (Figure 3.12a). None of the *A. caviae* strains were resistant to imipenem and few (3, 5%) were resistance to meropenem. Among the cephalosporins antibiotics studied, resistance to ceftriaxone (28, 45%) and cefotaxime (31, 50%), cefotaxime (22, 35.5%) and cefoxitin (22, 35.5%) were higher. The majority of *A. caviae* isolates were susceptible to cefotetan, chloramphenicol and kanamycin and half of the isolates were resistant to ciprofloxacin. Similarly, antibiotic resistance of *A. hydrophila* isolates recovered from the Akaki river is shown in Figure 3.12b. The majority of *A. hydrophila* isolates were resistant to penicillin such as amoxicillin (33, 100%), ampicillin (31, 94%), and the combination drug amoxicillin-clavulanate (24,72.7%). Resistance to tetracycline antibiotics such as doxycycline and tetracycline was found to be 84.8% and 78.8%, respectively. Carbapenem antibiotics resistance was higher in *A. hydrophila* than *A. caviae*. The prevalence of resistance to ertapenem, imipenem, and meropenem was 87.9%, 78.8% and 42.4% respectively. The majority *A. hydrophila* isolates were susceptible to antibiotics belonging to cephalosporines, macrolides and aminoglycosides. Most *A. veronii* isolates were susceptible to aminoglycosides, cephalosporines and macrolides (Figure 3.12c). However, all *A. veronii* isolates (49, 100%) were resistant to penicillin (amoxicillin, amoxicillin-clavulanate, and ampicillin). The majority of *A. veronii* isolates were resistant to tetracyclines, tetracycline (33, 67.3%), and doxycycline (44, 89.8%). The resistance prevalence of *A. veronii* to ceftriaxone, cefixime, cefotaxime was 24.5%, 22.2%, and 22.2 % respectively. All *A. veronii* strains were susceptible to ceftazidime, cefotetan and cefoxitin. The *Aeromonas* strains that were phenotypically resistant to carbapenem antibiotics were tested for carbapenemase production using CarbaNP hydrolysis assay. The majority of *A. hydrophila* (84.6%) and all *A. veronii* isolates (100%) that were resistant to carbapenem were carbapenemase producers. Few (9) *A. caviae* were tested for carbapenemase production and they were carbapenemase non-producer.

Based on the phenotypic antibiotic susceptibility results, 21 *Aeromonas* species were randomly selected from each site and subjected to whole-genome sequencing and bioinformatics. The draft genomes ranged in size from 4.4 Mbp to 5.0 Mbp (mean 4.7 Mbp). Mean GC content and coding sequence (CDS) were 60.0% and 4,570, respectively (Table 3.10). Sequence type was determined by *Aeromonas* PubMLST database using six alleles (*gltA*, *groL*, *gyrB*, *metG*, *ppsA*, and *recA*). It was found that the majority of alleles did not match any of the existing alleles in the *Aeromonas* database and therefore except for two isolates (ST-588 and ST-61), the *Aeromonas* isolates in the current study were new sequence types (Table 3.11).

To explore the mechanism of antibiotic resistance, the resistance genes were identified by CARD and ResFinder 4.1 (Center for Genomic Epidemiology). *Aeromonas* isolates in the current study contained diverse antibiotic resistance determinants, with a majority of the genes belonging to β-lactam and aminoglycoside resistance (Table 3.12). All classes of β-lactamase genes were detected in the current study with the highest prevalence of class A β-lactamases. Among the total isolates, 14 had at least one class A β-lactamase gene. In the correlation matrix, the β -lactam resistant genes were significantly correlated with *A. veronii* followed by *A. hydrophila* (Figure 3.13). There were very few β-lactamase genes detected in *A. caviae* compared to the other two species. However, surprisingly one *A. caviae* isolates (BAESBLEC_3) harbored 9 variants of bla_{TEM} ($bla_{\text{TEM-141}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{TEM-206}}$, $bla_{\text{TEM-209}}$, $bla_{\text{TEM-210}}$, $bla_{\text{TEM-214}}$, $bla_{\text{TEM-216}}$, $bla_{\text{TEM-33}}$ and $bla_{\text{TEM-34}}$). Extended-spectrum β-lactamase genes (ESBL) were frequently detected in the current study, and $bla_{\text{TEM-1B}}$ was the most common. Four class C β-lactamase genes were detected of which $bla_{\text{MOX-2}}$ was predominantly identified, followed by $bla_{\text{MOX-6}}$ and $bla_{\text{FOX-2}}$. Only two class D β-lactam resistance genes ($bla_{\text{OXA-1}}$ and $bla_{\text{OX-50}}$) were identified from four *A. veronii* and one *A. caviae* isolates. Seven *Aeromonas* isolates carried two chromosomally mediated class B β-lactam resistance genes.

Table 3 10:General genome features of *Aeromonas* isolates from Akaki river. The variables are determined by PATRIC bioinformatics tool.

Isolates	Contigs	GC (%)	Ge. Size (Mbp)	CDS	tRNA	rRNA
BAESBLEC_3	113	61.52	4.4	4,238	83	6
MKESBLEC_2	146	58.65	4.7	4,548	76	4
MKESBLEC_3	143	58.65	4.7	4,571	68	5
MKESBLEC_5	147	58.65	4.8	4,555	82	5
MKESBLEC_7	241	58.13	5,0	4,958	74	5
MKESBLKP_2	270	61.37	4.5	4,397	76	4
MKESBLKP_3	212	61.55	4.4	4,215	73	5
MKESBLKP_5	144	58.65	4.7	4,550	74	4
MKESBLKP_6	288	60.97	4.7	4,631	80	6
MKESBLKP_7	370	60.36	5.0	5,008	76	6
MKESBLKP_8	354	61.11	4.6	4,575	82	7
S5PS_3	38	61.47	4.7	4,479	84	4
S5VC_1	54	61.27	4.8	4,570	84	5
SGSH_1	88	58.29	4.9	4,736	83	5
SL1PS_3	83	58.37	4.8	4,664	78	5
SL1PS_5	37	61.49	4.7	4,488	85	4
SL1PS_6	30	61.67	4.7	4,320	77	5
SL1S_2	87	58.37	4.9	4,668	78	5
SL1VC_1*	78	64.46	10.9	10,247	145	7
SL1VC_2	81	61.43	4.8	4,506	85	4
SL1VC_3	95	58.29	4.9	4,731	78	5

*The genome size is extremely high and looks contaminated and therefore, excluded for consecutive analysis.

Table 3.11:Sequence types of *Aeromonas* isolates in Akaki river

Strain ID	**Isolates**	**Allel**						**ST**
		gltA	**groL**	**gyrB**	**metG**	**ppsA**	**recA**	
BAESBLEC_3	*A. caviae*	96	**162**	**160**	159	**163**	**158**	NS
MKESBLKP_2	*A. caviae*	96	179	**165**	198	174	207	NS
MKESBLEC_7	*A. caviae*	**167**	**461**	**54**	**65**	**173**	168	NS
MKESBLKP_3	*A. caviae*	**96**	179	**199**	**90**	163	94	NS
MKESBLKP_5	*A. caviae*	**352**	**461**	**453**	**490**	**60**	**368**	NS
MKESBLKP_8	*A. caviae*	96	233	93	96	96	158	588
MKESBLEC_2	*A. hydrophila*	**352**	**461**	**228**	**53**	**60**	**368**	NS
MKESBLKP_6	*A. hydrophila*	377	**22**4	**194**	445	174	207	NS
S5PS_3	*A. hydrophila*	**72**	**473**	**434**	**300**	**443**	**1**	NS
S5VC_1	*A. hydrophila*	**383**	**341**	**276**	**445**	**300**	**471**	NS
SL1PS5	*A. hydrophila*	**479**	**1**	**69**	**211**	**73**	271	NS
SL1PS_6	*A. hydrophila*	**482**	**29**	**373**	**478**	**380**	**453**	NS
SL1VC_2	*A. hydrophila*	**410**	**266**	**470**	**301**	**361**	122	NS
SL1VC_1	*A. hydrophila*	**482**	**29**	**373**	**478**	**380**	**453**	NS
MKESBLEC_3	*A. veronii*	352	461	453	490	60	368	61
MKESBLEC_5	*A. veronii*	**352**	**461**	**453**	**53**	**60**	**368**	NS
MKESBLKP_7	*A. veronii*	**348**	12	**163**	235	96	173	NS
SGSH_1	*A. veronii*	**226**	**125**	**126**	**143**	**525**	**351**	NS
SL1PS_3	*A. veronii*	**226**	**125**	**232**	**143**	**525**	**87**	NS
SL1S_2	*A. veronii*	**226**	**125**	**281**	**112**	**525**	**351**	NS
SL1VC_3	*A. veronii*	**226**	**125**	**281**	**112**	**525**	**87**	NS

•**Bold** values indicate alleles that did not match any of the existing alleles in the Aeromonas database (Aeromonas PubMLST). Nearest allele numbers are used. NS: New Sequence

The diversity of β-lactam resistance genes detected showed spatial variation between sampling sites. Isolates that were isolated from GE, upstream and AB, downstream of the river where there were no human activities contained fewer β-lactamase genes that were chromosomally encoded, bla_{ampH}, $bla_{\text{imi,}}$ and bla_{cph} genes. None of *Aeromonas* species that were isolated from these two sites harbored other classes of resistance determinants. Chloramphenicol acetyltransferase genes (*catB3* or *catB8*) were detected from strains that were isolated from anthropogenically impacted sampling sites (MK, BA, and ZE). The aminoglycoside resistance genes such as phosphotransferase encoded by *aph(6)-Ib*, *aph(6)-Id* and acetyltransferase encoded *aac(3)-IId* were detected in aminoglycoside-resistant isolates. Colistin resistance genes were detected in 4 *A. veronii*, 2 *A. hydrophila* and 1 *A. caviae* isolates and the variants were *mcr-3.17*, *mcr-3.7* and *mcr-3.25*. The nucleotide sequence of *mcr-3.7* and *mcr-3.25* genes had higher similarity with previously reported colistin resistance genes (NG_055661.1_A, NG_057484 and MG491669.1). Although *mcr-3.17* genes detected in the current study had 100% sequence identity, they had low identity with the nucleotide sequence of previously reported colistin resistance genes (Figure 3.14).

Table 3 12:Acquired antimicrobial resistance genes among *Aeromonas* isolates.

Strains ID	Strain	Amg[a]	βL[b]	Col[c]	Qui[d]	Others
BAESBLEC_3	*A. caviae*	*aph(3'')-Ib* and *aph(6)-Id*	*bla*CTX-M-15, *bla*MOX-6, *bla*TEM-141, *bla*TEM-1B, *bla*TEM-206, *bla*TEM-209, *bla*TEM-210, *bla*TEM-214, *bla*TEM-216, *bla*TEM-33 and *bla*TEM-34			*catA1*, *dfrA5 ere(A)*, *mph(A)* and *sul1*
MKESBLEC_2	*A. hydrophila*	*aac(3)-IId*, *aph(3'')-Ib* and *aph(6)-Id*	*bla*ampS, *bla*CEPH-A3, *bla*OXA-1, *bla*SFO-1 and *bla*TEM-1B	*mcr-3.17* and *mcr-3.7*	*aac(6')-Ib-cr*	*catB3*, *ARR-3*, *tet(C)*, *dfrB8* and *sul1*
MKESBLEC_3	*A. veronii*	*aac(3)-IId*, *aph(3'')-Ib*, and *aph(6)-Id*	*bla*ampS, *bla*CEPH-A3, *bla*OXA-1, *bla*SFO-1 and *bla*TEM-1B	*mcr-3.17* and *mcr-3.7*	*aac(6')-Ib-cr*	*catB3*, *ARR-3*, *tet(C)*, *dfrB8*, *mph(A)* and *sul1*
MKESBLEC_5	*A. veronii*	*aac(3)-IId*, *aph(3'')-Ib*, and *aph(6)-Id*	*bla*ampS, *bla*CEPH-A3, *bla*OXA-1, *bla*SFO-1 and *bla*TEM-1B	*mcr-3.17* and *mcr-3.7*	*aac(6')-Ib-cr*	*catB3*, *ARR-3*, *tet(C)*, *dfrB8*, *mph(A)* and *sul1*
MKESBLEC_7	*A. veronii*	*aadA1*, *aadA16*, *aph(3'')-Ib* and *aph(6)-Id*	*bla*ampS, *bla*CTX-M-3, *bla*OXA-1, *bla*TEM-1B and *bla*cphA4		*aac(6')-Ib-cr*	*catB3*, *catB8*, *ARR-3 tet(C) dfrA27mph(A)* and *sul1*
MKESBLK P_2	*A. caviae*	*aac(6')-Ib-Hangzhou* and *aadA16*	*bla*MOX-2 and *bla*PER-3		*aac(6')-Ib-cr* and *qnrVC6*	*catB3*, *ARR-3 tet(A)*, *dfrA27 mph(A)* and *sul1*
MKESBLKP_3	*A. caviae*	*aph(3'')-Ib* and *aph(6)-Id*	*bla*MOX-6			*dfrA1* and *sul1*
MKESBLKP_5	*A. caviae*	*aac(3)-IId*, *aph(3'')-Ib*, and *aph(6)-Id*	*bla*OXA-1, *bla*SFO-1 and *bla*TEM-1B	*mcr-3.17* and *mcr-3.7*	*aac(6')-Ib-cr*	*catB3*, *ARR-3*, *tet(C)*, *mph(A)*, *dfrB8* and *sul1*
MKESBLKP_6	*A. hydrophila*	*aac(6')-Ib3*, *aadA1* and *ant(2'')-Ia*	*bla*MOX-2 and *bla*VEB-1	*mcr-3.25* and *mcr-3.7*	*aac(6')-Ib-cr* and *qnrVC4*	*cmlA1*, *catB8*, *tet(C)*, *dfrA14*, *dfrA15* and *sul1*
MKESBLKP_7	*A. caviae*	*aac(6')-Ib-Hangzhou*, *aadA16*, *aph(3'')-Ib* and *aph(6)-Id*	*bla*MOX-2 and *bla*PER-3		*aac(6')-Ib-cr*	*catB3*, *mph(A)* and *sul1*
MKESBLKP_8	*A. caviae*	*aph(3'')-Ib* and *aph(6)-Id*	*bla*MOX-2			*tet(A)* and *sul2*
SGSH_1	*A. veronii*	*aph(3')-IIb*	*bla*ampH, *bla*OXA-50, *bla*PAO and *bla*cphA1			*catB7* and *fosA*
S5PS_3	*A. hydrophila*		*blaampH* and *bla*imiH			
S5VC_1	*A. hydrophila*		*bla*ampH and *bla*cphA4			
SL1VC_1	*A. veronii*		*bla*ampS, *bla*cphA4 and *bla*cphA6			
SL1PS_3	*A. veronii*		*bla*ampS, *bla*cphA4 and *bla*cphA6			
SL1PS5	*A. hydrophila*		*bla*ampH and *bla*cphA2			
SL1PS_6	*A. hydrophila*		*bla*ampH and *bla*cphA1			
SL1S_2	*A. veronii*		*bla*ampS, *bla*cphA4 and *bla*cphA6			
SL1VC-2	*A. hydrophila*		*bla*ampH, *bla*cphA2 and *bla*cphA7			
SL1VC_3	*A. veronii*		*bla*ampS, *bla*cphA4 and *bla*cphA6			

[a] aminoglycoside [b] β-Lactamase [c] colistin [d] fluoroquinolones *and* aminoglycoside

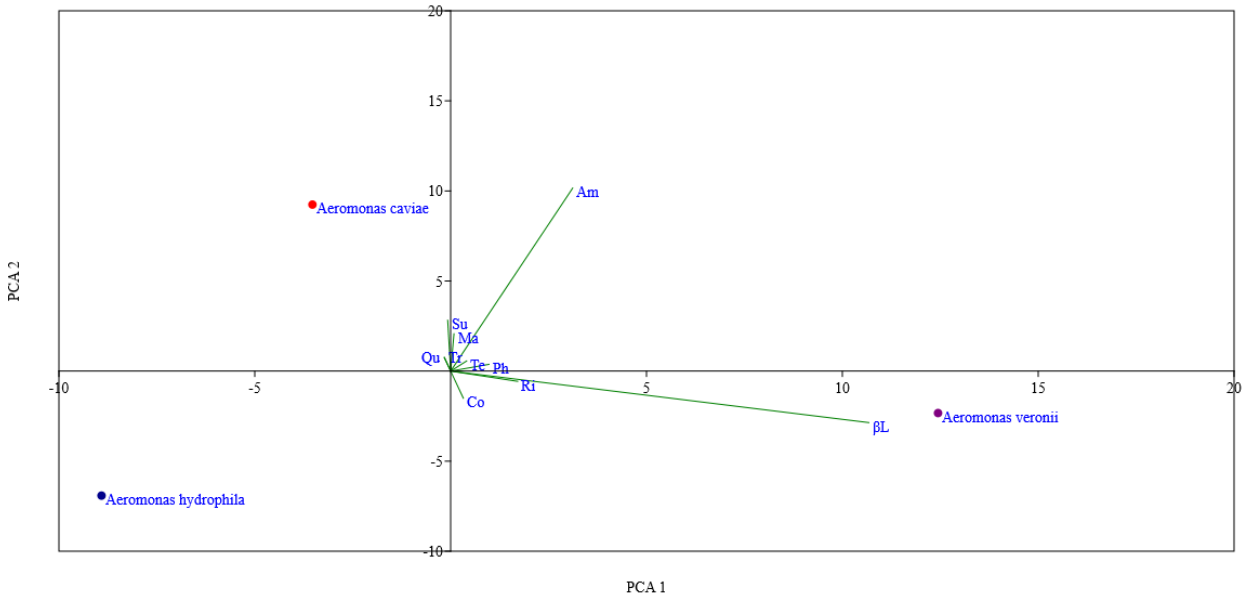

Figure 3.13: Correlation matrix biplots displaying correlation of antibiotic resistance genes detected and *Aeromonas* species based on the Bray–Curtis distance analysis. Abbreviations: Am, aminoglycosides; Qu, quinolone; Su, sulfonamides; Co, colistin; Ma, macrolide; Te, tetracycline; Tr, trimethoprim; Ph, phenicol; Ri, rifampicin and βl, β-lactam

Virulence Genes

All *Aeromonas* isolates were screened for virulence genes, in particular the toxins cytotoxic enterotoxin (*act*), heat-labile cytotonic enterotoxin (*alt*), heat-stable cytotoxic enterotoxin (*ast*), hemolysin (*hlyA*), and shiga-toxins (*stx-1* and *stx-2*). Except for Shiga toxin encoding genes, all tested virulence genes were detected in the majority of *Aeromonas* strains with some variation among the different species (Table 3.13). The prevalence of *alt* gene among *Aeromonas* species were 100%, 85.7% and 59.6% for *A. hydrophila*, *A. Veronii* and *A. caviae*, respectively. None of the *A. caviae* strains carried *act* gene.

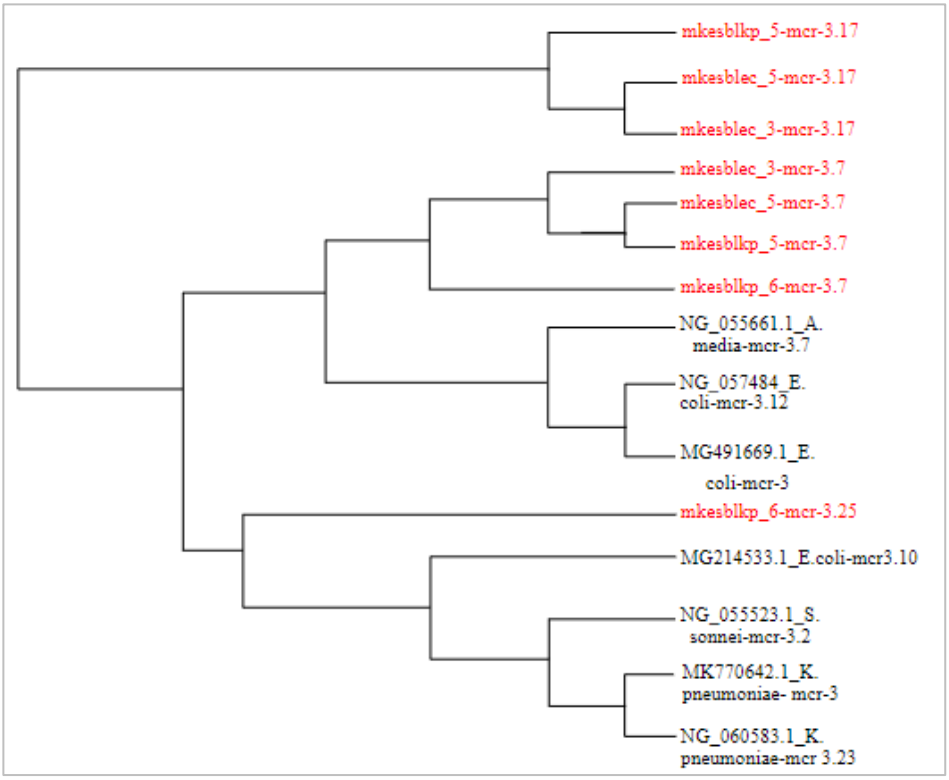

Figure 3.14: Cladogram showing phylogenetic comparison of colistin resistance, mcr genes in *Aeromonas* isolates to previously reported homologues. The Neighbor-joining tree was constructed using EMBOSS Clustal Omega with the DNA sequence of colistin resistance gene mcr in *Aeromonas* in the current study (red) and previously described *mcr* genes (black).

Table 3 13: Distribution of virulence genes among Aeromonas species in Akaki river

Species	Virulence genes					
	alt N (%)	*stx-1* N (%)	*stx-2* N (%)	*HlyA* N (%)	*ast* N (%)	*act* N (%)
A. hydrophila (n=33)	33 (100)	0 (0)	0 (0)	28 (84.8)	28 (84.8)	26 (78.8)
A. Veronii (n=49)	42 (85.7)	0 (0)	0 (0)	31 (63.2)	36 (73.4)	21 (42.8)
A. caviae (n=62)	37 (59.6)	0 (0)	0 (0)	31 (50)	45 (72.6)	0 (0)

3.4 Functional and Molecular features of *Pseudomonas aeruginosa* isolates in Akaki river, Ethiopia

The distribution and antimicrobial susceptibility levels of *P. aeruginosa* strains isolated from Akaki river were determined. A total of 21 *P. aeruginosa* strains were isolated from three sampling sites (MK, BA, and ZE) of the Akaki river. No *P. aeruginosa* were detected from GE upstream and AB downstream in the river. All strains were tested for their functional resistance patterns against 24 antibiotics belonging to different classes using disc diffusion. The antibiotic-resistant profiles are presented in Figure 3.15. The highest resistance was observed towards amoxicillin-clavulanate (21, 100%), kanamycin (21, 100%), cefotaxime (20, 95%), cefixime (90%), and erythromycin (100%). The majority of isolates were susceptible to carbapenem antibiotics, however, only two strains were resistant to ertapenem, while none were resistant to meropenem and imipenem. Significant numbers of *P. aeruginosa* isolates were resistant to cephalosporins. The highest prevalence of resistance was to cefoxitin (20, 95%), cefotetan (18, 85%), cefotaxime (20, 95%), cefixime (19, 90%), ceftriaxone (13, 60%), ceftazidime (20, 95%) and cefepime (17, 83%). The majority of *P. aeruginosa* strains were found to be resistant to penicillin antibiotics with the highest resistance to amoxicillin-clavulanate (21, 100%). Fluoroquinolone resistance was low among *P. aeruginosa* isolates except for nalidixic acid of which 95% were resistant. All isolates were resistant to tested tetracycline and aminoglycoside antibiotics.

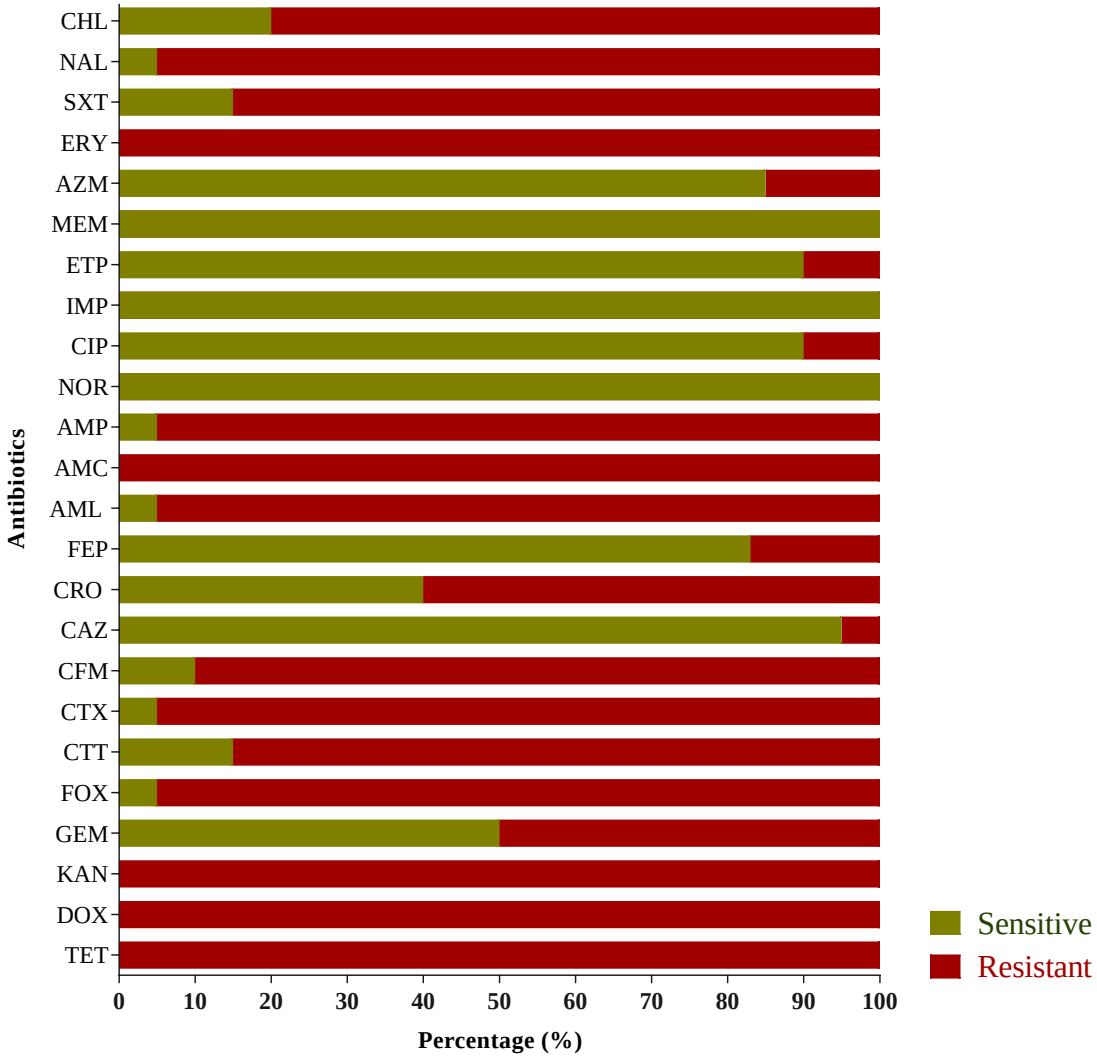

Figure 3.15: Phenotypic antimicrobial resistance of *P. aeruginosa* isolates derived from Akaki river. Result interpretation was carried out using the European Committee on Antimicrobial Susceptibility Testing (EUCAST-2019) standard. All strains showing ‘‘resistant’’ or ‘‘intermediate’’ behavior was subsumed under the category ‘‘resistant’’ and the others were classified as ‘sensitive’. Abbreviations: TET, tetracycline; DOX, doxycycline; KAN, kanamycin; GEM, gentamicin; FOX, cefoxitin; CTT, cefotetan; CTX, cefotaxime; CFM, cefixime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AML, amoxicillin; AMC, amoxicillin-clavulanate; AMP, ampicillin; NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; IMP, imipenem; ETP, ertapenem; MEM, meropenem; AZM, azithromycin; ERY, erythromycin; SXT, sulfamethoxazole / trimethoprim and CHL, chloramphenicol.

To determine the mechanism of resistance, distribution of high-risk clonal types, and serotypes of *P. aeruginosa*, 18 isolates were selected based on their antibiotic resistance patterns and subjected to whole-genome sequencing. The draft genomes ranged in size from 6.2 Mbp to 6.5 Mpb (mean 6.3 Mpb), the number of contigs ranged from 23 to 76 and the mean GC content was found to be 66.4% (Table 3.14). The 18 *P. aeruginosa* isolates belonged to 8 different sequence types (ST). The most abundant sequence types observed in the current study were ST-639 (7, 39%), followed by ST-558 (3, 16.6%) and ST-274 (3, 16.6%). The serotype of isolates was determined by the center for genomic epidemiology found that O4 (n=6), O3 (n=5), O6 (n=4), O1(n=2), and O9 (n=1).

Table 3 14:General genome features of P. aeruginosa isolates in Akaki river

Isolates	**Contigs**	**GC (%)**	**Genome Size (Mbp)**	**CDS**	**tRNA**	**rRNA**
BAP_2	45	66.38	6.46	6,127	59	3
BAP_3	46	66.39	6.46	6,127	60	3
BAP_4	58	66.30	6.48	6,131	59	3
BAP_5	56	66.30	6.48	6,124	58	3
BAP_6	67	66.29	6.48	6,143	57	3
BAP_7	47	66.38	6.47	6,123	58	3
BAP_8	52	66.30	6.48	6,126	58	3
BAP_13	76	66.29	6.46	6,145	59	3
MKESBL_4	23	66.50	6.26	5,886	59	3
MKP_1	58	66.41	6.37	5,999	60	3
MKP_2	54	66.52	6.27	5,875	60	3
MKP_3	70	66.26	6.39	6,071	60	3
MKP_4	34	66.59	6.22	5,835	59	3
MKP_6	43	66.40	6.38	5,989	61	3
MKP_5	44	66.49	6.31	5,932	57	3
MKP_7	44	66.41	6.38	5,988	59	3
ZP_1	41	66.48	6.31	5,923	57	3
ZP_5	44	66.49	6.31	5,932	57	3

The antibiotic resistance genes in the *P. aeruginosa* strains were determined by ResFinder and grouped into five classes (Table 3.15). The aminoglycoside-3'-phosphotransferase gene, *aph(3')-IIb* was detected in all *P. aeruginosa* isolates. Only one fluoroquinolone resistance gene, novel ciprofloxacin-modifying enzyme (*crpP*) which confers resistance to ciprofloxacin was detected in 15 *P. aeruginosa* isolates. A diverse group of β-lactamase genes was detected, and the most frequently identified resistance gene was bla_{PAO}, followed by $bla_{\text{OXA-486}}$. Few isolates carried $bla_{\text{OXA-50}}$ and $bla_{\text{OXA-396}}$. Four different subtypes of pseudomonas-derived cephalosporinase bla_{PDC} genes which confer resistance to carbapenem agents were detected in *P. aeruginosa*. The gene, $bla_{\text{PDC-3}}$ was the predominant subtype detected in the current study, followed by $bla_{\text{PDC-10}}$ (n=4). A fosfomycin resistance gene (*fosA*) and a chloramphenicol resistance gene (*catB7*) were common to all *P. aeruginosa* isolates.

Efflux pump genes conferring resistance to clinically relevant antibiotics were uniformly present in *P. aeruginosa* isolates. The efflux genes detected in the study are generally categorized into five efflux system superfamilies: resistance-nodulation-cell division family (RND), multi-antimicrobial extrusion protein family (MATE), ATP-binding cassette superfamily (ABC), small multidrug resistance family (SMR) and major facilitator superfamily (MFS). The majority of the efflux genes detected were from the RND (Figure 3.16). The distribution of efflux classes in *P. aeruginosa* showed some spatial variations between sampling sites. Efflux transporter genes from RND, ABC, MATE and SMR classes were detected in *P. aeruginosa* strains isolated from BA site and RND, ABC, MFS and SMR classes were identified from ZE sites. However, most of the isolates from MK sampling site contained all five classes of efflux systems. The predominant efflux mediated gene belonged to the multidrug resistance protein-coding genes, MexA, MexB and MexC. Multidrug efflux RND transporter outer membrane subunit *OpmB*, *OpmD*, *opmE*, *OpmH*, *OprJ*, *OprM* and *OprN* were also detected in all *P. aeruginosa*.

Table 3 15:Antimicrobial resistance genes among *P. aeruginosa* isolates.

Resistance Genes	BAP_2	BAP_3	BAP_4	BAP_5	BAP_6	BAP_7	BAP_8	MKESBL_13	BAP_13	MKP_1	MKP_2	MKP_3		MKP_4	MKP_5	ZP_1	ZP_5	MKP_6	MKP_7
Aminoglycoside resistance genes*																			
aph(3')-IIb	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■
Fluoroquinolone resistance genes*																			
crpP	■	■	■	■	■	■	■	■	■	■					■	■	■	■	■
Beta-lactamase resistance genes*																			
bla$_{OXA-486}$	■	■	■	■	■	■	■	■	■	■		■			■				■
bla$_{PAO}$	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■
bla$_{OXA-50}$											■							■	
bla$_{OXA-396}$													■	■		■	■		
bla$_{OXA-494}$													■	■					
bla$_{PDC-8}$	■	■				■													
bla$_{PDC-10}$								■		■					■				■
bla$_{PDC-3}$			■	■			■		■		■	■	■	■		■			
bla$_{PDC-5}$																		■	
Fosfomycin and chloramphenicol resistance genes*																			
fosA	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■
catB7	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■

*Shaded boxes indicate presence and white boxes absence of resistance gene.

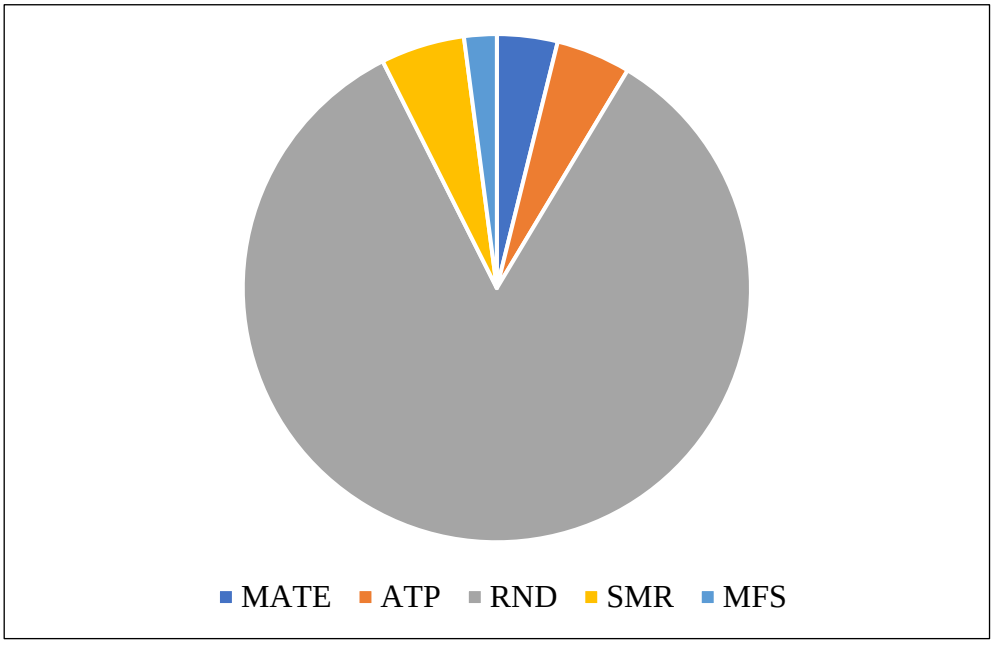

Figure 3.16: Proportion of efflux pumps in *P. aeruginosa* from Akaki river.

Abbreviations: MATE, Multidrug and Toxic compound Extrusion; ABC, ATP-binding cassette superfamily; RND, Resistance-Nodulation-cell Division; SMR, Small Multidrug Resistance; MFS, Major Facilitator Superfamily

3.5 Alteration of gene expression in *Pseudomonas aeruginosa* PAO1 grown in Akaki river waters

To determine the effect of Akaki river water on bacteria, *P. aeruginosa* PAO1 was grown in media prepared with Akaki river waters and compared to media prepared with MQ water, and the gene expression of selected genes associated with antibiotic/metal response (*mexA, oprM, cat, mexT, mexE,* mexX, *czcA* and *AmpC),*), oxidative stress (*dnaK, sigX, rpoS, recA, radA, oxyR, lexA, algU, groEL, groES, rpoH and pvdS*) and virulence factors (*rhL, ptxR, exoS, ppyR, mvfR, pilA, cupA, pcrV and lasB*) were assessed. The late log phase growth of PAO1 for RNA extraction was determined by growing PAO1 in the experimental and a control waters and it was found that the late log phase of PAO1 was 7.5 h.

The efflux pump genes were evaluated to determine how the Akaki River water alter their expression. Growth of PAO1 to Akaki river water significantly altered the expression of multidrug resistance efflux genes compared to the MQ control. The expression of antibiotic/metal-mediated genes has shown variation between the sampling sites (Figure 3.17-3.22). Among the tested four multidrug resistance efflux genes, *mexA* increasingly expressed in PAO1 grown in AL, MK, and AB water prepared medium. The fold change was relatively higher in *P. aeruginosa* grown in AL and AB water samples. Growth in BA water had no effect on the transcription of *mexA*. The river waters resulted in an increased expression of *mexT* gene, with the highest fold change in AB and lowest in BA. On the other hand, BA and AB water samples resulted in increased expression of *mexE* gene in PAO1. The metal cation efflux transporter gene, *czcA* increasingly expressed in *P. aeruginosa* grown in BA and AB water samples.

An increased expression of virulence factors will affect *Pseudomonas* pathogenicity, therefore the expression of nine virulence-associated genes (*rhL, ptxR, ppyR, pcrV, cupA1, mvfR,exoS, lasB* and *pilA*) were measured in the current study (Figure 3.18). Five virulence genes (rhL, *ptxR, pcrV, cupA1* and *exoS)* were increasingly expressed and four (*ppyR, mvfR, lasB* and *pilA*) were downregulated when *P. aeruginosa* was grown in the Akaki river water. The ATP-dependent RNA helicase, *rhL* was increasingly expressed in PAO1 grown in all water

samples, however, significant induction was observed in the bacteria grown in GE, AL, MK, and BA water samples. Although the majority of the water samples resulted from no alteration on *ppyR* which increases biofilm formation in *Pseudomonas* encoding and *mvfR* which is a transcriptional regulator of MvfR which plays a significant role in the pathogenesis of *P. aeruginosa*, they were significantly decreased in MK water sample. The expression of the gene that encodes elastase LasB, *lasB* was significantly down-regulated with all water samples exposure except GE. All the water samples revealed increased expression of the *exoS* gene with the highest fold change to GE and AL water samples.

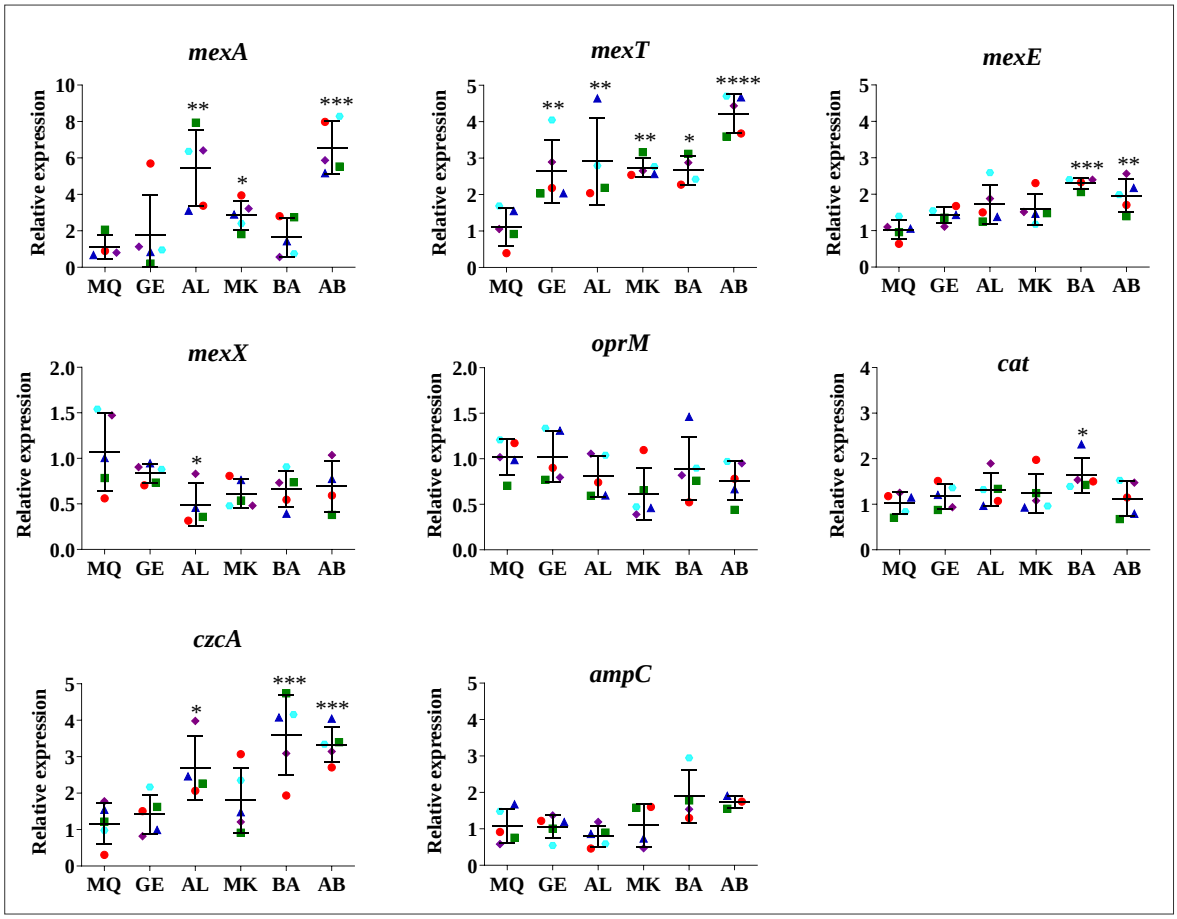

Figure 3.17: The expression of antibiotic/metal resistance genes in *P. aeruginosa* PAO1 grown in media prepared with Akaki river water compared to a MQ water control. Where * refers to p value between 0.01 to 0.05, ** refers to p value between 0.001 to 0.01 and **** refers to p value <0.0001 as compared to control.

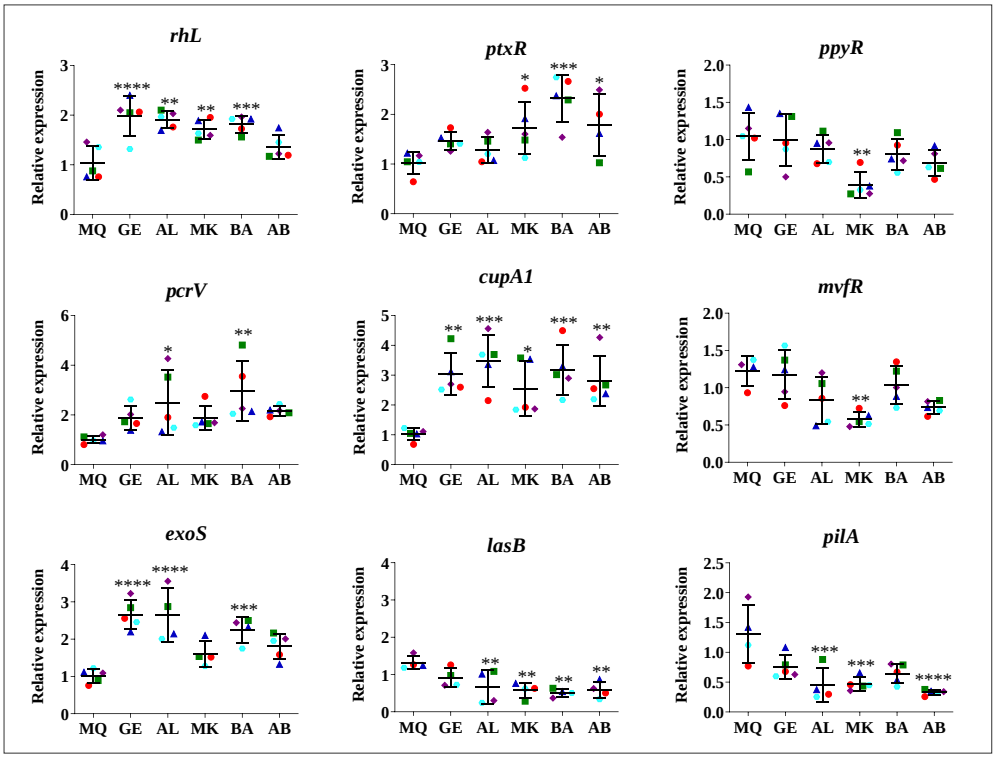

Figure 3.18: The expression of virulence associated genes in *P. aeruginosa* 01 exposed to Akaki river water compared to a control (MQ). Where * refers to p value between 0.01 to 0.05, ** refers to p value between 0.001 to 0.01 and **** refers to p value <0.0001 as compared to control.

To determine if the Akaki river waters promote specific stress responses in *P. aeruginosa*, the level of expression of 12 genes that are associated with oxidative stress was measured (Figure 3.19). The gene *sigX* was increasingly expressed in GE, AL, BA, and AB water samples. The highest fold change was observed in AL and BA treatment groups. The gene controlling the SOS network, *recA* was increasingly expressed only in the bacteria grown in BA and AL water samples, suggesting that these waters contain DNA damaging substances. Heat shock protein 60/chaperonin, *groEL* is involved in proper folding of many proteins was downregulated in PAO1 grown in all water samples with the lowest fold change in AL, BA, and AB. It has been observed that extra cytoplasmic-function sigma-70 factor gene, *pvdS* expression was extremely high in all treatment groups compared to a control MQ water. Sigma 70 is a global regulator that plays a significant role in the bacterial response to environmental stress. The fold change was differential varied among the sampling sites. The highest fold change

expression of *pvdS* was observed in PAO1 grown in GE, followed by AL, BA, and AB. The remaining stress genes (*rpoS*, *radA*, *groES* and *rpoH*) associated with a general stress regulator, encodes protein to repair DNA damage, and regulation of genes involved in environmental stress, respectively were unaffected in all treatment groups. In general, it was observed that none of antibiotic/metal resistance-associated genes expression downregulated up on exposure to Akaki river water. Except for few genes similar trends were also observed in other classes of genes.

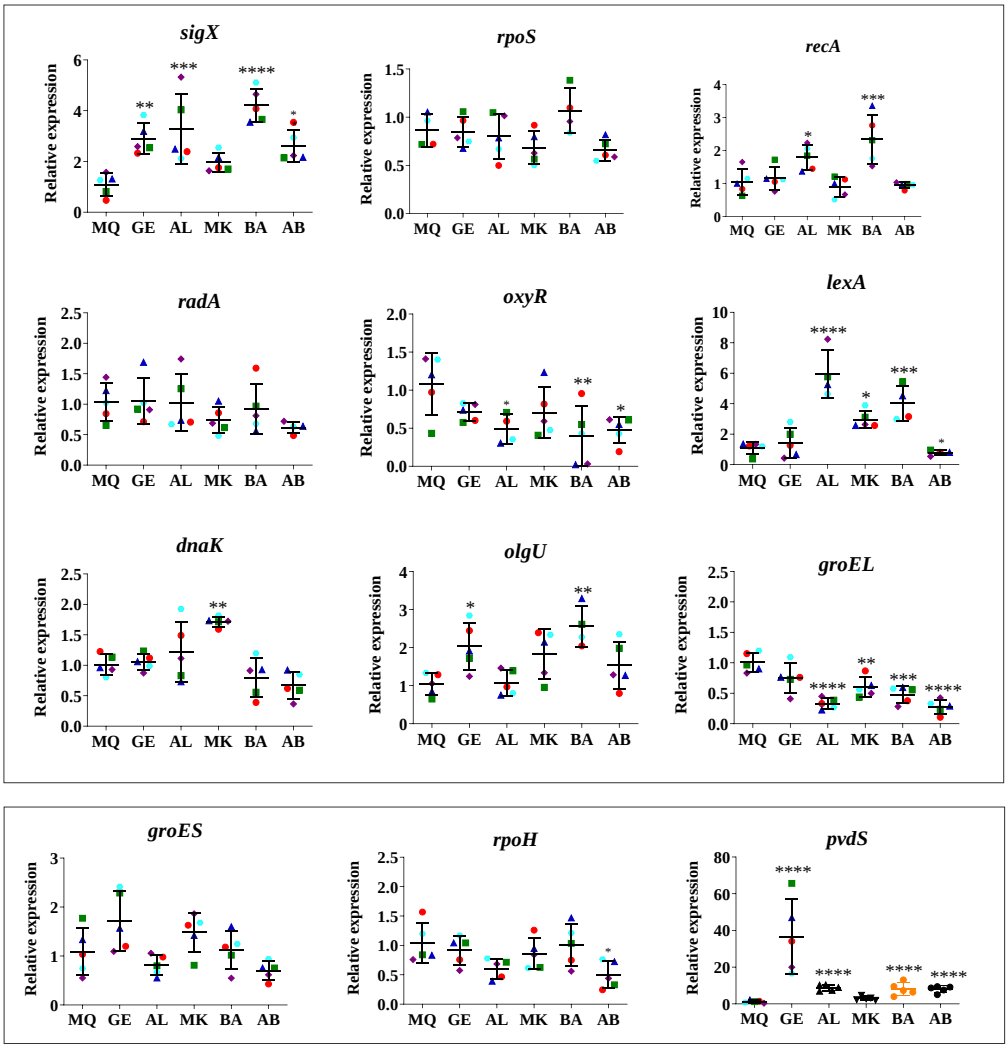

Figure 3.19: The expression of stress response genes in *P. aeruginosa* 01 exposed to Akaki river water compared to a control (MQ). Where * refers to p value between 0.01 to 0.05, ** refers to p value between 0.001 to 0.01 and **** refers to p value <0.0001 as compared to control.

3.6 Toxicogenomic analysis of Akaki river water using *Caenorhabditis elegans*

To determine toxicogenomic effects of Akaki river water, the model nematode organism *C. elegans* were exposed to waters collected from the anthropogenically impacted sites, MK and BA of the Little Akaki River and the expression of genes associated with different classes was measured. Except for manganese (Mn), all tested heavy metals were below the permissible limit in both water samples. Manganese (guideline limit of water quality for drinking and irrigation 200µg/l) (Jeong *et al.*, 2016) was above the standard limit in both sites. It was found that Mn was relatively higher in MK than in BA waters with a concentration of 2085.15 µg/l and 1307.03 µg/l, respectively. Mn pollution of the river may arise from effluent from steel and iron manufacturing industries situated along the Akaki river. The bacteria isolated from both water samples were *Acinetobacter lwoffii, Bacillus cereus,* and *Solibacillus silvestris*. *Bacillus flexus, Bacillus badius, Lysinibacillus boronitolerans, Lysinibacillus sphaericus,* and *Ochrobactrum intermedium* were identified only in BA sampling site.

Stress response genes were altered in *C. elegans* when exposed to Akaki river waters. Of the 11 stress response genes tested 8 were up- or down-regulated upon exposure to the two Waters (Figure 3.20). An increased expression of heat shock protein-16.2 *(hsp-16.2*), *hsp-16.48,* and *hsp-16.1* in the nematodes was observed when exposed to both Waters, whereas stress-induced protein-1 (*sip-1*) expression was significantly decreased compared to the control. The expression of heat shock protein-70 (*hsp-70*) was significantly down-regulated only in the nematodes exposed to BA water. No statistically significant change was observed in the expression of *hsf-1* gene upon exposure to either water sample. The expression of superoxide dismutase (*sod-1*) and cytochrome P450-35A2 (*cyp-35A2*) which are involved in converting superoxides into less toxic substances and detoxification of xenobiotics respectively were significantly increased in exposure to both water samples whereas glutathione-S-transferase-4 (*gst-4*), another gene associated with detoxification of reactive oxygen species was downregulated. Furthermore, *gst-4,* had a lower expression in nematodes exposed to BA than MK water was significantly down-regulated compared to the control. No significant change was observed in catalase 2 (*cat-2*) and protein skin head-1 (*skn-1*) expression upon exposure to either water sample.

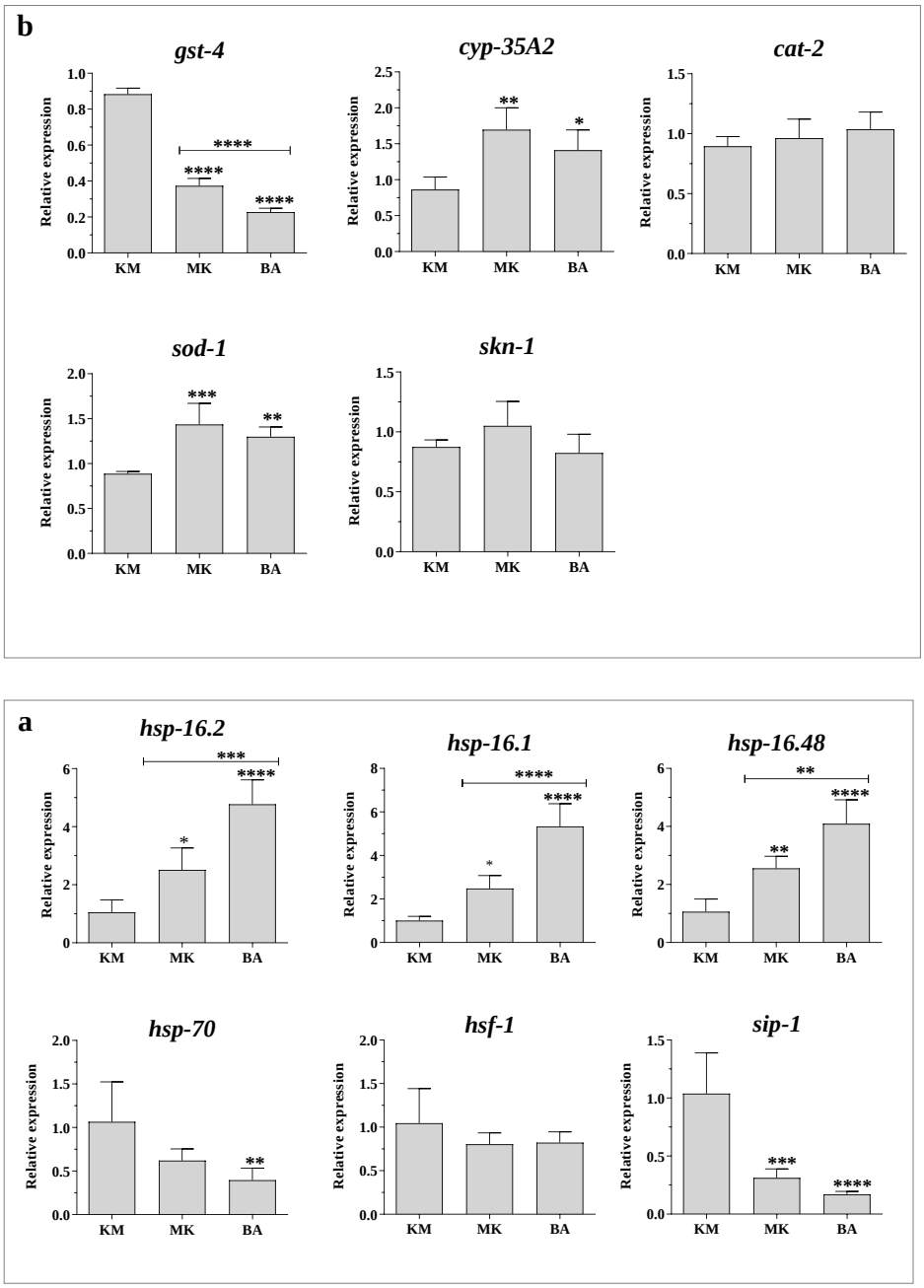

Figure 3.20: The expression of genes associated with (a) heat shock and (b) oxidative stress genes in *C. elegans* exposed to Akaki river water samples (MK and BA) samples compared with a control. One-way analysis of variance followed by Tukey's post-test was used to compare groups. Where * refers to $p < 0.05$, ** refers to $p < 0.01$, ***refers to $p < 0.001$ and ****refers $P < 0.0001$.

Differential expression of metal response genes was observed in *C. elegans* exposed to Akaki river waters. From the 10 metal response genes assessed, 3 genes had increased expression and 3 decreased expressions by at least one of the waters (Figure 3.21). Only nuclear-localized metal responsive family member-1(*numr-1*) was significantly up-regulated in nematodes exposed to both water samples with higher fold changes compared to the control, MK (6.2-fold change), and BA (18.5-fold change). Cd-inducible lysosomal protein-1 (*cdr-1*) was more expressed in BA water exposed nematodes, with a fold change of 2.2 but not in MK water for which no significant change was observed. Ferritin heavy chain homolog1 (*ftn-1*) was up-regulated only in nematodes exposed to MK water. Three metal response genes, metallothionein-1 (*mtl-1*), P-glycoprotein-5 (*pgp-5*), cation diffusion facilitators-2 (*cdf-2*), were down-regulated in response to both water samples. No significant fold change was seen in the expression of *mtl-2,* hypoxia-induced factor-1 (*hif-1*) and arsenite inducible protein 1(*aip-1*), and metal tolerance factor-1 (*hmt-1*) in nematodes exposed to either water sample. However, a significant difference was observed between the two water samples in the expression of *hmt-1*. The fold change of *hmt-1* expression in nematodes exposed to MK water was higher than in BA water exposed nematodes. Similarly, the expression of *ftn-1* in BA and *cdr-1* in MK water exposed nematodes was not significantly different from the control.

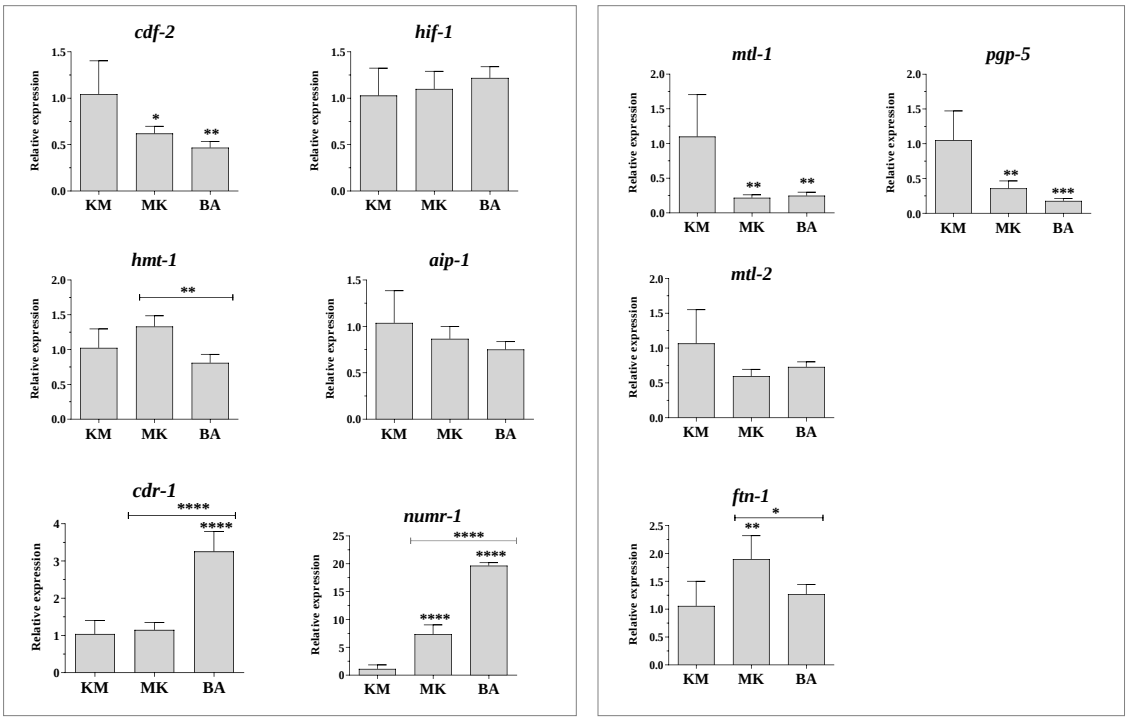

Figure 3.21: The expression of metal response genes in C. elegans exposed to Akaki river water samples (MK and BA) samples compared with a control. One-way analysis of variance followed by Tukey's post-test was used to compare groups. Where * refers to p <0.05, ** refers to p < 0.01, ***refers to p< 0.001 and ****refers P < 0.0001.

Genes associated with development and innate immune system were altered in *C. elegans* up on exposure to Akaki river water. Among the developmental genes analyzed in the current study, vitellogenin-6 (*vit-6*) and abnormal dauer formation protein 12 (*daf-12*) were highly expressed with different fold changes upon exposure to MK and BA water samples (Figure 3.22). The fold changes of *vit-6* in nematodes exposed to MK and BA water were 3.1- and 6.5-times baseline, respectively. The fold change of *daf-12* did not significantly vary between nematodes exposed to MK and BA water; 1.6 and 1.7 times respectively. Gene *daf-16* was significantly down-regulated in nematodes exposed to both water samples. However, none of the water samples had a significant change in *vit-4* gene expression. Compared to the two water samples, *vit-6* was significantly overexpressed in nematodes exposed to BA than MK water. Five innate immunity-associated genes were evaluated in Akaki-water exposed *C. elegans*. The toll-like receptor-1 (*tol-1*), Toll and Interleukin 1 Receptor-1 (*tir-1*) and C-type

lectin-60 (*clec-60*) were significantly up-regulated with varying fold changes (Figure 3.23). Antibacterial factor related-2 (*abf-2*) and lysozyme-7 (*lys-7*) genes were down-regulated in exposure to both water samples. The expression of *tir-1* was significantly more expressed in BA water than MK water exposed nematodes.

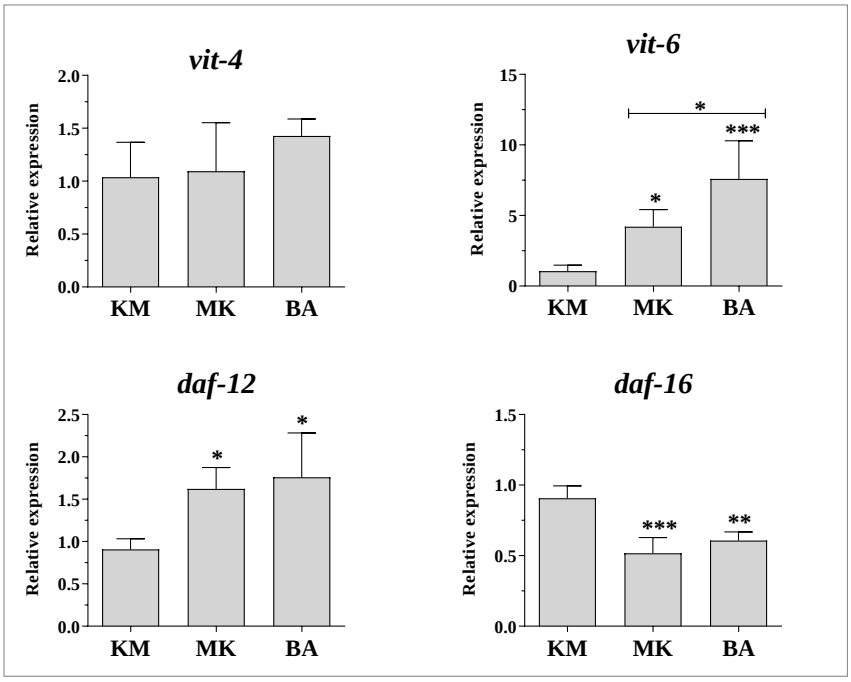

Figure 3.22: The expression of developmental genes in C. elegans exposed to Akaki river water samples (MK and BA) samples compared with a control. One-way analysis of variance followed by Tukey's post-test was used to compare groups. Where * refers to p <0.05, ** refers to p < 0.01, ***refers to p< 0.001 and ****refers P < 0.0001.

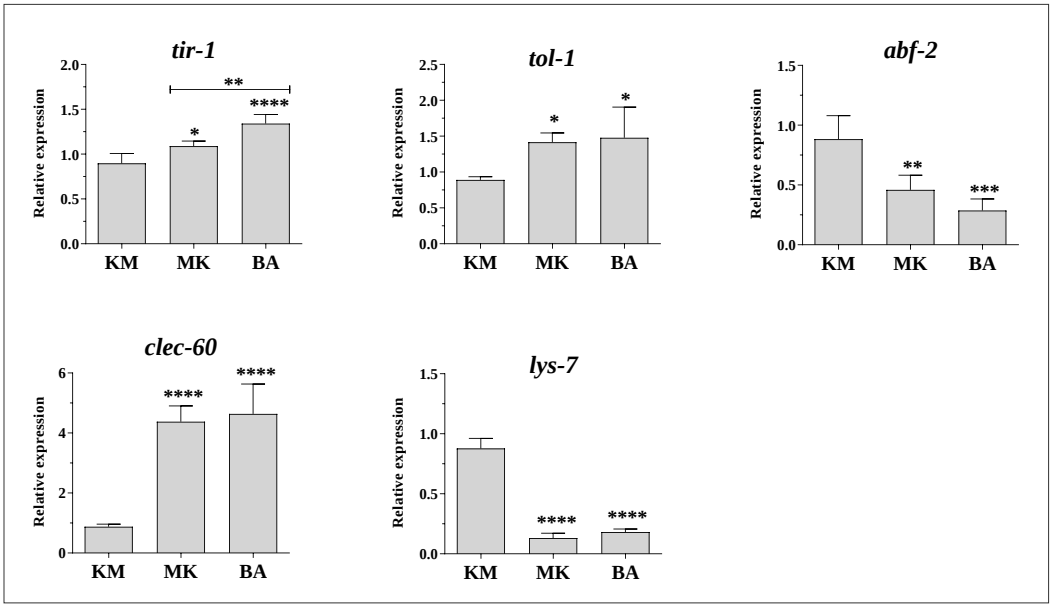

Figure 3.23: The expression of innate immune genes in C. elegans exposed to Akaki river water samples (MK and BA) samples compared with a control. One-way analysis of variance followed by Tukey's post-test was used to compare groups. Where * refers to p <0.05, ** refers to p < 0.01, ***refers to p< 0.001 and ****refers P < 0.0001.

Chapter 4: DISCUSSION

4.1 Antibiotic resistance genes in bacterial communities in Akaki rivers

ARGs are emerging environmental pollutants because of the continual discharge of antibiotic-resistant microbes (Luo and Zhou, 2008, Yang *et al.*, 2018), together with pharmaceutical and chemical pollutants into the environment. The diversity and the level of ARGs are related to the presence of antibiotic-resistant bacteria in the aquatic environment (Baker-Austin *et al.*, 2006). It is critical to establish a better understanding of the distribution, diversity, and level of ARGs and the associated clinically important bacteria in the aquatic environment to control the spread of these microbial pollutants. However, the diversity of ARGs and the bacterial community that possibly harbour resistance genes are not well studied in Sub-Saharan Africa. As in many regions, the spread of antibiotic-resistant bacteria in clinical settings is well documented in Ethiopia (Moges *et al*. 2014). A cross-sectional study in Addis Ababa has shown that prescriptions of high doses of broad-spectrum antibiotics, prolonged treatment, and increased prevalence of empirical treatment facilitate the emergence of resistance in health care facilities (Worku and Tewahido 2018). It has been shown that broad-spectrum antibiotic therapy favours the development of resistant bacteria (Cižman and Plankar Srovin, 2018). However, the community-acquired resistance, as well as the contribution of environmental factors, is not well-known in Ethiopia.

Rivers in Addis Ababa are severely impacted by increasing population and industries (ie. tanneries, breweries, wineries, distilleries, pharmaceutical, and liquor factories) situated along the Akaki river that discharges untreated effluents directly into the river or its tributaries (Yohannes and Elias 2017; Aschale *et al*. 2015). Anthropogenic activities have a significant role in the dissemination and abundance of antibiotic-resistant microbes and their resistance genes (ARGs) in rivers of major cities (Chen *et al*. 2019). Discharge from factories could provide selective pressure for the evolution of resistance among the microbial communities in the Akaki river, establishing a persistent resistome in the environment. In the current study, distribution, and level of resistance genes against β-lactam, aminoglycoside, fluoroquinolone, macrolide, vancomycin, and erythromycin antibiotics were evaluated in Akaki River, together with the density of clinically relevant bacteria. In total, 61 of the 84 genes tested were detected in the river, including 38 β-lactam, 4 aminoglycoside, 9 fluoroquinolone, 6 macrolide, 1

multidrug resistance efflux pump, 2 tetracycline efflux pump, and 1 vancomycin resistance genes.

In the current study, we found ESBL genes widely distributed in the Akaki rivers water. This is likely due to the rapid dissemination of the genes among the bacterial community since they are frequently plasmid-encoded (Ceccarelli *et al.*, 2019) and thus are easily transferred within the bacterial community (Ceccarelli *et al*., 2019, Li *et al.*, 2019). However, the distribution significantly varied between sampling sites. The Gefersa reservoir, upstream of the Little Akaki river contained significantly fewer β-lactamase genes, with only *bla*$_{\text{OXA-10}}$ and *bla*$_{\text{OXA-58}}$ detected. *bla*$_{\text{OXA-10}}$ which is commonly reported in *P. aeruginosa* isolates, however, due to horizontal gene transfer, it has also been found in Enterobacteriaceae (Maurya *et al.*, 2017). Most of *bla*$_{\text{OXA-10}}$ derived ESBL confer resistance to third-generation cephalosporin and aztreonam (Naas *et al.*, 2008). Since the levels of *bla*$_{\text{OXA-10}}$ are very low upstream of the river, it could be that it is intrinsically present in the bacterial community as previously reported (Yang *et al.*, 2019, Mahnert *et al.*, 2019) or contamination by wild birds derived resistant bacteria with low level (Darwich *et al.*, 2019). Anthropogenically impacted sites are more prone to pollution by bacteria harbouring *bla*$_{\text{OXA-10}}$ and thus exhibiting higher levels as seen in the Little Akaki (MK, BA) and Big Akaki (ZE) river sites. The β-lactamase genes distribution pattern of the three sites (MK, BA, and ZE) was more closely correlated, suggesting that anthropogenic activities accelerate the dissemination of β-lactam resistance genes and microbes to be able to tolerate selective environmental stress. In the MK site, there is an irrigation system with fertilizers and pesticides that discharges into the river. The presence of fertilizers and pesticides could trigger the persistence of resistance genes since microbes resistant to pesticides are also often resistant to antibiotics (Ramakrishnan *et al.*, 2019, Zhou *et al.*, 2017b, Liu *et al.*, 2017). Agricultural activities are a major reservoir of genetic markers of resistance (Chen *et al.*, 2013).

Previous studies have shown that anthropogenic activities are major environmental factors for resistance dissemination in the aquatic environment (Uyaguari-Díaz *et al.*, 2018, Tripathi and Cytryn, 2017, Berglund, 2015). The sites where a higher abundance of antibiotic resistance genes was detected also had higher dissolved organic carbon (data not shown) that supports

high dissolved organic carbon accelerates the accumulation of resistance genes (Ma *et al.*, 2017). Our physicochemical (data not shown) and toxicogenomics data also revealed, the middle catchment of the river was relatively contained high levels of heavy metals and trigger stress response genes in *Caenorhabditis elegans* and *P. aeruginosa* PAO1. Therefore, the toxicity of these sites could have a positive correlation with the persistence of drug-resistant bacteria and resistant genes.

The diversity of the β-lactam resistance genes between the anthropogenically impacted sites showed some variations. For instance, the New Delhi metallo-beta-lactamase 1 ($bla_{\text{NDM-1}}$) gene was detected only in BA (Little Akaki river). The gene is located on a self-transmissible plasmid that can easily be transferred between the same or different bacterial species. Bacteria such as *K. pneumonia, E. coli, Acinetobacter* species, and *Enterobacter* species carry the gene. Discharge from hospitals frequently contain bacteria that are positive to $bla_{\text{NDM-1}}$ (Islam *et al.*, 2017), however, in this study, it was found in a site not associated with hospitals suggesting it may either be present in the local community or other contributing sources. Poultry farming at this site may contribute to the persistence of the $bla_{\text{NDM-1}}$ gene as poultry farming are hotspots for the spread of resistant pathogens (Dandachi *et al.*, 2018).

Health care facilities and their effluent are among the major sources of ARG transmission to the environment (Devarajan *et al.*, 2016). The hospital site (ZE) in the current study contained unique β-lactamase genes (bla_{KPC}, bla_{SHV}, and $bla_{\text{per-2}}$) which are potentially discharged from hospitals. The bla_{KPC} is encoded on transferable plasmids and bacteria that have KPC enzymes confers resistance to all β-lactam antibiotics (Anderson *et al.*, 2007). The abundance of genes in the hospital site was relatively higher than in other sites. This suggested that the hospitals discharged their untreated wastes directly to the Akaki river or its tributaries and thus contribute to the high abundance. It could be also contamination by patient-derived pathogens which already carried resistance genes. Our results are in line with other reports that have shown effluents from hospitals result in an increased abundance of ARGs in their receiving river (Nasri *et al.*, 2017, Khan et al., 2019).

The sediment collected from the ZE site contained high diversity and abundance of resistance genes. This may be related to the severe anthropological pollution at this site than others. For instance, the sediment at this site received pollutants from hospitals and diagnostic and research laboratories may contain antibiotic or antibiotics resistance bacteria. In addition, the abundance and diversity of bacterial communities also correlated with the increased abundance of ARGs (Han *et al.*, 2018, Khan *et al*., 2019), and in the current study, we found the abundance of *A. baumannii* which is intrinsically resistant to many antibiotics effective against Gram-negative bacteria (Manchanda *et al.*, 2010) was highest in hospital site. Hospital-based cross-sectional studies have shown the emergence of carbapenemase-producing *Enterobacteriaceae* in Ethiopia (Beyene *et al.*, 2019, Legese *et al.*, 2017). The finding suggested that hospital effluent polluted the environment and may facilitate the spread of carbapenemase-producing bacteria as the water is used for different purposes such as cloth and car washing, domestic purposes, and irrigation for raw eat fruits and vegetables. Much fewer β-lactamase genes were detected downstream of the river. This could be a result of reduced or no selection pressure for resistance development exists. Unique carbapenem resistance genes ($bla_{\text{IMP-2}}$ and $bla_{\text{IMP-5}}$) detected in the Aba-Samuel reservoir are more likely due to contamination from animals such as birds, since the site is far from the other sites associated with anthropogenic activities, being 25 and 52 km downstream of BA and ZE, respectively. Birds are reported to have a significant role in the emergence and transmission of antimicrobial-resistant bacteria (Sjölund *et al.*, 2008, Altizer *et al.*, 2011) and have been shown to have a significant number of carbapenem resistance bacteria among migratory birds in China (Liao *et al.*, 2019b).

Of the 5 aminoglycoside resistance genes tested, all were found, except *aacC4* and only *aadA1*was present in all sampling sites. *aadA1* is a Streptomycin 3"-adenylyltransferase that mediates resistance to the antibiotics streptomycin and spectinomycin. The gene is distributed in a wide range of bacteria including *Aeromonas* spp., *Citrobacter* spp., *Shigella* spp. (Zhang *et al.*, 2009) and *A. baumannii* (Khoshnood *et al.*, 2017). The persistence of the gene in all sampling sites in the current study is likely due to the persistence of the *Aeromonas* and *Citrobacter* species. The level of aminoglycoside resistance genes was highest in areas associated with hospitals and thus are likely due to hospital discharge containing resistant

bacteria or residual antibiotics that trigger resistance in the aquatic environment. Despite this, a retrospective study in Addis Ababa indicated aminoglycosides are the least prescribed antibiotics in Addis Ababa (Worku and Tewahido, 2018). A similar study of hospital discharge has shown the highest levels of aminoglycoside resistance genes (Keul *et al*., 2017). The gentamycin-resistant gene, *accC1* is present in different bacterial communities in the environment (Heuer *et al*., 2002, Zhang *et al*., 2009) and was persistent in all the sampling points except the Gefersa reservoir upstream of the Little Akaki River.

Fluoroquinolone antibiotics are widely used in the world because of their efficacy against both Gram-negative and Gram-positive bacteria (Schindler *et al.*, 2017). The consumption of fluoroquinolones in Addis Ababa correlates with the emergence of resistance, as often seen in countries where consumption of fluoroquinolone is high, resistance is also high (Redgrave *et al.*, 2014). In Ethiopia, fluoroquinolone is the second most prescribed antibiotic in health care facilities (Worku and Tewahido, 2018, Gutema *et al.*, 2018) and a meta-analysis report has shown a significant prevalence of fluoroquinolone-resistant in both Gram-positive and Gram-negative bacteria (Sisay *et al.*, 2018). However, the role of the environment on the persistence and spread of fluoroquinolone resistance is not reported in Ethiopia. We determined the presence of 11 plasmid-mediated fluoroquinolone resistance genes and found *AAC(6)-Ib-cr* and *qnrS* were persistently detected in all sampling sites of the rivers. *Escherichia, Aeromonas,* and *Acinetobacter* are common bacteria in aquatic environments to develop resistance to fluoroquinolones and resistance is most frequently due to the presence of *AAC(6′)-1b-cr* gene, which is a transferable gene encoding for aminoglycoside acetyltransferase that neutralizes both aminoglycosides and fluoroquinolones antibiotics. The *qnrS* gene has been reported mostly from *Aeromonas* and *Vibrio* species isolated in an aquatic environment (Poirel *et al.*, 2012, Osinska *et al.*, 2016). The high abundance of *qnrS* gene in the hospital site (ZE) is most likely due to the presence of human-derived pathogens or the persistence of ciprofloxacin. The fluoroquinolone efflux pump resistance gene, *qepA*, was detected only in the hospital site. The gene is rarely reported from clinical isolates, however, significant isolates from animal sources harbour the gene. Although efflux pumps are chromosomal encoded (Poole, 2007), *qepA* is a plasmid-mediated gene (Poirel *et al*., 2012) so it can be horizontally transferred to pathogenic bacteria.

Of the 2 vancomycin resistance genes tested, only *VanB* was detected in the sites associated with hospitals. *Enterococci* and methicillin-resistant *Staphylococcus aureus* are a major source of vancomycin resistance genes (Varela *et al.*, 2013). The presence of the gene only in the Big Akaki river (ZE) site indicated hospital effluents are an important source of vancomycin resistance genes. A similar report has also shown the presence of vancomycin resistance gene from hospital discharges (Basode *et al.*, 2018, Khan *et al*., 2019).

It has been reported that sediments in the aquatic environment act as the pools of antibiotic-resistant bacteria and ARGs, mainly in rivers receiving effluents containing microbes and drug residues from sewage (Heß *et al.*, 2018). However, the Akaki river sediment contained few resistance genes with some spatial variations between sampling sites compared to the water samples. Most ARGs types detected in the sediment were shared by the river water. This low prevalence of antibiotic resistance could be due to the absence of resistant pathogens in sediment as observed in low bacterial diversity and densities in the current study. There were a relatively high diversity and level of ARGs in sediment collected from the site near hospitals (ZE). This could be due to the different nature of the sediments as well as the source of pollutants. The sediment in the hospital site is characterized as sandy/clay that could help the bacteria easily to proliferate. It has been reported that clay sediments facilitate the persistence of resistant pathogens as they have a capacity of high adsorption for ARGs (Guo *et al.*, 2020). Effluents from hospitals may contain resistant pathogens and settle to the bottom of the river as it flows slowly compared to the Little Akaki river. Our result is inconsistent with other reports (Guo *et al*., 2020) who reported comparable diversity and level of ARGs in both water and sediment of streams and lakes.

4.2 Functional and Molecular features of *Escherichia coli* isolates from Akaki river

The current study investigated the occurrence of potentially pathogenic strains of *E. coli*, their phenotypic antibiotic resistance levels, and resistance mechanisms recovered from Akaki Rivers. *E. coli* is one of the most frequently studied bacteria, and it is a common microbe in the human and animal gut. Determining the antibiotic resistance levels and distribution of virulence-associated genes of *E. coli* isolates in the environment gives basic information on how the environment contributes to the spread and persistence of resistant and virulent strains. The majority of isolates in the Akaki River were resistant to most of the antibiotics studied. It was observed that 100% of the *E. coli* isolates were resistant to at least three of the antibacterial agents tested, and few isolates were resistant to imipenem and meropenem. The presence of resistant *E. coli* in the current study shows the role of the aquatic environment as a conduit of resistant strains and ARGs. The result is consistent with previously reported findings in other aquatic environments in Iraq (Alwash and Al-Rafyai, 2019, Tuem *et al*., 2018). Since most antibiotic-resistant strains were identified in the anthropogenically impacted sites, it could be due to the selective pressures imposed by the release of antibiotics or ARGs found in discharge from clinical settings or households. In the previous study, it was observed higher diversity and abundance of ARGs in the rivers with similar sites from metagenomics samples. Previous studies suggested that environmental factors in surface water have played a significant role in the evolution and spread of antibiotic-resistant bacteria (Dhawde *et al*., 2018). For instance, the presence of antibiotics, heavy metals, and organic pollutants play a significant role in the emergence of resistance in the aquatic environment. Sub-inhibitory concentrations of antibiotics in aquatic environments produce sufficient selective pressure for resistance evolution (Baker-Austin *et al*., 2006). In addition, bacteria that are resistant to heavy metals can also promote resistance to antibiotics since they have similar mechanisms of action in most cases (Ding *et al.*, 2019). Significant numbers of resistant strains in anthropologically impacted sites could be due to the presence of these driving forces in the environment.

Strains isolated from the site near residential and irrigation areas were resistant to a wide range of antibiotics and contained diverse groups of resistance genes. A potential factor could be selection for resistance by discharge from agriculture irrigation. Since farmers in this site use open irrigation, chemicals such as fertilizers and pesticides directly enter the river that could trigger bacterial resistance to pesticides and antibiotics. Widespread use of pesticides in agricultural activities has significantly correlated with antibiotic resistance evolution since they shared the same target site, involving genes for multidrug efflux pumps, and plasmid or chromosome mediated resistance (Webber and Piddock, 2003, Chen *et al.*, 2018). The results were consistent with another report (Araújo *et al.*, 2017) which shows a significant correlation between antibiotic-resistant strains and anthropogenic activities. The current antimicrobial resistance (AMR) prevention and control strategies such as reducing antibiotics use and controlling misuse of antibiotics are not sufficient to stop resistance in the healthcare setting or environment. The presence of other pollutants such as heavy metals, fertilizer, pesticide, and organic pollutants in the environment also contributes to the evolution of resistance in the environment (Burridge *et al.*, 2010). The use of commercial organic and inorganic fertilizers in agricultural activities increase the abundance and diversity of antibiotic resistance genes, and thus resistant bacteria (Zhou *et al.*, 2017a).

Recurring faecal pollution from nearby households, poultry farms, and hospitals is also a potential source of already resistant bacteria, and this increases the resistant strains by providing human-derived *E. coli* strains. Therefore, the detected resistance to different antibiotics in *E. coli* recovered from site MK was probably because of extensive use of fertilizers for irrigation together with human-derived antibiotic-resistant strains from household discharge. The water in this site is used for irrigation for raw vegetables and can thus be a potential source of bacterial contamination that can harm humans through direct contact or the ingestion of contaminated food. The *E. coli* isolates from upstream (GE) and downstream (AB), exhibiting low human activities, were susceptible to the majority of antimicrobials.

Reports have shown that there is a variation in the resistance prevalence among the different phylogenetic groups of *E. coli*. Phylogenetic group D had highest the resistance levels (Mosquito *et al.*, 2015) whereas B2 and B1 had the lowest (Pereira *et al.*, 2013). However, in another report, the highest prevalence of resistance was observed in A, B2, and B1 (Alwash and Al-Rafyai, 2019). In the present study, there was no significant difference in the prevalence of resistance between phylogenetic groups within the 27 sequenced *E. coli* isolates. This could be due to all are selected based on their multidrug resistance levels or the sample size was too small. However, we observed that phylogroup B1 and D were resistant to greater numbers of antibiotics.

In the current study, *E. coli* isolates carried different virulence determinants, including toxins and adherence factors. Most of the isolates harbored enteroaggregative heat-stable enterotoxin 1 (EAST1). EAST1 is produced by enteroaggregative *E. coli* strains and are significantly associated with a sporadic outbreak of diarrhea in humans (Dubreuil, 2019) and widely distributed among diarrheagenic *E. coli* (Konno *et al.*, 2012) and enterotoxigenic *E. coli* (ETEC), which are the most common bacterial causes of diarrhea. In accordance with our finding, a high prevalence (69%) of *E. coli* strains carrying *EAST1* isolated from surface water has been reported by Sidhu and colleagues (Sidhu *et al.*, 2013). The majority of the strains that contained this virulence gene were isolated from MK and ZE sites. This could be due to human-derived strains contaminating the waters. It was observed that EAST1 co-occurred with F6 and F18 fímbriae. The combination of virulence genes is one of the main factors determining the clinical outcomes in *E. coli* infection (Sidhu *et al.*, 2013). The second most commonly identified toxin gene in the current study was for the heat-labile (LT) toxin. LT is a powerful enterotoxin produced by enterotoxigenic *E. coli* which causes diarrhea in young children, especially in developing countries and travelers from developed nations to endemic areas. The result was in contrast with previous reports of *E. coli* isolates from surface water in Germany (Stange *et al.*, 2016) in which no isolates contained *LT* gene, and in Australia (Sidhu *et al*., 2013) reported only 2% of isolates harbored *LT* gene. Shiga toxin-producing *E. coli* strains were detected in the current study although the frequency for *stx-1* and *stx-2* was low, with 6% and 1.8 %, respectively. Shiga toxin-producing *E. coli* strain causes serious, and

sometimes fatal human disease. Our finding is only slightly higher than the report in Austria among *E. coli* isolates from drinking water (Halabi *et al.*, 2008, Masters *et al.*, 2011) and in contrast with a report by Ram and colleagues in India (Ram *et al.*, 2009) that showed no isolates harboring either of the Shiga toxins.

Adherence-associated virulence determinant is an important factor in the pathogenesis of *E. coli* (Benevides-Matos *et al.*, 2015). Our findings showed that the frequency of adhesins, F18, F4, and F6 was 58.3%, 28.6%, and 12.5%, respectively. F18 antigen is one of the most important fimbrial virulence determinants associated with diarrhea in both humans and animals (Vidotto *et al.*, 2009). Although an *in vivo* study to determine whether the virulence genes in the isolates capable of expressing pathogenicity and causing disease is required, the presence of both toxin and adherence associated virulence genes in *E. coli* strains in Akaki river increases the risk of water-borne diarrhea diseases since the water is used for irrigation, domestic and recreational purposes. The frequent acute watery diarrhea outbreaks in Addis Ababa could potentially be associated with pathogenic strains of *E. coli* derived from Akaki River since the water is used for irrigation purposes to grow raw eaten crops and vegetables (Woldetsadik *et al.*, 2018). In addition to that, the community in downstream uses the river water for domestic purposes, including drinking, cooking food, shower and washing clothes.

The *E. coli* isolates that were sequenced in the current study were divided into six phylogenetic groups (A, B1, B2, C, D, and F). Phylogenetic A and B1 were predominantly detected. It has been reported that in most cases *E. coli* strains isolated from aquatic environments belonged to phylogenetic group A and B1 (Figueira *et al.*, 2011). Our results are in line with the report by Ghaderpour *et al* (2015) from surface waters in Malaysia (Ghaderpour *et al.*, 2015). Our finding is, however, in contrast with previous reports that showed isolates belonging to the B2 and D phylogenetic groups that were predominant in Al-Hillah River in Babylon Province, Iraq (Alwash and Al-Rafyai, 2019). A significant number of isolates also belonged to phylogroup B2 and D which are associated with extraintestinal infections (Johnson and Stell, 2000).

The majority of resistance determinants identified in *E. coli* isolates belonged to the β-lactamase genes and four of which (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1B}}$, *ampC1,* and *bla*$_{\text{OXA-1}}$) were more frequently detected. We report that 85% of isolates harbored at least one β-lactamase gene. These resistance determinants belonged to the extended β-lactamases which are known mechanisms conferring resistance to extended-spectrum cephalosporins (Kola and Gastmeier, 2003) and can be plasmid or chromosomally mediated. Previous reports have shown the presence of extended-spectrum β-lactamase producing *E. coli* in surface water (Blaak *et al.*, 2015). The presence of ESBL genes in *E. coli* from the aquatic environment is quite alarming because they can be easily transferred among bacterial species including other human pathogens with the help of mobile genetic elements (plasmids, integrons, insertion sequences, and transposons). Mobile genetic elements play a significant role in the dissemination of antibiotic resistance among environmental and human bacteria (Pérez-Etayo *et al.*, 2018). *E. coli* isolates in the present study, carried at least 10 mobile genetic elements. This could facilitate the transfer of the ESBL genes from environmental strains to human pathogens and increases the risk of community-acquired ESBL producing bacterial infections.

CTX-M-15 producing *E. coli* is the most widely distributed strain worldwide and associated with community and hospital-acquired infections (Cantón and Coque, 2006). In the current study, CTX-M-15 subtype was the second most frequently identified ESBLs and was consistently present in *E. coli* strains that were isolated from anthropogenically impacted sites. The result is in accordance with the report from France where they reported 69% of *E. coli* isolates carried *bla*$_{\text{CTX–M}}$ gene (Bollache *et al.*, 2019). The absence of the gene in strains that were isolated downstream in the river could be attributed to the increased dilution under flowing water. The nucleotide sequence of β-lactam resistance genes identified in the current study had sequence identity with the previously reported genes. For instance, the nucleotide sequences of *bla*$_{\text{CTX-M-15}}$ gene detected in the current study showed close identity with previously reported *bla*$_{\text{CTX-M-15}}$ gene in *E. coli* (NZ_CM015666.1, MT188708.1, and MT188705.1), *K. pneumonia* (MN688549.1) and *Aeromonas* species (MT188707.1). However, all *bla*$_{\text{CTX-M-15}}$ genes detected in the current study had low sequence identity with the *bla*$_{\text{CTX-M-15}}$ gene detected in *K. pneumonia* (CP039952.1) that was identified from veterinary settings. This clonal similarity of *bla*$_{\text{CTX-M-15}}$ genes indicated that the global

distribution of *bla*$_{\text{CTX-M-15}}$ within clinical and environmental isolates as the gene is disseminated with the help of mobile genetic elements (Canton *et al.*, 2012, Hooban *et al.*, 2020). Previous reports showed a wide distribution of *bla*$_{\text{CTX-M-15}}$ gene in *E. coli* from the aquatic environment (Hassen *et al.*, 2020, Hooban et al., 2020) and an increased prevalence of community-acquired urinary tract infections caused by Gram-negative bacteria-harboring *bla*$_{\text{CTX-M-15}}$ gene (Cahill *et al.*, 2019).

There was a high heterogenicity within the *E. coli* sequence types carrying ESBL genes. A high-risk pandemic clone that frequently carries the ESBL ST131was detected in one sampling site. This sequence type plays a significant role in the global dissemination of CTX-M-15 type resistance, carries the *bla*$_{\text{CTX-M-15}}$ resistance gene. Other sequence types such as ST46, ST405, ST746, ST120, ST998, ST1722 and ST224 also harbored *bla*$_{\text{CTX-M-15}}$ gene in the present study. *E. coli* ST10 and ST224 were previously reported as the main cause of nosocomial infections (Walther *et al.*, 2014, Heuer *et al*., 2002). *E. coli* ST1308 is a known drug-resistant pathogenic lineage and ESBL producing sequence type (Elias *et al.*, 2019). In the current study one isolate was assigned to ST1308 harboring *bla*$_{\text{NADM}}$, *ampc1* and seven subtypes of *bla*$_{\text{TEM}}$. One of the predominant sequence types of *E. coli* was ST69. This sequence type has been reported globally and associated with blood stream and urinary tract infections (Wang *et al.*, 2016). In addition, the main concern associated with this sequence type is the evolution and wide spread of antibiotic resistance (Wang et al., 2016). In the current study, this sequence type was resistant to 10-15 antibiotics studied. All ST69 strains were isolated from only ZE sampling site and possibly derived from health care facilities.

The carbapenemase encoding gene, New Delhi metallo-beta-lactamase (*bla*$_{\text{NDM-1}}$) was detected in one strain that was isolated in MK sampling site. Carbapenemase-producing bacteria cause a serious health risk due to the limited treatment options (Cahill *et al*., 2019). Most strains harboring *bla*$_{\text{NDM-1}}$ can resist a wide range of β-lactams, including nearly all carbapenem antibiotics. This enzyme was first identified in clinical isolates (Yong *et al.*, 2009). However, in recent years, it has also been rarely reported among environmental isolates (Khan *et al.*, 2018). Since the site is anthropogenically impacted by household discharge and irrigation, it was potentially human-derived. Although the gene is detected in a single *E. coli*

isolate, the presence of *bla*$_{\text{NDM-1}}$ in the aquatic environment is a matter of public health concern. One of the factors for the dissemination of *bla*$_{\text{NDM-1}}$ among environmental or clinical isolates is its presence on mobile genetic elements and incompatible groups of plasmids (Walsh *et al.*, 2011). The isolate contained 18 mobile genetic elements and one of which was an *IncC* plasmid. *IncC* plasmids are key contributors to the proliferation and dissemination of resistance genes (Ambrose *et al.*, 2018), and *bla*$_{\text{NDM-1}}$ is significantly associated with this plasmid (Walsh *et al*., 2011). Therefore, there is a possibility to transfer *bla*$_{\text{NDM-1}}$ more easily to other bacteria, including pathogens that may potentially infect human and causes community-acquired infections.

Fluoroquinolone antibiotics are commonly used in Ethiopia to treat infections caused by both Gram-negative and Gram-positive bacteria. However, because of frequent empirical treatments and non-prescription dispensing in drug stores (Worku and Tewahido, 2018), there is a growing trend of fluroquinolone-resistant bacteria in clinical settings (Mekuria *et al.*, 2018). In the current study, the prevalence of fluoroquinolone-resistant *E. coli* was 11.9%, 18%, and 26.7 % to norfloxacin, ciprofloxacin, and nalidixic acid, respectively. We also observed that isolates which were resistant to ciprofloxacin in the current study were also resistant to multiple antibiotics which is in line with a previous report (Alam *et al.*, 2006). In the whole-genome sequencing data, we found that fluoroquinolone resistance was due to the presence of mutation on the topoisomerase genes, *gyrA, parC,* and *parE*. In addition, unknown mutations were also identified but their effects were not directly obvious. Some of them can cause nalidixic acid and ciprofloxacin resistance when associated with *gyrA* mutations. The topoisomerase mutations were observed in all isolates that were phenotypically resistant to at least one fluoroquinolone antibiotic tested except for one strain and no mutations were observed in all strains that were susceptible to fluoroquinolones. Since fluoroquinolones are synthetic antimicrobial agents widely used in human medicine (Adachi *et al.*, 2013), they are not normally present in natural environments. When fluoroquinolones are released into the aquatic environments from different sources like effluents from hospitals or pharmaceutical companies, they remain stable for a long time and have strong potential for binding to sediments where they can exert selection pressure for resistance (Adachi *et al*., 2013). Mutation on the quinolone resistance determining region is not common in environmental *E.*

coli isolates compared to clinical isolates. The concentration of ciprofloxacin in Little Akaki River for instance found to be, 5mg/l (data not shown) higher than the lowest reported minimal selective concentration for resistance mutation. The minimum concentration of ciprofloxacin to develop resistance mutation is 0.1 µg/l (Gullberg *et al.*, 2011). The presence of mutations in *E. coli* in the current study indicates the presence of fluoroquinolone antibiotics in the Akaki Rivers leading to mutation. These mutations were not detected in *E. coli* strains that were isolated in the river downstream, however, this could be due to the dilution effect. Previous studies have revealed a high abundance of resistance mutation in *gyrA* and *parC* in *E. coli* isolated from aquatic environments known to have a history of fluoroquinolone pollution (Johnning *et al.*, 2015). Mutation on the *gyrA* gene was more frequently detected in the current study, followed by *parC*. The result is consistent with a study done in Sweden (Johnning *et al*., 2015). The *gyrA* mutation was the substitution of S83L and D87N amino acids. Both substitutions are significantly associated with fluoroquinolone resistance in *E. coli* and confer higher levels of resistance compared to other types of substitutions (Yoshida *et al.*, 1991). Alteration on the S83L amino acid was detected in all fluoroquinolones resistant *E. coli* strains. It was reported that this amino acid is the most frequently altered in clinical *E. coli* isolates (Uchida *et al.*, 2010, Hopkins *et al.*, 2005).

Although the most important mechanism of resistance to fluoroquinolones in Enterobacteriaceae is due to mutations in DNA gyrase and DNA topoisomerase IV genes (Jacoby, 2005), plasmid-mediated quinolone resistance (PMQR) is also steadily increasing (Vasilaki *et al.*, 2008). PMQR genes were detected in 10 *E. coli* strains in the current study and the predominant was *qnrS1,* followed by *aac(6')-Ib-cr*. The ability of plasmid-mediated fluoroquinolone resistance genes to spread by horizontal gene transfer among different bacterial species constitutes a major concern for AMR control (Poirel *et al.*, 2012). In the previous study, we have shown that there was a higher abundance of *qnrS1*, followed by *aac(6')-Ib-cr* in bacterial communities of the Akaki river system. This indicated that these plasmid-mediated resistance genes could be transferred among diverse group of bacterial species. It has been also reported that the environments outside the healthcare facilities are a reservoir of PMQR (Poirel et al., 2012). The presence of fluoroquinolone resistance mutation

and plasmid-mediated resistance genes in *E. coli* isolated from the Akaki river systems highlights the need for an intervention to the control the release of resistant microbes and antibiotics into the aquatic environment.

In general, these findings highlight the presence of virulent and antibiotic-resistant *E. coli* strains in the Akaki River. The presence of virulent strains in surface water could pose a significant public health risk to associated populations. Resistance in most cases was associated with anthropogenic activities along the river. Our finding emphasizes the need for an efficient intervention strategy to control the levels of antibiotic-resistant pathogens in the aquatic environment.

4.3 Functional and molecular features of *Aeromonas* strains in Akaki river

Aeromonas spp. are emerging gastrointestinal pathogens associated with different clinical syndromes including gastroenteritis, cellulitis, wound infections, urinary tract infections, and severe conditions such as meningitis and septicemia (Lamy et al., 2009). Human infections caused by *Aeromonas* spp. most commonly occur in community settings, although reports also show an increasing prevalence of nosocomial infections. Gastrointestinal illness associated with *Aeromonas* spp. infections include acute watery diarrhea, dysenteric diarrhea, chronic diarrhea, and traveler's diarrhea (Elorza *et al.*, 2020). Complications such as small bowel obstruction (Block *et al*., 1994), acute renal failure (Filler *et al*., 2000) and hemolytic-uremic syndrome (Fang *et al*., 1999) can also be caused by toxin-producing *Aeromonas* spp. In recent years, *Aeromonas* spp. have become important bacterial wound pathogens related to exposure to polluted environmental water. *A. hydrophila*, *A. caviae*, and *A. veronii* are commonly implicated in human intestinal infections (Elorza *et al*., 2020). *Aeromonas* spp. from the environment and healthcare facilities show increasing trends of antibiotic resistance, including to β-lactams. *Aeromonas* spp. related gastroenteritis outbreaks have been reported from different part of the world. However, in Ethiopia there is no report of *Aeromonas* associated diarrhea despite frequent acute watery diarrheal outbreaks in Addis Ababa and other parts of the country. This could be due to poor isolation and identification techniques for *Aeromonas* species, or no attention was given to this group of bacteria. In this study we

investigated the phenotypic and molecular features of three *Aeromonas* species, *A. hydrophila*, *A. caviae*, and *A. veronii* isolated from Akaki River.

The overall resistance phenotype of *Aeromonas* spp. in the current study showed the highest level of resistance to penicillin, similarly with previous studies (Maravić et al., 2013, Elbehiry *et al.*, 2019). High resistance levels were observed among the three *Aeromonas* spp. to penicillin, however, all *A. veronii* strains were resistant to penicillins, whereas this was not observed in the other two species. The prevalence of tetracycline resistance in the present study was in agreement with other reports of environmental and clinical *Aeromonas* isolates (Igbinosa and Okoh, 2012, Skwor *et al.*, 2014, Aravena-Román *et al.*, 2012). However, it contrasts with other reports of clinical (Ko *et al.*, 1996, Ghenghesh *et al.*, 2013) and environmental isolates collected from some rivers in Thailand (Usui *et al.*, 2016). This discrepancy could be because of the different selection pressures present in the water for resistance evolution among *Aeromonas* spp. Since the Akaki river is a major recipient of wastes from households and health care facilities, resistant strains potentially contaminate the rivers and contribute to the observed differences.

The evolution and spread of carbapenem-resistant bacterial pathogens are a public health threat of the 21st century. Carbapenem antibiotics are an important treatment option for life-threatening infections (Adams *et al.*, 2017). In the current study, it was found that resistance to carbapenem antibiotics was high, with different prevalence rates among *Aeromonas* spp. The majority of *A. hydrophila* and *A. veronii* were resistant to the three carbapenem antibiotics tested (meropenem, imipenem and ertapenem) and the prevalence of carbapenem resistance among *A. veronii* strains was the highest. None of *A. caviae* isolates were resistant to imipenem and only 5% were resistant to meropenem. It has been reported that carbapenem resistance in *Aeromonas* is commonly mediated by metallo-β-lactamase (CphA), encoded by the bla_{cphA} gene and the distribution is species dependent. The gene is frequently found in *A. hydrophila* and *A. veronii*, but not in *A. caviae* (Wu *et al.*, 2012), therefore, the low prevalence of resistance in *A. caviae* to carbapenem antibiotics in the present study may be because of the absence of bla_{cphA} gene. In the whole genome sequence data, none of the *A. caviae* carried this resistance marker. In the majority of isolates, resistance to carbapenems was due to the

production of carbapenemase as confirmed by CarbaNP test. Resistance prevalence of *Aeromonas* spp. to carbapenems in the current study was higher than the clinical isolates (Rosso *et al.*, 2019). It has been also reported that none of the *Aeromonas* strains that were isolated from cholera-like illnesses were resistant to meropenem (Mohan *et al.*, 2017). This indicated that the environmental factors such as the presence of diverse bacterial communities and chemical pollutants provided higher selection pressure favoring the persistence of these carbapenemase-producing strains. In a similar study, antibiotics resistance was significantly higher in *Aeromonas* strains isolated from water than those from clinical samples (Li *et al.*, 2015). The high prevalence of resistance to ertapenem in the current *Aeromonas* isolates was comparable with a report by Igbinosa *et al* (2017) (Igbinosa *et al.*, 2017). The resistance mechanism to ertapenem can slso be by other than the carbapenemase enzyme like in Enterobacteriaceae in which primary resistance to ertapenem is most common due to expression of β-lactamases such as an AmpC β-lactamase or an extended-spectrum β-lactamase (ESBL) combined with porin loss (Hawser *et al.*, 2011, Hawser *et al.*, 2012).

Resistance to cephalosporine antibiotics, in general, was found to be low among all *Aeromonas* species in the current study. The prevalence of resistance to cephalosporines was higher in *A. caviae* than in the other two species. For instance, a significant number of *A. caviae* were resistant to ceftriaxone, cefoxitin, cefotaxime and cefixime. Resistance to cephalosporines in *Aeromonas* is mediated by class B, C or D β-lactamases (Chen *et al.*, 2012). The difference in the prevalence of resistance to this group of antibiotics among the different species could be due to the presence of species-specific resistance genes. Previously it has been reported that the distribution of class B, C, and D β-lactamases among *Aeromonas* is species-specific. For instance, class B and C in *A. hydrophila*, class C and D β-lactamase in *A. caviae* and B and D β-lactamases in *A. veronii* are widely distributed (Chen *et al*., 2012). In another hybridization study using class C cephalosporinase genes, bla_{cepS} revealed all *A. veronii* and the majority of *A. hydrophila* were hybridized-positive for class C cephalosporinase gene, bla_{cepS} (Walsh *et al.*, 1997).

The virulence determinants associated with diarrhea were assessed among the three *Aeromonas* species. The six cytotoxic enterotoxins virulent determinants were selected as targets because of their implication as crucial virulence factors in diarrheal disease (Sha *et al.*, 2002). Cytotoxic enterotoxin encoding genes were detected in the majority of *Aeromonas* isolates. The heat-labile cytotonic enterotoxin (*alt*) was the predominant toxin gene detected, followed by a heat-stable cytotonic enterotoxin (*ast*). The gene, *alt*, encodes a heat-labile enterotoxin toxin (LT) that cross-reacts with the Shiga-like enterotoxin. It has been reported that *alt* and *ast* genes are detected in diarrheagenic isolates (Albert *et al.*, 2000), some environmental and healthy control strains (Silva *et al.*, 2017). Contrary to our study, in northern Africa, none of the *Aeromonas* species isolated from untreated drinking water from wells, diarrheal patients, and healthy control carried the *alt* virulence determinant (Ghenghesh *et al.*, 2014). The described differences could be linked to differences in geographical location. The presence of *ast* is not only toxicity associated with infection but also associated with intoxication (Kingombe *et al.*, 2010). The distribution and prevalence were similar to the report by Sen *et al* (2004) who reported most of *Aeromonas* strains isolated from US drinking water utilities were found to be positive for *alt* and *ast* virulence markers (Sen and Rodgers, 2004). In a previous study, it was also reported that more clinical isolates harbored *alt* and *ast*, but not the environmental isolates (Albert *et al*., 2000).

The majority of *A. hydrophila* and *A. veronii* in the current study harbored cytotoxic enterotoxin (*act*) gene and none of *A. caviae* isolates carried the gene. The gene *act* has hemolytic and cytotoxic effects and is lethal to mice (Sha *et al.*, 2009). The prevalence of *act* gene in the current study was higher than the report by Ghenghesh *et al* (2014) among *Aeromonas* isolates in Libya (Ghenghesh *et al*., 2014). They have reported only 36% of *Aeromonas* isolates from water harbored *act* gene. In another study, the distribution of *act* genes between environmental and clinical isolates was comparable, 65.5 and 62.5% of clinical and environmental strains harbored the *act* gene respectively (Li *et al.*, 2015). In the current study, *act* was not detected among *A. caviae* isolates, which is in agreement with a previous report in Norway (Hoel *et al.*, 2017) that showed no *act* gene detected in *A. caviae* strains isolated from fresh Retail Sushi. In general, the presence of multiple virulence determinants

in *Aeromonas* isolates in Akaki Rivers is a potential public health risk to residents in Addis Ababa and downstream communities.

A total of 21 *Aeromonas* isolates were subjected to whole-genome sequencing for in-depth genomic analysis. Diverse groups of antibiotics resistance genes were detected in the three species with some spatial variations among sampling sites. Strains that were isolated from the upstream and downstream of the river contained fewer ARGs, which belonged to only the β-lactamases. No resistance gene belonging to aminoglycosides, quinolone, tetracycline, macrolides, and other groups were detected in those strains that were isolated from both upstream and downstream sampling sites (GE and AB). The β-lactamase genes in these isolates belonged to bla_{ampS} and bla_{cphA} variants. Only three strains that were isolated from the middle catchment (MK, BA, and ZE) sampling sites harbored bla_{cphA}. bla_{cphA} is chromosomally mediated in most wild-type *Aeromonas* spp. and its expression results in the production of a metallo-β-lactamase with the ability to hydrolyze carbapenems. However, not all strains harboring bla_{cphA} gene can express the carbapenemase activity (Rossolini *et al.*, 1995). In our study, it was found that all strains that were isolated from both sites and carried bla_{cphA} gene were also carbapenemase producers and phenotypically resistant to at least one carbapenem antibiotic. This gene variant was detected only in *A. veronii* and *A. hydrophila,* but not in *A. caviae*. This result is concordant with previous reports (Wu *et al*., 2012, Sinclair *et al.*, 2016). Other classes of ARGs were identified in *Aeromonas* strains that were isolated from anthropogenically impacted sites. For instance, the aminoglycoside resistance genes, aminoglycoside phosphotransferase *aph(3'')-Ib* which is mediated by mobile genetic elements (plasmids and transposons) and chromosomes in some bacterial species such as *P. aeruginosa* (Hächler *et al.*, 1996) was detected only in strains that were isolated from anthropogenically impacted sites. This could be due to the diverse bacterial community in anthropogenically impacted sites, it may be possible *Aeromonas* species acquired these genes through horizontal gene transfer.

The majority of the β-lactamases genes identified belonged to class A-β-lactamase. Fourteen class A-β-lactamase genes were detected and the most prevalent was bla_{TEM} with nine different variants. It was consistent with previous reports that showed that class A β-lactamase

genes were more frequently detected in *Aeromonas* species isolated from urban wastewater treatment plants (Piotrowska *et al.,* 2017). Most of the detected class A-β-lactamase genes in the current study were extended-spectrum β-lactamases. This highlights that the highest phenotypic resistance of *Aeromonas* species to penicillin could be due to the expression of ESBL genes.

In a few isolates, we also identified genetic markers conferring resistance to colistin. The mcr variants identified among *A. caviae* and *A. veronii* were similar, whereas one of the mcr variants (*mcr-25*) was detected only in *A. hydrophila*. Colistin resistance mcr genes have been found on either conjugative plasmid (Liu *et al.,* 2016) or ColE-type plasmids (Alba *et al.,* 2018). Previous reports have shown that *Aeromonas* and other bacterial species isolated from rivers contained different variants of colistin resistance genes (Tuo *et al.,* 2018). The presence of *Aeromonas* isolates harboring the colistin resistance gene in Akaki Rivers is of a great public health concern as these isolates may contribute to the potential transmission of colistin resistance genes to other bacterial pathogens. The nucleotide sequence of the two mcr variants (*mcr3.7* and *mcr-3.2*) in the current study share high identity with previously reported colistin resistance genes identified from different bacterial species. For instance, the *mcr-3.7* identified in the current study shared 99.4% identity with *mcr* detected in *E. coli* in Brazil (NG_057484) (Kieffer *et al.,* 2018), MG4916669.1), 99.75% in *A. media* in Germany (NG_055661.1), 95.35% in *K. pneumonia* in Thailand (NG_060583.1). The other variant (*mcr-3.17*) nucleotide sequence had lower similarity with previously reported *mcr* genes (NG_06058.1).

4.4 Functional and Molecular features of *Pseudomonas aeruginosa* isolates from Akaki river

Pseudomonas aeruginosa is an opportunistic pathogen widely distributed in both clinical and environmental settings. Because of its metabolic versatility, it can survive in a variety of aquatic environments such as rivers, lakes and drinking water (Mena and Gerba, 2009, Hall *et al*., 1998). *P. aeruginosa* can cause skin and eye infections in healthy individuals and life-threatening illness in immunocompromised individuals and burn and surgical patients. The presence of *P. aeruginosa* in the surface water has been associated with infection outbreaks (Mena and Gerba, 2009). Due to its ability to resist a wide range of antibiotics, it has become a major cause of nosocomial infections (Kang *et al.*, 2003, Pobiega *et al.*, 2016). Although *P. aeruginosa* is known to harbor intrinsic, it also acquires resistance genes associated with mobile genetic elements including plasmids and transposons, that can help transfer resistance determinants between bacterial families (Pfeifer *et al.*, 2010). Determining the distribution of *P. aeruginosa*, its antibiotic resistance patterns and mechanism of resistance in the aquatic environment is crucial to mitigate the spread of resistance in the human-environment interface. This study intended to gain insight into the distribution of antibiotic resistant *P. aeruginosa*, and its resistance genes in Akaki River system.

A total of 21 *P. aeruginosa* strains were isolated from three anthropogenically impacted sites in the Akaki River and tested for their antibiotic susceptibility patterns. To identify the genetic determinants of the antibiotic resistance, 18 isolates were sequenced. MLST result showed that the *P. aeruginosa* isolates in this study comprised of eight sequence types (ST), with the most frequent being ST639 (6, 33.4%), ST274 (3, 16.6%) and ST558 (3, 16.6%). ST639 is associated with carbapenem-resistant *P. aeruginosa* isolates from clinical settings and reported in burn patients (Yin *et al.*, 2018). However, in the current study, ST639 was susceptible to all the carbapenem antibiotics tested. The ST274 is frequently reported in Cystic Fibrosis (CF) patients and a growing list of CF epidemic clones (López-Causapé *et al.*, 2013, Kidd *et al.*, 2012). This ST is a high-risk clone circulating in European countries in CF patients (López-Causapé *et al.*, 2017, Ocampo-Sosa *et al.*, 2015), and also described as an intestinal colonizer of healthy individuals (Valenza *et al.*, 2015). Two *P. aeruginosa* strains that were

isolated from site near the hospitals belonged to ST116. This sequence type identified as the second most common clone responsible for cystic fibrosis (Smart *et al.*, 2006, Bai *et al.*, 2019). The presence of this sequence type in the Akaki River could be derived from healthcare facilities situated along the river. *P. aeruginosa* isolated in the current study included five serotypes (O1, O3, O4, O6 and O9), although the most common were O4, O3, and O6. The environmental isolates examined are typical serotypes among clinically relevant *P. aeruginosa*, in particular serotypes O1, O6 (Lu *et al.*, 2014, Faure *et al.*, 2003) and O4 (Faure et al., 2003). *P. aeruginosa* ST-O4 was significantly associated with mortality in a previous report (Faure et al., 2003). In another similar study, O4 was not normally detected from environmental settings (Radó *et al.*, 2017). This discrepancy could be due to the differences in the sources of contamination.

Almost all *P. aeruginosa* isolates from Akaki River were found to be multi drug-resistant. The highest resistance observed in the isolates was against penicillins, and 1st, and 2nd generation cephalosporines. The highest resistance prevalence of *P. aeruginosa* in this study to these group of antibiotics was due to the presence of β-lactamase genes. Resistance to β-lactam antibiotics is mainly mediated by inactivating enzymes called β-lactamases encoded by a diverse group of genes (Stefani *et al.*, 2017). Resistance in *P. aeruginosa* results from different mechanisms such as enzyme-mediated drug inactivation, efflux systems, target site inactivation and uptake inhibition (Pang *et al.*, 2019). The WGS data showed that the majority of the isolates carried at least three β-lactamase genes. The predominant β-lactamase gene was *bla*$_{\text{PAO}}$, followed by *bla*$_{\text{OXA-486.}}$ It has been reported that the gene *bla*$_{\text{PAO}}$ was detected among β-lactam resistant strains of *P. aeruginosa* isolated from urinary tract infections (Hussain *et al.*, 2017). The highest resistance prevalence against penicillin in the current study is in line with the study done by Khan *et al* (2007) who reported 100% of *P. pseudomonas* isolates from the river, ocean, clinical and animal isolates in Japan were resistant to ampicillin and amoxicillin-clavulanate (Khan *et al.*, 2007). However, the prevalence of resistance to β-lactam antibiotics in the current study is higher than the report by Pirnay *et al* (2005) in a Belgian river (Pirnay *et al.*, 2005) and by Miladi *et al* (2020) in various clinical specimens (Miladi *et al.*, 2020). The difference in the results with Miladi *et al* (2020) could be due to the different

sources of samples. For instance, in the current study, surface water was used while Miladi *et al* (2020) used urine sample. Although aquatic environments are rarely contaminated by antibiotics compared to clinical settings, presence of other triggering factors such as metals could make the environmental strains more resistant especially if the resistance is mediated by efflux systems. This is supported by the whole genome sequence results in which genes associated with efflux pumps were widely distributed among *P. aeruginosa* isolates.

Aminoglycosides are active against *P. aeruginosa* (Ratjen *et al.*, 2009), however, a significant number of isolates were resistant in the current study. Resistance to aminoglycosides in *P. aeruginosa* is mediated by reduced permeability, increased efflux, or enzyme modification, although, enzyme modification is the most important mechanism of resistance (Ratjen et al., 2009). In the current study, all isolates carried the aminoglycoside phosphotransferase gene, *aph(3')-IIb*. This gene is widely distributed among aminoglycoside-resistant *P. aeruginosa* clinical isolates (Kashfi *et al.*, 2017). This genetic element can be exchanged between different bacterial tax in the presence of selective pressures in the environment such as chemical pollutants including antibiotic residues (Hall and Collis, 1998). All isolates were susceptible to imipenem and meropenem, which is in accordance with a previous report by Suzuki *et al* (2013) in which 100% of *P. aeruginosa* isolates from Kiyotake River and Yae River, Japan were susceptible to these two carbapenems (Suzuki *et al*., 2013). However, it is in contrast to the report by Amsalu *et al* (2020) who showed resistance to carbapenem was 25% among *P. aeruginosa* isolates from healthcare-generated wastewater in Australia (Amsalu *et al.*, 2020). This difference could be because of the inclusion of healthcare-generated wastewater samples in their study.

Efflux system is one of the mechanisms of antibiotic resistance in bacteria and it has different families and sub-families. The proteins belonging to the RND family of efflux pumps play a key role in the antibiotic resistance of *P. aeruginosa* (Li and Nikaido, 2009). Most of the efflux systems genes detected in this study belonged to the RND family. The wide distribution of multiple efflux-associated resistance genes in *P. aeruginosa* isolated from the Akaki River suggests that the water may have factors that cause selective pressure for resistance. This is supported by our gene expression assays in *P. aeruginosa* PAO1 exposed to water samples

collected from the Little Akaki river in which transcriptional change of antibiotics/metal resistance, stress response, and virulence-associated genes were assessed.

Differential expression of multi-drug efflux system genes such as *mexA*, *mexT* and *mexE* in the current study was observed. Although in some cases the response to water collected from the upstream reservoir also causes increased expression of genes, the fold change in the middle catchment and downstream of the river was higher. This showed that there was a different types and levels of pollutants of the river as there are high human activities in the middle catchment. The *mexA* gene is one of the MexAB-OprM efflux systems in *P. aeruginosa* constitutively expressed multidrug efflux pump (Fernández and Hancock, 2012). Exposure of *P. aeruginosa* to different stressing chemicals leads to overexpression of the MexAB-OprM efflux pump (Sobel *et al.*, 2005). Therefore, the increased expression of the gene that belonged to the multidrug efflux system in the current study could be due to the substances that are toxic to microbes and the differential expression particularly in the upstream of the river was due to the difference in the levels of toxic substances. The metal cation efflux transporter gene, czcA was increasingly expressed in all water samples except GE, and significantly high in AL, BA, and AB. It has been reported that *czcA* is one of the gene conferring resistance to heavy metals such as cadmium, cobalt, and zinc in different microbes (Janssen *et al.*, 2010, von Rozycki and Nies, 2009, Hu and Zhao, 2007, Intorne *et al.*, 2012) including *pseudomonas* species (Hu and Zhao, 2007). This gene also causes cross-resistance selection by either heavy metals or antibiotics. Perron *et al* (2004) reported that resistance to Zn, Cd, and Co mediated by *czcA* in *P. aeruginosa* is also resistant to carbapenem such as imipenem (Perron *et al.*, 2004). Environmental pollutants such as detergents, organic solvents and antibiotics induce multiple antibiotic resistance operons (Rickard *et al.*, 2004) which regulate the expression of large numbers of genes including those involved in the efflux system (Alekshun and Levy, 1999). It was also found that *P. aeruginosa* isolated from the Akaki River were resistant to a diverse group of antibiotics and resistance was mainly mediated by the RND family of multidrug efflux pumps. This finding showed the likelihood of the Akaki River water to trigger resistance in bacterial communities and particularly through the efflux system. The chemical analysis data showed that, almost all tested heavy metals were within the permissible

limit, and that indicates that the sub-inhibitory concentration could play a significant role for stress-induced antibiotic resistance. The results were in line with a similar study using different strains that showed the exposure of *P. aeruginosa* to wastewater resulted in significant induction of MexCD-OprJ efflux pump (Schwartz *et al.,* 2015).

Environmental stress is caused by either depletion of nutrients or the presence of toxic compounds such as antibiotics and heavy metals result in phenotypic or genotypic alteration in bacteria (Storz and Hengge-Aronis, 2011). Phenotypic changes include expression of protective heat shock proteins that protects subsequent challenges to the same chemical or cross-resistance against other chemicals such as against antibiotics (Velkov, 1999, Rowan, 1999). In the current study significant alterations in the stress-associated genes were observed. The extra-cytoplasmic function sigma factor encoding gene, *sigX* was significantly induced in PAO1 exposed to all water samples except MK. One of the adaptation mechanisms of bacteria to the environment is sensing and responding to extracellular conditions. In bacteria one of the common ways to respond is activation of genes in response to the environment is the use of the extracellular sigma factors (Potvin *et al.*, 2008). The other sigma factor analyzed in the current study was iron starvation sigma factor *pvdS* which was extremely upregulated in all experimental groups. Under iron limitation, this gene is involved in the transcription of pyoverdine and virulence genes in *P. aeruginosa* (Tiburzi *et al.*, 2008). The induction fold change was highest in GE water exposed PAO1, followed by AL, BA, and AB respectively. The gene is down-regulated by the presence of excess iron in the growth environment (Rombel *et al.,* 1995). Some studies have shown a relationship between iron concentration and antibiotic resistance in *P. aeruginosa*. Increasing iron concentration decreases antibiotics resistance in *P. aeruginosa* against some antibiotics such as ampicillin, gentamicin, norfloxacin and ofloxacin (Yeom *et al.*, 2010, Kreamer *et al.*, 2015). The increased expression of the gene in the current study could be explained by that low concentration of iron and that could induce antibiotic resistance in bacterial communities in the Akaki river.

In *P. aeruginosa*, there are two major quorum-sensing systems, las and rhl, which regulate virulence gene expression (Pesci, 1999). The rhl system regulates the synthesis of rhamnolipid which has hemolytic property (Koch *et al.*, 1991). In the current study, significant induction of *rhl* gene in PAO1 exposed to Akaki River water was observed. The expression was more prominent in GE exposed PAO1. Virulence gene expression in bacteria are affected by several factors such as nutrient starvation and the presence of toxic compounds in the growth environment (Paytubi *et al.*, 2017). Increasing temperature and depletion of extracellular iron are main signals for bacterial invasion and enable turning on virulence expression and resulting promotion and proliferation of bacteria in an unconducive environment (Lam *et al.*, 2014). Significant induction of virulence genes such as *rhl*, *exoS, cupA1, pcrv* and *ptxR* in the current study could be due to depletion of nutrients or presence of other xenobiotic pollutants in the river water. The differential expression of such genes among sampling sites could most likely be due to the different pollution levels. For instance, the fold change of *exoS* gene was highest upstream of the river which was supposed to be no pollution. The gene is increasingly expressed in bacteria grown in low-cation environment since reporter gene was inducible in a low-cation condition. It has been also reported that environmental strains of *P. aeruginosa* expressed much more *exoS* gene compared to clinical isolates (Yahr *et al.*, 1995). It can be concluded that toxic substances in the Akaki river water induces expression of virulence associated genes.

4.5 Toxicogenomic analysis of Akaki River water using *Caenorhabditis elegans*

C. elegans is frequently used model organism for environmental toxicity assessment because of its short life cycle, rapid generation time, and easy maintenance and allowing predictions of toxicity in higher animals (Leung *et al.*, 2008). The current study measured the expression of 30 genes from different classes in *C. elegans* exposed to Little Akaki water to determine the biological effects on the nematodes as proxy indicators of potential toxicity to humans.

Oxidative stress is disproportion between antioxidants and free radicals. Free radicals such as reactive oxygen species affect cellular components in organisms and contribute to the development of several diseases including Alzheimer's and Parkinson's. Organisms respond to this condition by regulating different genes (Finkel, 2011). *C. elegans* regulate numerous genes to overcome the stress conditions for adaptation and toxicity. Of the five oxidative genes analyzed, *cyp-35A2* and *sod-1* were significantly up-regulated whereas *gst-4* was down-regulated compared to untreated nematodes. Gene *cyp-35A2* is involved in xenobiotic stress response in *C. elegans*. The increased expression of the gene was in line with the report by Ji-Yeon *et al* (2006) in nematodes exposed to heavy metals (Roh *et al.*, 2006), organophosphorus pesticide, and fenitrothion (Roh and Choi, 2011). Its induction could be associated with combined effects of the different heavy metals, organic or other environmental pollutants. Superoxide dismutase (*sod*) has a significant role in the lifespan of *C. elegans* by removing superoxide free radicals (Doonan *et al.*, 2008) and it has different isoforms. The antioxidant, *sod-1*, was significantly induced upon exposure to both water samples. The finding is in contrast with a study in Swedish environmental water samples in which significant change was not observed (Kumar *et al.*, 2015). This could be due to differences in combinations and levels of pollutants in the waters. However, they reported laboratory reconstituted heavy metal exposure significantly induces the expression of *sod-1* (Kumar *et al*., 2015). Mn toxicity contributes to the induced expression of the gene since Mn enhances the accumulation of reactive oxygen species (Martinez-Finley *et al.*, 2013). In both water samples, Mn concentration was above the standard limit, and relatively higher in MK site. The fold change of the gene in nematodes exposed to MK water sample was higher than BA exposed nematodes, although it was not significant.

Heat shock proteins are a group of proteins produced by several cells in response to stressful conditions. Exposure of *C. elegans* to harmful environments leads to activation of heat shock response, upregulation of expression of heat shock proteins genes (Kwon *et al.*, 2004). The expression of six heat-shock protein genes was measured of which *hsp-16.2, hsp-16.48* and *hsp-16.1* were significantly up-regulated whereas *sip-1* was significantly down-regulated in response to both water samples. A significant induction of only small heat shock protein genes was observed. Among the small heat shock proteins, *hsp-16.1, hsp-16.2, hsp-16.41* and *hsp-16.48* were activated only in response to heat shock, heavy metals, methanol, and ethanol (Wilson and Kakouli-Duarte, 2009). The two small heat shock proteins, *hsp-16.1* and *hsp-16.2* are involved in resistance to increased oxidative stress (Park *et al.*, 2009, Jones *et al.*, 1996). A spatial variation was observed between the two sites on the expression of the gene, with a higher fold change in BA water exposed nematodes. The increased expression of the genes in response to environmental water is in line with another study in nematodes exposed to River sediment with high level of Cd (Tejeda-Benitez *et al.*, 2016). Like *hsp-16.2, hsp-16.1* expression level was significantly higher in BA than MK exposed nematodes. The high concentration of Mn in the water possibly significantly activates high levels of heat shock proteins. This finding shows that small heat shock protein could be a potential target biomarker for environmental risk assessment.

Metals at low levels are essential for the survival of organisms. However, when they appear at excessive levels, they have a harmful effect by generating free radicals and reactive oxygen species (Kordas, 2010). They are major contaminants of the aquatic environment and thus influence human health (Tchounwou *et al.*, 2012). *C. elegans* gene expression assay can be a good bioindicator for the presence of metals and metal pollution (Huang *et al.*, 2015, Roh *et al*., 2006). Ten metal-associated genes were measured in exposed *C. elegans*. The gene *numr-1,* which is involved in resistance to different heavy metal toxicity (Sahu *et al.*, 2013, Liao *et al.*, 2002), was significantly induced upon exposure to both water samples. Induced expression of the gene in response to laboratory reconstituted heavy metal exposure has been previously reported (Sahu *et al*., 2013, Yu *et al.*, 2016, Kumar *et al*., 2015). The increased expression could be due to the compiled effects caused by complex heavy metal mixtures in the water. The Cd-inducible lysosomal protein-1 (*cdr-1*) gene which is observed in intestinal cells in

response to cadmium exposure (Liao *et al*., 2002, WHO, 1998) was induced only in nematodes exposed to BA water. The difference between the two sites could be due to the presence of a relatively higher levels of Cd in the BA site. The gene is up-regulated in the nematodes exposed to laboratory reconstituted Cd in other studies (Yu *et al*., 2016, Cui *et al.*, 2007). The transcription of *pgp-5* in *C. elegans* is induced by both bacterial infection and heavy metal exposure and its regulation is triggered by *tir-1* (Hintze and Theil, 2006). In the current study, the expression of the gene was downregulated in response to both water samples. The findings are in contrast with the report by Kurz *et.al* (Kurz *et al.*, 2007) which report induction of *pgp-5* in *C. elegans* during infection and heavy metal stress. This discrepancy could be due to the different levels or combination of metals; and they exposed the nematode to laboratory metal cocktails and not environmental water samples. The other heavy metal responding genes coding for ferritin protein which protects cells from iron toxicity is *ftn-1* (Hintze and Theil, 2006). The gene was significantly up-regulated in *C. elegans* exposed to MK water sample only. The high level of iron in MK sites contributes to the induced expression of the gene in nematodes. It can be concluded that the low level of heavy metals together with other xenobiotic pollutants resulted in induction of heavy metal associated genes.

The lifespan of *C. elegans* is determined by the regulation of different genes in response to environmental conditions (Hu, 2007). When the conditions are not conducive to growth at the L1 stage, the nematode proceeds into the dauer stage, which is dormant, sexually immature, stress-resistant, and long-lived and it is controlled by *daf-12* and *daf-16* genes (Antebi *et al.*, 1998). The increased expression of *daf-12* in this study revealed that the water samples were not conducive to the growth of *C. elegans*. Our findings were in contrast with a similar study done in Sweden in *C. elegans* exposed to water from one sampling site (Kumar *et al*., 2015). The presence of a high level of Sr in the Akaki river water has also an effect on *C. elegans* development. Strontium slows down the development of *C. elegans* and extends the life span by altering Ca^{2+} influx signaling (Güner *et al.*, 2018). An increased expression of *daf-16* indicate an increasing lifespan of the nematode (Henderson and Johnson, 2001) and it is negatively regulated by *daf-2*, so *daf-2* mutant nematodes showed increased expression of *daf-16* and increased lifespan. In the current study, *daf-16* was down-regulated. This could be

because the gene was negatively regulated by *daf-2* or bacteria in the sampled water could down-regulate its expression as does *P. aeruginosa* (Evans *et al.*, 2008).

C. elegans is an important model to study innate immunity (Alper *et al.*, 2007). It responds to infections by secreting different antimicrobial molecules (Kato *et al.*, 2002, O'Rourke *et al.*, 2006). In the current study, we measured the transcription of six innate immunity genes and found that the expressions of *tir-1, clec-60* and *tol-1* were significantly up-regulated in exposure to both water samples, whereas *abf-2* and *lys-7* were down-regulated. The gene *clec-60* is essential in the nematode and is regulated in response to microbial infection and microbial product exposure (Sivamaruthi and Balamurugan, 2014). The increased expression of the gene could be due to the bacteria in the water sample as well as microbial products. Our finding was in line with other studies upon exposure to bacteria (Sivamaruthi and Balamurugan, 2014, Bolz *et al.*, 2010). However, the result is in contrast with the study done in Sweden upon exposure to environmental water samples (Kumar *et al*., 2015). This difference could be due to the water in Sweden likely do not contain much bacteria that the immune response genes would not be activated. Toll-like receptor-1 is crucial for pathogen avoidance in *C. elegans* (Tenor and Aballay, 2008). In addition to pathogen clearance, the gene also regulates the expression of *abf-2* and *hsp-16.41* (Tenor and Aballay, 2008). The gene was significantly up-regulated in both water exposures. Although our finding was different from the study using Swedish environmental water samples, it was in-line with laboratory reconstituted metal exposures (Kumar et al., 2015). The other innate immune gene which is involved in microbial defense (Liberati *et al.*, 2004) and organophosphate exposure (Lewis *et al.*, 2013) in *C. elegans* is *tir-1*. Exposure of *nematodes* to Akaki River water caused an increased expression of *tir-1*. Similar to *tol-1, tir-1* was up-regulated to laboratory reconstituted metals but not to environmental water samples in another study (Kumar et al., 2015). The induction of innate immunity genes could be associated with bacterial infection and their products. Some bacterial isolates like *Acinetobacter lwoffii* could have potential nematocidal activities and might activate innate immune-associated genes like *Acinetobacter johnsonii* (Tian *et al.*, 2016).

The *lys-7* gene was down-regulated in response to both water samples. This gene has an important role in fighting against pathogens in *C. elegans* and is increasingly expressed in response to different microbial infections such as *Microbacterium nematophilum* and *Serratia marcescens* (O'Rourke et al., 2006). However, the gene was significantly downregulated despite the presence of many bacteria in the current study. The result is in agreement with another study (O'Rourke et al., 2006) which report increased expression of *lys-7* in *C. elegans* after infection with *M. nematophilum*. Some microbes such as *P. aeruginosa* are known to suppress *lys-7* expression in *C. elegans* so this could contribute to the visible difference in the different studies (Evans et al., 2008). Alternatively, *lys-7* is located downstream of *daf-16* (Miyata *et al.*, 2008) which is down-regulated in the current study and thus could not correctly regulate *lys-7* gene. Genes coding lysozyme proteins are significantly downregulated by a range of bacterial infections in *C. elegans*, for instance, *Ochrobactrum* species infection in *C. elegans* leads to lysozyme reduction (Cassidy *et al.*, 2018). The presence of *Ochrobactrum intermedium* in the water possibly mediates reduction of *lys-7* expression.

It can be concluded from this study that although the levels of most metals were below the permissible limit, the compiled effect resulted in transcriptional changes in *C. elegans*. The use of toxicogenomic risk assessment of water using *C. elegans* could be an alternative approach to determine synergistic or individual pollutant effects at the genetic level.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

These studies together report the widespread levels of antibiotic resistance genes and potential bacterial pathogens in both waters and sediments of the Akaki river system in Ethiopia. The river is a conduit of major clinically relevant ARGs, conferring resistance to carbapenems, 3rd and 4th generation cephalosporins and quinolones, which can be potentially transferred among environmental and pathogenic bacteria in the river contributing to an overwhelming public health risk. The persistence of ARGs in the Akaki river system indicates the environment outside the health care facilities has a significant role in the spread of antibiotic-resistant strains in the community. Most of the resistance genes detected were plasmid-mediated which have an increased potential to be transferred among bacteria including pathogens that can infect humans and animals using the water source. Anthropogenic activities like land irrigation, hospitals, and factories along the river contribute to the diversity and abundance of ARGs since many do not treat waters before releasing them to the river, thus this problem requires a multi-organizational response.

The present investigation showed the presence of virulent and antibiotic-resistant *E. coli* strains in the Akaki river. Toxin-associated virulence determinants were present in the majority of *E. coli* isolates suggests that they are potential human pathogens. The presence of virulent and resistant *E. coli* strains in the water could pose a serious health risk to the public. Numerous *E. coli* in the Akai River were resistant to many clinically relevant antibiotics tested. The β-lactamase genes were the most frequently detected resistance genes, followed by aminoglycosides. Dissemination of ESBL genes, mainly *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{OXA-1}}$, and *bla*$_{\text{TEM-1B}}$ were observed in environmental bacterial pathogens. Majority of the isolates harbored *IncFIB* and *IncFII* replicons that can carry and transfer antibiotic resistance and virulence genes. Our findings highlight the need for an efficient intervention strategy to control antibiotic resistant *E. coli* in the aquatic environment. The majority of *Aeromonas* isolates in the Akaki river contained enterotoxins associated with diarrhea and they were resistant to diverse group of antibiotics tested. β-lactamase genes were predominant resistance genes found in *Aeromonas* spp, in particular *bla*$_{\text{TEM-1B}}$ which was the most common ESBL

detected. Three variants of colistin resistance genes (*mcr-3.17, mcr-3.7* and *mcr-3.25*) were also detected in a few *Aeromonas* isolates. Although a few enzyme-mediated resistance genes were detected in *P. aeruginosa,* efflux pumps were widely distributed in these isolates from the Akaki river. Furthermore, it can be concluded from the toxicogenomic study that although the levels of most metals were below the maximum permissible limit, they initiated transcriptional changes in *C. elegans* and *P. aeruginosa* PAO1. The toxicogenomics study in *C. elegans* shows that there is also other pollutants that can together with the presence of enteric pathogens increase the potential health risk of the community using the water. Pollutants in the Akaki river potentially induce antibiotic resistance especially efflux mediated resistance as observed in *P. aeruginosa* PAO1 gene expression assay.

Recommendations

To effectively implement an antimicrobial stewardship program, a one-health approach is needed, therefore, the role of the environment in the generation and spread of ARGs must also be considered. Furthermore, governmental regulations are needed to control antimicrobial-resistant bacteria and their resistance genes as pollutants in the aquatic environment. This study emphasizes the importance of considering the aquatic environment in the antimicrobial resistance surveillance system in Ethiopia for effective control of the spread of antibiotic-resistant bacteria and resistance genes. Therefore, multidisciplinary collaboration is essential to sharing knowledge and finding solutions to mitigate the impact of antimicrobial resistance on the global one health perspective. The farmers using the Akaki river for irrigation should use the water after treatment. Awareness should be created in the downstream community not to use the river water for domestic purposes and alternative sources of clean water need to be made available. A wider selection of microbes including *Aeromonas* spp. should be considered in the routine bacteriological screenings in gastrointestinal disease. More government investment into research that investigates the directionality of transmission of pathogens in humans and environmental interface from a one-health perspective.

LIMITATION OF THE STUDY

The research does not show the clonal relationship of environmental and clinical isolates and thus is not able to conclude the directionality of transmission of pathogens. All isolates were not sequenced and analyzed for generalizability.

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Annexs

Annex I: Microbial DNA qPCR Arrays

- √ Collect water sample from five sampling sites by immersing the container 15-20 cm below the surface of the river water in sterile bottles in the flow of the stream. Collect a sediment sample from the same sites
- √ Transport the sample to AHRI at 4^{o}C
- √ Prefilter the water sample through 25µm isopore polycarbonate filter
- √ Filter again the flowthrough by 0.22 µm isopore polycarbonate filter
- √ Store the filter at -20℃ or proceed to DNA extraction.
- √ Weight 500mg wet weight of sediment sample from each site
- √ Extract DNA by Qiagen DNeasy PowerWater Kit (Qiagen, USA) for water sample and FastDNA SPIN Kit (MP Biomedicals) for sediment sample
- √ Measure the quality and concentration of DNA by NanoDrop Spectrophotometer (DeNovix, USA)
- √ Stored at -20°C until shipped to Örebro University, Sweden.
- √ Ship the samples to Örebro University in dry ice to prevent degradation.
- √ Prepare the Reaction mix preparation for 96-well formats as follow
    - Microbial qPCR Mastermix: 1275 µl
    - Microbial Genomic DNA: 1µg
    - Microbial DNA-Free Water: variable according to the concentration of DNA sample
    - Total volume per sample: 2550 µl
- √ Preparation for No Template Control
    - Microbial qPCR Mastermix :1275 µl
    - Microbial DNA-Free Water: 1275 µl
    - Total volume per sample: 2550 µl
- √ Remove the Microbial DNA qPCR Array plate from its sealed bag
- √ Add 25 µl of reaction mix to each well
- √ Tightly seal the Microbial DNA qPCR Array with the optical thin-wall 8-cap strips
- √ Place one plate in the real-time thermal cycler

- √ Run qPCR thermocycling conditions for SYBR Green as follow
    - Initial PCR activation step for 10 min at 95°C followed by 40 cycles of 95ºC for 15 s denaturation and 60°C for 2 min annealing and extension.
- √ Calculate the ΔCT for each well using the cycler's software and for profiling ΔΔCT method was used to calculate the fold change.

Annex II: Bacterial Isolation
Water Sample Sample Collection

Materials

- ♣ Long-handled plastic dipper
- ♣ 1L sterile polyethylene bottle
- ♣ Cold chain box/with ice/
- ♣ Glove
- ♣ Leveling marker
- ♣ GPS

Sampling

- ♣ Submersing the bottle with long-handled plastic dipper 15-20cm from the surface (pointing the mouth of the bottle toward the direction of the river flow)
- ♣ Store and transport samples in cold chain

Sample Processing

- Filter the water samples through 25µm polyester filter to remove debris
- Prepare serial dilution of water samples (10 fold dilution series from 10^{-1} to 10^{-8})
- Concentrated the flow-through onto a 0.45µm membrane using a handheld vacuum.
- Remove the membrane filter and place it facing up onto the surface of adequate medium, Chromocult Coliform Agar,Thiosulfate-Citrate-Bilesalts-Sucrose, MacConkey agar, SS agar and Pseudomonas agar
- Incubate at 30°C for 24hrs
- Count *E. coli* and Total coliforms on Chromocult® Coliform Agar and calculate colony forming unit per 100mL

- Subculture colonies on respective media
- Store suspected pure colonies in Skim milk media at -80^{o}C for subsequent experiments

Annex III: Bacterial Identification by MALDI-TOF MS

Required materials.

MALDI disposable plate

MALDI Matrix prepared with acetonitrile and trifluoroacetic acid

Toothpick stick (Autoclaved)

Pipette and 1µL tips (1µl)

Procedure:

1. Pick overnight grown single colony with sterile toothpick stick and apply a thin layer on to the spot plate
2. Overlay the spot with 1µl of the matrix and let it dry for 1min at room temprature
3. Ship the plate to Orebro for analyzing
4. Analyze the spots with MALDI-TOF MS
5. Record the results on to the excel template

Annex IV: Antibiotics Susceptibility Testing

- √ Touch 4 to 5 of bacterial colonies with sterile plastic loop. Emulsify the colonies in 5 ml of sterile saline until the turbidity is approximately equivalent to that of the McFarland No. 0.5 turbidity standard.
- √ Dip a sterile swab into the bacterial suspension and inoculate the surface of an Muler Hinton agar plate.
- √ Place antibiotic disks using dispenser on the agar surface.
- √ Invert the plates and incubate at 37°C for 18 to 24 hours.
- √ Measure and record zone of inhibition and interpret as resistant, intermediate, and susceptible according to the EUCAST standard.

List of antibiotics disks and their concentration used in the current study for Antibiotic Susceptibility Testing

Class	**Antibiotics**	**Abb.**	**Disc Concn. (µg)**
Tetracycline	Tetracycline	TET	30
	Doxycycline	DOX	30
Aminoglycoside	Kanamycin	KAN	30
	Gentamicin	GEM	10
Cephalosporins	Cefoxitin	FOX	30
	Cephotetan	CTT	30
	Cefotetan	CTX	30
	Cefotaxime	CFM	30
	Ceftazidime	CAZ	5
	Ceftriaxone	CRO	30
	Cefepime	FEP	30
Penicillin	Amoxicillin	AML	25
	Amoxicillin-clavulanate	AMC	44/24
	Ampicillin	AMP	10
Fluoroquinolone	Norfloxacin	NOR	5
	Ciprofloxacin	CIP	5
	Nalidixic Acid	NAL	30
Penems	Imipenem	IMP	10
	Ertapenem	MEM	10
	Meropenem	ETP	10
Macrolides	Azithromycin	AZM	15
	Erythromycin	ERY	15
Sulfonamides	sulfamethoxazole / trimethoprim	SXT	23.75/1.25
Phenicols	Chloramphenicol	CHL	30

Annex V: Detection of Virulence Genes in *E. coli* and Aeromonas Species

Polymerase Chain Reaction

1. Subculture from frozen colony on Tryptic Soya Agar (TSA) and incubate overnight at 37℃
2. Inoculate 3-4 colonies on Tryptic Soya Broth (TSB) and incubate overnight at 37℃
3. Transfer 1mL of bacterial suspension to clean Eppendorf tube and extract DNA by Genelut bacterial DNA extraction kit (for *E. coli*) or Phenol- chloroform DNA extraction method (for *Aeromonas*)
4. Level PCR tube for the respective samples and calculate saple volume for DNA 80ng/µL
5. Master mix preparation
    - ✓ For conventional PCR add:
        - 2.5 µL 10X Taq buffer
        - 0.5 µL dntps
        - 1 µL primer mix (both forward and reverse)
        - Template
        - 0.125 µL Taq DNA polymerase and
        - Nuclease free water until 25 µL (Total reaction volume)
    - ✓ For m-PCR
        - 25 µL 2x QIAGEN Multiplex PCR Master Mix
        - 5 µL 10x primer mix, 2 µM each primer
        - RNase-free water
        - Template DNA
        - Total reaction volume 50 µL

6. **Run PCR with thermocycling conditions.**
    - ✓ **For conventional PCR**
        - Initial denaturation for 30s at 95℃ and then 35 cycles were applied individually as follows, 60seconds at 72℃ (extension), and final extension for 10 minutes at 72℃.
    - ✓ **For m-PCR**
        - Apply first thermal cycles for 15 minutes at 94℃, and then 40 cycles individually as follows: 30 seconds at 94℃ (denaturation), 90 seconds at 60℃ (annealing temperature), 90 seconds at 72℃ (extension), and final extension for 10 minutes at 72℃.

Gel Electrophoresis

1. Prepare 2% of agarose solution by mixing 1gm of agarose powder with 50mL 1X TAE buffer.
2. Heat with microwave till the agarose completely dissolved
3. Let the agarose solution cool down to 50℃
4. Add 3µL of Ethidium bromide.
5. Pour the agarose into a gel tray with the well comp and let it solidify (~20-30min)
6. Add 5µL (Depends on the concentration of DNA) and 1µL loading dye (6X loading dye) to PCR tube and mix properly.
7. Place the agarose gel into gel box
8. Fill the box with 1X TAE (Make sure it covers the agarose gel)
9. Carefully load 3µL of ladder into the first loan of the gel
10. Load 5 µL samples accordingly.
11. Run the gel at 80V for 45 min
12. Read the gel by UV machine.

Table 1: Oligonucleotide primer sequences for amplification of virulence genes in *E. coli*

Category	Gene	Primers		Size
		Forward (5'-3')	**Reverse (5'-3')**	
Toxin	EAST1	CCA TCA ACA CAG TAT ATC CGA	GGT CGC GAG TGA CGG CTT TGT	111
	STa	TCTTTCCCCTCTTTTAGTCAG	ACAGGCAGGATTACAACAAAG	166
	LT	ATT TAC GGC GTT ACT ATC CTC	TTT TGG TCT CGG TCA GAT ATG	281
	Stx1	CGC TGA ATG TCA TTC GCT CTG C	CGT GGT ATA GCT ACT GTC ACC	302
	Stx2	GGCACTGTCTGAAACTGCTCC	TCGCCAGTTATCTGACATTCTG	255
Adhesin	F4	GGT CGC GAG TGA CGG CTT TGT	CCA CTG AGT GCT GGT AGT TAC AGC C	792
	F6	TCT GCT CTT AAA GCT ACT GG	AAC TCC ACC GTT TGT ATC AG	333
	F18	GTG AAA AGA CTA GTG TTT ATT TC	CTT GTA AGT AAC CGC GTA AGC	510

Table 2: Oligonucleotide primer sequences for amplification of virulence genes in *Aeromonas* Species

Gene	Forward (5'-3')	Reverse (5'-3')	Size
act	AGAAGGTGACCACCACCAAGAACA	AACTGACATCGGCCTTGAACTC	232
hlyA	GGCCGGTGGCCCGAAGATACGGG	GGCGGCGCCGGACGAGACGGG	597
alt	CCA TCC CCA GCC TTT ACG CCA T	TTT CAC CGA GGT GAC GCC GT	338
ast	ATG CAC GCA CGT ACC GCC AT	ATC CGG TCG TCG CTC TTG GT	260
stx1	ATA AAT TGC CAT TCG TTG ACT AC	AGA ACG CCC ACT GAG ATC ATC	180
stx2	GGC ACT GTC TGA AAC TGC TCC	TCG CCA GTT ATC TGA CAT TCT G	255

Annex VI: Phenol Chloroform DNA Extraction Protocol

1. Incubate bacterial colony overnight in broth medium
2. Transfer 1ml bacterial suspension to 1.5 ml Eppendorf tubes and centrifuge at 6000 rpm for 5 min
3. Decant the supernatant and re-suspend the pellet in 3ml TE buffer
4. Aliquot and store at -80^{o}C for DNA extraction or continue to next steps
5. Centrifuge Eppendorf tube at 8000 rpm for 2 minutes and remove the supernatant
6. Suspend pellet in 70 ul of 1X TE and 330 ul of GES lysis buffer (500 mM Guanidium thiocyanate, 100 mM EDTA, 0.5 % (w/v) *N*-lauryl-sarcosine) and mix with hands for 20 seconds
7. Incubate at room temperature (RT) for 60 minutes
8. Add 170 ul of cold ammonium acetate (4M, pH 5.2), mix with hands for 10 seconds and incubate at RT for 10 mins
9. Add 100 ul of Phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, Phenol pH=8) and mix thoroughly with hand for 10 seconds
10. Centrifuge at full speed for 5 mins
11. Carefully transfer the upper phase to new eppendorf tube without disrupting the interface and repeat PCI step with the upper phase until white interface disappears (usually 3 times)
12. Add 300 ul Chloroform:Isoamylalcohol (24:1) to the upper phase, mix for 10 seconds
13. Centrifuge at full speed for 5 minutes
14. Carefully transfer the upper phase to new Eppendorf tube and repeat Chloroform: Isoamylalcohol step
15. Add 300 ul of Isopropanol to upper phase and mix for 5 seconds.
16. aIncubate overnight in -20^{o}C (or 1 hour in -80^{o}C)
17. Centrifuge at full speed for 15 min at 4^{o}C

18. Discard the supernatant and add 1ml of ice-cold 70% ethanol to the pellet and mix with pipette
19. Centrifuge with full speed for 5 min at 4^{o}C
20. Repeat Ethanol wash step
21. Discard the supernatant
22. Dry the DNA pellet at 40^{o}C for 10 minutes or until all the ethanol evaporates (do not over-dry)
23. Resuspend the DNA pellet in 60 ul Nuclease-free water (incubate at 40^{o}C for 5 minutes if it is difficult to dissolve over-dried pellet)
24. Measure the quality and concentration of DNA

Annex VII: Gene Expression in *C. elegans*

LB plates/LB broth preparation (for OP50 *E. coli* culture)

- √ Dissolve LB broth powder as indicated in required volume of water.
- √ Autoclave
- √ Turn on the water bath at 56 ^{o}C for 30 min
- √ Distribute the LB-agar mixture to petri dishes
- √ Let them solidify at room temperature.

NGM plates preparation (*C. elegans culture*)

1. For 300 ml NGM agar plates mix NaCl (0.9 g), Peptone (0.75 g), Agar (5.1 g) and dH_2O (300ml)
2. Autoclave at Program 4 / 121 ^{o}C for 2 hours
3. Put in a water bath at 56 ^{o}C for 30 min
4. Add the rest chemicals/reagents:
    - Cholesterol (5 mg/ml in EtOH) : 0.3 ml
    - 1M $CaCl_2$: 0.3 ml

- 1M $MgSO_4$: 0.3 ml
- 1M potassium phosphate buffer (pH= 6/ NaOH) : 7.5 ml

5. Distribute NGM agar medium as follows:
    - 7 ml to small petri dishes
    - 15 ml to regular petri dishes
6. Let them solidify at room temperature.
7. Randomly select one plate and incubate for 24h for sterility
8. Keep at 4°C

M9 Buffer preparation

1. For 1 liter M9 buffer mix KH_2PO_4 (3 g), Na_2HPO_4 (6 g), NaCl (5 g), 1 M $MgSO_4$ (1ML) and water (1L)
2. Sterilize by autoclaving.
3. Keep at room temperature

OP50 preparation:

- √ *Inoculate E coli OP50* in LB medium and incubates at 37°C overnight at constant shaking at 200 rpm.
- √ On the following day harvest E. coliOP50 and wash in M9 buffer twice
- √ Pour 500 µl of OP50 suspension in 90mm Petri plates and let it dry at RT

Bleach solution preparation

- ✓ For 6 ml bleach solution mix properly:
    - 20% NaClO,
    - 360µl 50% NaOH
- 4.5ml H_2O

C. elegans Exposure and RT-PCR Procedure

1. Wash Adult *C. elegans* in M9 buffer in 15 ml centrifuge tubes to get rid of the OP50 at 2500 rpm in centrifuge.
2. Add 3ml each of M9 buffer and bleach solution to the worms. Shake vigorously for 1 minute 50 seconds.
3. Wash 2-3 times with M9 buffer followed by bleaching step again for 50 sec.
4. Wash the released eggs with M9 2-3 times,
5. Suspend in 10 ml of M9 and keep in tube rotator overnight for hatching.
6. Collect L1 worms by centrifugation.
7. Prepare test wells by adding 500 µl of the experimental water and a control K medium test and material and 20 µl of the *E. coli* suspension to each well. Five replicates for each water sample and control.
8. For gene expression assay add approximately 1000 synchronized L1 to each well and incubate at 20ºC for 48-hrs.
9. Harvested worms by centrifugation at 2500 rpm and suspend in 700µl of Trizol reagent and store at -80 ºC overnight.
10. In the following day thaw the samples, and homogenized by bead beating to lyse the samples
11. Isolate RNA by Direct-zol™ RNA MidiPrep Kit (Zymo research, USA) following manufacturer's instruction.
12. Measure the quality and extracted RNA using spectrophotometer (DeNovix, USA) at 260 nm
13. Run cDNA synthesis with 1µg of RNA/ sample using qScript cDNA synthesis kit (Quanta Biosciences, USA)
14. Quantify gene expression by SYBR Green qPCR kit (Kappa Biosciences, USA) following manufacturer's guidelines. qPCR will be run with the thermocycling conditions for the qPCR consisted of a denaturation step for 5 min at 95ºC followed by 40 cycles of 95ºC for 2 s and 60ºC for 30 s in CFX96^{TM} Real-Time system (Bio-Rad Laboratories, Canada).
15. Calculate the fold change using ΔΔCT method and analysis fold change difference between control and experimental groups.

Annex VIII: *Pseudomonas aeruginosa* PAO1 exposure and RT-PCR

Media Preparation

1. Prepare nutrient broth with experimental water and a control (MQ)
2. Filter through 0.2µm membrane filter
3. Spread 100µL nutrient broth on TSA plate and incubate at 30℃ for 24hr for sterility checking
4. Keep at 4 ℃

Growth curve determination

1. Prepare a standard suspension of PA01(OD~0.11)
2. Add 200µL of experimental water and a control to each well of 96 well plate (4 replicate to each)
3. Add 10µL of bacterial suspension to each well
4. Read OD at 0hr and incubate at 30℃
5. Read the OD every 30min for 2hr and then every 1hr for 12 hr incubation
6. Construct the growth curve graph

Exposure and Gene Expression

1. Inoculate 3-4 colonies of PAO1 into nutrient broth and incubate overnight at 30℃ with 200 RPM
2. Adjust the OD~0.6 and add 300µL suspension to 3mL of nutrient agar prepared with experimental water and a control (~ 0.5McFarland standards)
3. Incubate at 30℃ for 7:30hr
4. Transfer 1mL into sterile Eppendorf tube
5. Centrifuge at 6000xg for 3min at 4^{o}c
6. Extract RNA by NucleoSpin RNA II (Macherey-Nagel, Du¨ren, Germany) according to the manufacturer's instruction
7. Measure the concentration and the quality of RNA by NanoDrop Spectrophotometer (DeNovix, USA) at 260 nm
8. Measure the quality and concentration of RNA

9. Remove genomic DNA by treating RNA samples with DNase (Heat and Run gDNA removal kit)
10. Prepare cDNA by qScript cDNA synthesis kit (Quanta Biosciences, USA)
11. Select reference gene.
12. Measure gene expression by SYBR Green qPCR kit
13. Calculate the fold change using ΔΔCT method and analysis fold change difference between control and experimental groups.

Annex IX: Material transfer agreement

Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by Addis Ababa University Department of Microbiology, Immunology and Parasitology, Ethiopia (and Örebro University, School of Science and Technology, Örebro Life Science Center-Biology, Sweden in all transfer of research material (samples, derivatives, and specimens) related to the protocol, "Phenotypic and Molecular Characterization of Potential Pathogenic Bacteria in Akaki River and their Biological Effect on Model Organism, Addis Ababa, Ethiopia"

Provider: Berhanu Yitayew, Addis Ababa University, Department of Microbiology Immunology and Parasitology, Ethiopia

Recipient: Professor Per-Erik Olsson, Örebro University, School of Science and Technology, Örebro Life Science Center-Biology, Sweden

1. Provider agrees to transfer to recipient designated (provider) the following research materials /specimen: pellets from water sample, Bacterial colony and Bacterial DNA

The research material will only be used for research purposes as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions. This research material will not be used for commercial purposes such as screening, production or sale for which a commercialization license may be required. Recipient agrees to comply with all National and International guidelines rules and regulations applicable to the Research Project and the handling of the Research Material.

a) Are the research materials of human origin?

Yes ☐ No ☑

b) If yes, are they collected according to the details in the protocol and in adherence to National Research Ethics Review Committee (NRERC) and _____ _____Ethics Review Committee recommendations and their approval?

Yes ☐ No ☐

2. This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project") described with specificity as follows: "Phenotypic and Molecular Characterization of Potential Pathogenic Bacteria in Akaki River and their Biological Effect on Model Organism, Addis Ababa, Ethiopia"

3. In all presentations or written publications concerning the research project, recipient will seek agreement of provider and acknowledge provider's contribution of this research material unless requested otherwise.
4. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the research material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of completion of the project.
5. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the recipient under conditions agreed to in the protocol on shipment of the samples.

This research material is provided as a service to the research community. IT IS BEING SUPPLIED TO RECIPIANT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF ERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right of third parties.

6. The recipient shall notify the provider in witting of any intention, improvement, modification discovery or development to the material or the information made by recipient or parties, collaborating with recipient, here in after referred to as "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
7. The under-signed provider and recipient expressly certify and affirm that the contents of any statements made herein are truthful and accurate.
8. Any additional terms (use an attached page if necessary): None.
9. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise.

The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement

Signature page

For Recipient:

Recipient's Investigator

Professor Per-Erik Olsson

Signature

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DECLARATION

I undersigned declare that this PhD Thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for the Thesis has been duly acknowledged.

Principal investigator: Berhanu Yitayew Sahilu

Signature: _____

Date of submission: _____

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2. Prof. Daniel Asrat

   Signature _____ Date_____