MICROBIAL DIVERSITY AND ANTIMICROBIAL RESISTANCE PROFILE OF BACTERIAL ISOLATES FROM RAW NILE TILAPIA (*Oreochromis niloticus*) MARKETED FOR HUMAN CONSUMPTION IN SELECTED SUB-COUNTIES OF NAIROBI, KENYA

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Microbial Diversity and Antimicrobial Resistance Profile of Bacterial Isolates from Raw Nile Tilapia (*Oreochromis niloticus*) Marketed for Human Consumption in Selected Sub-Counties of Nairobi, Kenya

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2024

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my lovely parents, Mr. Gabriel Mumbo Wanyama and Mrs. Clementine Wanyama and my siblings.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial resistant
API	Analytical Profile Index
ARB	Antibiotic resistant bacteria
BLAST	Basic local alignment search tool
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and agriculture organization
Ha	Hectares
HGT	Horizontal gene transfer
KNBS	Kenya national bureau of statistics
kg	Kilogram
LB	Luria-Bertani
MAR	Multipleantibioticresistanceindices
MDR	Multidrug-resistant
MEGA	Molecular evolutionary genetics analysis
ml	Milli-litres
NCBI	National center for biotechnology information
%	Percentage
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
spp.	Species

TAE	Tris-acetic-EDTA
TVC	Total Viable Count
UV	Ultra violet
µl	Microlitres
WHO	World health organization

ABSTRACT

Nile tilapia (*Oreochromis niloticus*) is a rich source of protein and is increasingly being consumed in urban Kenya. However, fish from multisource pollution waters can harbor antimicrobial resistant (AMR) bacteria that can be transferred to humans through eating or contact of contaminated fish. The ability of AMR transfer can cause the rapid establishment of multidrug resistance (MDR) in bacteria from fish thus creating a food-borne risk to human health. Frequent assessment/monitoring of bacterial contamination and antimicrobial resistance in aquatic products is crucial in reducing the passage of clinically important AMR from fish to humans. This study aimed at determining microbial diversity and antimicrobial resistance profile of bacterial isolates from raw Nile tilapia (*O. niloticus*) for human consumption in Nairobi County, Kenya. A total of 68 *O. niloticus* fish with an average weight of 300.12 ± 25.66 g and body length of 23.00 ± 0.82 cm were randomly sampled from retail markets in five sub-counties of Nairobi County. Bacterial isolates were obtained from the flesh and gills and characterized by morphological and biochemical techniques. Polymerase chain reaction and sequencing were used for identification and evaluation of microbial diversity. Antimicrobial susceptibilities of the isolates were tested by Kirby-Bauer agar disc diffusion method as per the criteria of Clinical Laboratory Standards Institute (CLSI) 2018. Inhibition zone diameters around the discs were measured to the nearest millimeter and classified as resistant, intermediate or susceptible as per the criteria of CLSI 2021.The multi-drug resistant (MDR) isolates were identified by 16S rRNA sequencing and phylogenetic analysis using the Bayesian inference method. The MDR isolates were subjected to PCR-based screening for the detection of gentic markers that code for drug resistance and antibiotic resistance genes. Correlation coefficient was used to analyze the relationship between phenotypic resistance pattern to antibiotics and the presence of antibiotic resistance genes. Data generated was statistically analyzed using Minitab 17.1 software. Tests were conducted at a significance level of 0.05 where probability less than 0.05 was considered significant. From 68 fish samples collected, 106 presumptive bacteria were isolated using selective media.These bacteria were grouped into three. Group 1 represented *E. coli* and *Salmonella* spp., group 2 were *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus* and group 3 represented *C. freundii, Klebsiella* spp., and *Enterobacter* spp. Bacterial contamination was detected in fresh Nile tilapia fish; group 1(42/68, 61.8%), group 2 (44/68, 64.71%) and group 3(20/68, 29.41%). The prevalence of contamination of the fish samples with *Salmonella* and *E.coli* species was 26.47% and 35.29% respectively. In group 2, the most prevalent bacteria were *Proteus* spp. (44.12%), with the rest of the bacterial species registering a prevalence of 10.29%, 4.41%, 2.94%, and 2.94% for *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus*, respectively. Prevalence of presumptive bacteria in group 3 was 4.41%, 16.17%, 8.82% for *Citrobacter freundii, Klebsiella* spp., and *Enterobacter* spp., respectively. Overall phenotypic resistance in group 1 ranged from 5.5% for ceftazidime, chloramphenicol, meropenem, nitrofurantoin and streptomycin and 22.2% for penicillin-G (*Salmonella* species). For *E. coli* phenotypic resistance ranged from 4.2% for ceftazidime and chloramphenicol and 25% for rifampicin.

Multi-drug resistance was observed in three *Salmonella* species and two *E. coli* isolates. The 16S rRNA sequence alignment and phylogenic trees confirmed the identified MDR isolates as *S. typhimurium* and *E. coli*. The presence of β-lactamases, tetracycline, sulfonamide, trimethoprim and aminoglycosides were detected in all the identified MDR isolates. For group 2, AMR was detected in all the bacteria species and were also resistant to atleast one antibiotic except Cefepime (30µg). Additionally, 86.36%, (38/44) of the isolates exhibited multidrug resistance, with higher multiple antibiotic resistance indices (MAR index>0.3) indicating that fresh *O. niloticus* fish were highly contaminated with MDR bacteria. The 16S rRNA sequence alignment, BLASTn analysis, and phylogenetic trees confirmed the identified MDR bacterial isolates as *Proteus mirabilis* and other *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus*. In group 3, antibiotic sensitivity test (AST) showed resistance of the isolates to antibiotics like vancomycin, rifampicin and meropenem. *Citrobacter freundii* was highly resistant to vancomycin, rifampicin and meropenem (3, 100%). *Klebsiella* spp., recorded a resistance of 36.4% to Ampicillin/Cloxacillin and meropenem. *Enterobacter* spp., revealed a high resistance of 50% to rifampicin. None of the isolates showed resistance to chloramphenicol, nitrofurantoin and cefepime. In this study, the overall highest multiple antibiotic resistances (MAR) index recorded in all the bacteria was 0.64 indicating high use or misuse of antibiotics in aquaculture. The following MDR bacterial isolates were identified; *Salmonella* spp., *E. coli*, *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, *V. parahaemolyticus*, *C.freundii*, *Klebsiella* spp., and *Enterobacter* spp. Antimicrobial resistance was detected in all the bacteria species, with each isolate resistant to at least one antibiotic except Cefepime (30µg). PCR amplifications confirmed the presence of multiple antibiotic resistance genes namely *bla*TEM-1, *bla*CMY-2, *tet*A, *tet*C, *Sul*2, *dfr*A7, *str*A, and *aad*A which belonged to β-lactamases, tetracycline, sulfonamide, trimethoprim, and aminoglycosides in MDR bacterial isolates. There was strong correlation between antibiotic-resistant genes and phenotypic resistance to antibiotics of MDR bacteria. This study provides valuable information on the patterns of antibiotic resistance of bacterial pathogens isolated from Nile tilapia fish marketed for human consumption within Nairobi County. The study therefore concluded that raw Nile tilapia fish acts as reservoirs of MDR bacteria and this calls for continuous monitoring and surveillance of bacterial status and hygienic handling of fish during harvesting, transportation and marketing.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

The Nile tilapia (*Oreochromis niloticus*) is one of the most widely cultured fish species in Africa. Even though the Nile tilapia are known to tolerate poor water quality and eat a wide range of food organisms, they cannot withstand high-water temperatures. They also experience spawning due to early sexual maturity before reaching market size. Nile tilapia aquaculture has been embraced by most farmers in Kenya (FAO, 2017). The demand for Nile tilapia in local and international markets has stimulated farmers to intensify their production. Intensive fish production has led to increase in diseases due to high stock densities and poor water quality causing the fish to be susceptible to infections caused by parasites and bacteria (Shoemaker *et al*., 2006; Pulkkinen *et al*., 2010).

Presence of infectious diseases is as a result of changes in ecology, mostly related to human activities, like agricultural practices, environmental degradation, transfer of organisms or technology (Patz *et al*., 2000; Dobson & Foufopoulos, 2001; Jones *et al*., 2008; Murray & Peeler, 2005). In aquaculture, it has been established that agents of most emerging diseases originate from another host species or from another geographical area. However, some studies have shown that changes in a given geographical area can cause unexpected outbreaks from native diseases (Dobson & Foufopoulos, 2001). Bacterial pathogens of economic importance in tilapia include: *Streptococcus iniae,* a gram-positive bacteria that is responsible for reduced production in intensive culture (Shoemaker *et al*., 2000; 2001), *Aeromonas, Edwardsiella, Flavobacterium, Mycobacterium, Vibrio* and *Streptococcus* species. Other species are *Plesiomonas, Klebsiella* and *Pseudomonas* (Cipriano, 2001; 2014; Abowei & Briyai, 2011).

Study by Petronillah *et al*. (2014) revealed that the use of manure from livestock in aquaculture contributed to the increase of pathogenic bacteria that affected the health of the surrounding community and its environs. Transmission of pathogenic bacteria from fish to humans would be as a result of handling the fish or from the fish consumption. Pathogenic bacteria to human that were isolated from fish included *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Enterococcus faecalis*. The study also confirmed that all the bacteria species that had been recovered from fish were as well detected in the initially collected samples of water. The presence of bacteria from enterobacteriaceae family in fish indicated that the aquatic environment had been polluted by faecal contamination representing a potential hazard to humans.

Even though environmental bacteria may pass their resistant genes to aquatic environment, over the years antimicrobial agents have been used in fish farming, resulting to development of antimicrobial-resistant bacteria in aquatic environment and the fish. The accessibility of antimicrobial drugs over the counter in a number of developing countries without the supervision of a veterinarian has contributed to pathogenic bacteria of fish and humans to exhibit antimicrobial resistance. The use of a different varieties of antimicrobial agents, including non-biodegradable antimicrobial agents has ensured their presence in aquatic system for long periods of time contributing to increase of antibacterial resistance in fish pathogens, emergence of antimicrobial resistant bacteria in aquatic environments, and also increasing the potential to transfer these resistant genes to pathogenic bacteria of terrestrial animals and humans (Miller & Harbottle, 2017).

Aquaculture farming is among the fastest-growing sectors in the food production/industry, providing fish for human consumption as a source of protein and fatty acids (Anderson *et al*., 2017). Nile tilapia (*Oreochromis niloticus* Linnaeus, 1958) is the most popular fish in Kenya due to its palatability and economic price and as a result there has been rapid expansion of aquaculture by most farmers (FAO, 2017; Obwanga *et al*., 2020). In addition, the demand for Nile tilapia in local and international

markets has intensified its production by farmers in Kenya. Nile tilapia was first cultured in Kenya in 1924 to boost the livelihoods of communities and to improve nutrition (Adeleke *et al*., 2020; Shrestha *et al*., 2018) and is currently the most cultured fish species representing about 90 % of the national aquaculture production (Obiero *et al*. 2022). However, contamination is one of the main challenging factors either in the ponds or during harvesting or marketing, which can be a source of pathogens and may be a potential source of infection to humans (Kromhout *et al*., 1985). Contaminated fish are unsuitable for human consumption since they can be a source of pathogenic bacteria.

Despite the high nutritional quality that links fish consumption to positive health effects in humans, the unsanitary conditions at fish farms and the occurrence of superbug bacteria in fish products have been reported as worrisome observations. This could pose a threat to human health, especially at this times when the demand of Nile tilapia as a source of animal protein in Kenya seems to be on the increase (Obiero *et al*. 2022). Aquatic ecosystems are vulnerable to many contaminants such as chemicals and drug residues as they are the recipients of run-offs from agricultural or livestock farms and healthcare facilities (Bashir *et al*., 2020, Patel *et al*., 2019). Intensive fish farming has increased the uncontrolled use of antibiotics in the treatment of infections and as growth promoters resulting in the emergence of resistant bacteria strains (Smith, 2008). This is of importance to human health as fish and fish products may be an important vehicle for the dissemination of antibiotic-resistant pathogenic bacteria to other bacteria or directly to humans. Fish and fish products contaminated with human pathogens have been reported in many countries with *Salmonella* species, *Escherichia coli*, *Staphylococcus* species, *Vibrio* species, and *Pseudomonas aeruginosa* being the most important pathogens (Herrera *et al*., 2006; Onyuka *et al*., 2011).

Bacterial contamination is responsible for more than 600 million cases of foodborne illnesses, with 420,000 fatal infections annually (World Health Organization, 2015). This is a huge economic burden to low- and middle-income countries because the estimated cost of treatment is about USD 110 billion per year. Pathogenic bacteria mainly contribute to food-borne infections due to production of toxins in foods,

especially animal-derived foods (Addis & Sisay, 2015). Fish has been identified as one of the reservoirs of pathogenic bacteria linked to human illnesses (Novotny *et al*., 2004). Fish is an integral part of the human diet for many generations and is promoted because of its health benefits, providing a rich source of animal protein, omega-3 fatty acids, vitamin D, selenium, and iodine (Tørris *et al*., 2018). Of particular interest is Nile tilapia (*Oreochromis niloticus*), which has become more popular as indicated by increasing levels of consumption (Obiero *et al*., 2022). However, both undercooked and raw fish can expose consumers to different types of pathogenic bacteria, either from their postharvest handling or their original aquatic environment, storage as well as conditions during processing (Vázquez-Sá nchez *et al*., 2012; Baliere *et al*., 2015). Therefore, pathogenic bacteria can be introduced into fish at any point throughout the production and supply chain. Foodborne pathogens such as *Plesiomonas shigelloides, Salmonella* spp., *Aeromonas* spp., *Proteus* spp., *Yersinia, Shigella, Enterobacter, P. aeruginosa, S. aureus, V. parahaemolyticus, V. cholerae, Bacillus, Klebsiella, Serratia* spp., and pathogenic *Escherichia,* are of importance in fish (Novotny *et al*., 2004) because they are responsible for foodborne illnesses such as diarrhea, gastroenteritis, typhoid fever, and dysentery. These illnesses pose significant health risks, including death, to consumers (Obande *et al*., 2017).

Outbreaks of fish-associated food poisoning are caused by the consumption of raw or insufficiently heat-treated fish contaminated with *Vibrios* from the water environment (*Vibrio* spp.) or terrestrial sources (*Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Pseudomonas* spp.), (Novotny *et al*., 2004). *Vibrios* are Gram-negative bacteria and ubiquitous in aquatic environments such as aquaculture, marine, and estuarine, either free-living in water, sediments or associated with shrimps and fishes (Vezzulli *et al*., 2010). Pathogenic *Vibrios* of major public health importance are *Vibrio vulnificus, V.parahaemolyticus*, and *V. cholerae. Vibrios* species have been observed to be associated with deadly cholera outbreaks globally (WHO, 2021a). For example, the consumption of dried fish was linked with a cholera epidemic in a village (Ifakara) in southern Tanzania in 1997 (Acosta *et al*., 2001). An outbreak of cholera in Germany in

2001 was associated with fresh fish imported from Nigeria (Schurmann *et al*., 2002). Therefore, continuous monitoring of Vibrios in fish is important for food safety control. *S. aureus*, a Gram-positive bacterium, is the most prevalent foodborne pathogen of the genus *Staphylococcus* and among the leading causes of food contamination, and spoils food by producing lethal enterotoxin (Akbar & Anal, 2014).

S. aureus has been reported in fish and fishery products (Vaiyapuri *et al*., 2019). *S. aureus* is not a natural microbiota of fish and therefore its presence in fish is associated with unhygienic handling by fish handlers, processors or sellers, cross-contamination during transport and storage, and contamination by workers, due to the presence of this pathogen in the microbiome of most humans (Saito *et al*., 2011; Hammad *et al*., 2012; Sergelidis *et al*., 2014; Badawy *et al*., 2022). *Proteus mirabilis* is a Gram-negative, rod-shaped bacterium found in the environment, animal microbiota and humans (Drzewiecka, 2016), and an important zoonotic pathogen that causes infections in animals and humans. It causes human urinary tract infections and extraintestinal infections such as respiratory, ear, eye, nose, skin, and wound infections (Sanches *et al*., 2019). *P. mirabilis* causes food poisoning through consumption of contaminated food, and high incidence has been reported in various countries (Gong *et al*., 2019). Therefore, presence of *P. mirabilis* in fish is a threat to the health of the consumer, especially when the specific strain possesses a variety of virulence factors that contribute to human infections. *P. aeruginosa* is an opportunistic bacterium with the ability to inhabit animals, soil, water, and plants, from which it is easily transmissible. It is one of the major causes of bacterial diseases in fish and there is growing evidence of *P. aeruginosa* in foodborne infections (Bricha *et al*., 2009; Nawaz & Bhattarai, 2015; Virupakshaiah & Hemalata, 2016). Various *Pseudomonas* species are multidrug resistant and resistance is likely to evolve over time, which explains why the number of effective antibiotics is decreasing and this may pose threat to public health (Algammal *et al*., 2020; Ayman *et al*., 2022). It is therefore important to determine the role of *O. niloticus* fish as a reservoir of *P. aeruginosa*.

Citrobacter freundii, Klebsiella and *Enterobacter* are Enterobacteriaceae bacteria collectively called coliforms. Coliform bacteria are facultative anaerobic rod-shaped Gram-negative non-spore forming motile or non-motile bacteria that ferment lactose to acid and gas at 35–37°C. These coliforms are considered to be hygiene indicators (WHO, 2011; Halkman & Halkman 2014: Daoliang & Shuangyin 2019; Tominaga & Ishii 2020).

Human pathogens such as *Citrobacter* and *Klebsiella* species when isolated from fish and fish products give an indication about environmental faecal pollution of fish. These bacteria may have been found to survive and multiply in the gut, mucus and tissues of fish and render fish to act as a potential vector of human disease (Wogu & Maduakol, 2010). *C. freundii* is capable of causing diarrhoea and related infections in humans (Samonis *et al*., 2009; Bai *et al*., 2012; Liu *et al*., 2017). The main virulence determinants found in diarrhoea-causing *C. freundii* are toxins; heat stable toxins, Shiga-like toxins and a cholera toxin B subunit homolog (Bai *et al*., 2012).

Three species in the genus *Klebsiella* are associated with illness in humans: *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Klebsiella granulomatis*. In recent years, *Klebsiella* have become important pathogens in nosocomial infections. Infections with *Klebsiella* spp. occur in the lungs, causing inflammation, hemorrhage and necrosis (Nordmann *et al*., 2009). *Klebsiella* spp., possess capsules composed of complex acidic polysaccharides that protects them from phagocytosis by polymorphonuclear granulocytes of the host. They also produce multiple adhesions that help them to adhere to host cells making it easier to infect the host. Lipopolysaccharides (LPS) also contribute to their pathogenicity (Qureshi, 2009).

Enterobacter species are non-spore-forming, flagella-containing, urease positive, and lactose fermenters that cause nosocomial infections, soft tissue infections, urinary tract infections (UTI), osteomyelitis, respiratory infections and endocarditis etc. Pathogenicity of these bacteria depends on use adhesins to bind to host cells, lipopolysaccharide (LPS) capsule that aid bacteria in avoiding opsonophagocytosis (Davin-RegliandPagès, 2015).

Foodborne infections have led to continued use of antibiotics globally leading to antimicrobial resistance in bacteria that were initially sensitive to antibiotics (Doyle, 2015). Indiscriminate use of antimicrobials in aquaculture remains to be the major cause of multidrug resistant bacteria since most aquaculture farmers lack knowledge about the non-biodegradable antibiotics. It is estimated that thousands of deaths occur annually due to Antibiotic resistance and the figure is likely to go higher globally (Momtaz *et al*., 2013; Adzitey, 2020). It is estimated that by 2050, antibiotic resistance might lead to ten million deaths annually (Bengtsson-Palme *et al*., 2018; Praveenkumarreddy *et al*., 2020). Thornber *et al*. (2020) stated that a number of factors favour antimicrobial resistant bacteria in aquaculture; high stocking densities that leads to stress and infections, use of various chemicals, contamination of aquaculture with waste/untreated water, occupational human exposure to AMR bacteria, etc. Antimicrobial-resistant bacteria, including zoonotic and human pathogens have been reported from various aquaculture farms (Cabello *et al*., 2013; Miranda *et al*., 2013; Watts *et al*., 2017).

The rapid emergence of antimicrobial resistance (AMR) in the global ecosystem has become a threat to human, animal and environmental health (Acar *et al*., 2009; WHO, 2020; Ferri *et al*., 2017). Human, animal and environmental reservoirs contribute to AMR. Aquaculture production has been identified as a hot spot for the development of AMR, and transfer of drug-resistant microorganisms between food producing animals and humans (Lulijwa *et al*., 2020; Dewi *et al*., 2022). In particular, fish are reservoirs of zoonotic disease, infecting both the host and humans through food-borne disease or direct contact at the aquaculture facility (Gauthier, 2015).

Studies have reported the presence of antibiotic-resistant *Salmonella* species, *Escherichia coli* in fish and fish products (Onyuka *et al*., 2011; Budiati *et al*., 2013; Le *et al*., 2015). Antibiotic resistance is growing and has affected critically important classes of bactericidal antibiotics used to treat bacterial infections in humans. One of the most important resistance mechanisms in Gram-negative bacteria against beta-lactam antibiotics is induced by the production of beta-lactamases (Bali *et al*., 2010). Extended β-lactamases (ESBL) are plasmid-mediated β–lactamase enzyme recognized for their

remarkable ability to hydrolyze penicillin, 3rd and 4th generation cephalosporins and monobactams except for carbapenem and cephamycin (Hassuna *et al*., 2020). These enzymes emerged from blaTEM-1, blaTEM-2 and blaSHV as narrow-spectrum parents. Recently, blaCTX-M, a new class of ESBL genes, appeared to have gained global attention. The rates of CTX-M producing bacteria have increased worldwide and the situation is more complicated as these enzymes confer co-resistance to other commonly used antibiotic classes (Chandel *et al*., 2011; Xie *et al*., 2020). In aquaculture, a variety of these antibiotics are authorized for use, resulting in the emergence of ESBL-producing Gram-negative bacteria.

The occurrence and increase of bacterial strains resistant to routinely used antibiotics in fish hatcheries and their possible human health implications is calling for intensified surveillance systems. There is limited scientific data on the antimicrobial resistance of food pathogens in fish from retail markets in Kenya. Therefore, monitoring the prevalence of antibiotic resistance microorganisms is necessary to provide knowledge about the magnitude of the problem and help government authorities to evaluate the effectiveness of the control measures.

The study also aimed to determine the prevalence and genetic markers coding for drug resistance potential of foodborne bacterial pathogens in retailed fresh *O. niloticus* fish in markets in Nairobi, Kenya. It also examined the antibiotic resistance of the isolated bacteria species and determined the genetic diversity of MDR bacteria. The findings provide prerequisite information supporting the need for control and prevention of outbreaks associated with exposure to the pathogenic bacteria in Kenya. This study also characterized *Salmonella* spp., *E. coli, Proteus* spp., *S. aureus, P. aeruginosa, V. cholerae, V. parahaemolyticus, Citrobacter freundii, Klebsiella* and *Enterobacter* strains isolated from retail markets of Nairobi County, Kenya, in terms of prevalence, antimicrobial resistance and genetic diversity.

1.2 Problem Statement

The intensive fish production has contributed to emergence of many different diseases because of polluted water and high stock densities causing fish to be susceptible to bacterial infections (Shoemaker *et al*., 2006; Pulkkinen *et al*., 2010). Despite using antimicrobials in aquaculture, antimicrobial resistance (AMR) has been observed in some bacteria causing reduced Nile tilapia production and this is because of microbial diversity in the pathogenic bacteria. The presence of antibiotic resistance in bacterial pathogens is a very big problem in aquaculture. Lack of supervision in the use of chemotherapeutics and antibiotics in aquatic farms as a dietary supplement or to curb fish diseases has contributed to antimicrobial resistant bacteria and resistant plasmids (Miranda *et al*., 2013).The emergence of R-plasmids having virulent factors has enhanced antibiotic resistance in pathogenic bacteria (Sørum, 2006).

Infections as a result of antibiotic resistance have been established making it difficult to have a standard antimicrobial treatment. In aquaculture, it is a common practice to use large amounts of antibiotics as a prophylactic measure. When these drugs remain in the aquatic environment a longer time, resistance to antimicrobials can occur. These resistant genes may be transferred to other bacterial species including human bacteria that are pathogenic (Sørum, 2006). Recreational fisheries, human populations, and other animal rearing systems near aquatic environment have highly contributed to the emergence of antimicrobial resistance (Miranda *et al*., 2013; Cabello *et al*., 2016; Cabello *et al*., 2013).

Transfer of resistant genes to susceptible bacteria is a major problem in aquaculture since bacteria isolates from the different groups originate from different sources into aquatic environment resulting into a high genetic diversity of bacteria isolates hence the presence of multi-drug resistant bacteria (Wamala *et al*., 2018). Transfer of these antibiotic resistant strains can lessen medical treatment options in the market hence increasing deaths from initially treatable diseases (Watts *et al*., 2017).

Further more antimicrobial resistant *E. coli* and *Salmonella* isolates have previously been reported in fish from Lake Victoria (Onyango *et al.*, 2009; Onyuka *et al.*, 2011), which eventually end up in retail markets. Contamination of fish occurs during harvesting, transportation in dirty boats, storage, and use of contaminated water (Onyango *et al.*, 2009; Onyuka *et al.*, 2011). Nairobi County being a cosmopolitan area compared to other counties in Kenya was selected for study.

1.3 Justification

Nile tilapia (*Oreochromis niloticus*) is a rich source of dietary protein and is increasingly being consumed in urban Kenya. In addition, it is cultivated in many regions in Kenya due to its high productivity and adaptation to different culture conditions. However, contamination of fish in the water systems can be a source of food-borne pathogens and could be a potential source of infection to consumers. The determination of diversity of bacterial pathogens in raw Nile tilapia should be checked regularly in order to undertake effectual plan in curbing diseases caused by bacteria. Observation of the microbial quality of fish is also vital in enhancing safety food in the market.

Resistance to antimicrobials is a serious concern experienced in aquaculture farming (Miller & Harbottle, 2017). The capability of fish pathogenic bacteria to resist the effect of new generation antimicrobials reminds the importance of supervision on the use of antimicrobials as well as regular observation and surveillance programs. This study will provide valuable information on patterns of antimicrobial resistance by diverse pathogenic bacteria from Nile tilapia fish, hence proposing effective treatment to consumers.

1.4 Research Questions

(i) What are the antimicrobial resistance patterns of bacteria found in raw fish?
(ii) What is the molecular diversity of multidrug resistant bacteria in raw fish from selected retail outlets in Nairobi County?

(iii) What are the genetic markers coding for drug resistance on the bacterial isolates
(iv) What are the high MAR Indices of MDR bacteria?
(v) What is the relationship between phenotypic resistance pattern to the antibiotics for MDR bacteria and the presence of antibiotic resistance genes

1.5 Objectives

1.5.1 General Objective

To determine the molecular characterization and antimicrobial resistance patterns of microbial isolates present in raw Nile tilapia (*Oreochromis niloticus*) from selected retail outlets in Nairobi County, Kenya.

1.5.2 Specific Objectives

(i) To determine the antimicrobial resistance patterns of bacteria found in raw fish.
(ii) To evaluate molecular diversity of multidrug resistant bacteria in raw fish from selected retail outlets in Nairobi County.
(iii) To determine genetic markers coding for drug resistance on the bacterial isolates
(iv) To determine MAR Indices of MDR bacteria
(v) To determine the relationship between phenotypic drug resistance patterns for MDR bacteria and the presence of antibiotic resistance genes

1.6 Null Hypotheses

(i) There are no antimicrobial resistance patterns of the most prevalent bacteria found in raw fish.
(ii) There is no molecular diversity of bacterial flora in raw fish from selected retail outlets in Nairobi County.
(iii) There are no genetic markers that code for drug resistance present in antibiotic-resistant bacteria isolates.
(iv) There are no high MAR Indices in MDR bacteria.

(v) There is no relationship between phenotypic resistance pattern to the antibiotics for MDR bacteria and the presence of antibiotic resistance genes.

CHAPTER TWO

LITERATURE REVIEW

2.1 Distribution of Nile Tilapia in Africa

Geographically *Oreochromis niloticus* is found in the tropical and subtropical regions of Middle East and Africa (Figure 2.1). This species is mostly found in Niger and River Nile basins, lakes Victoria, Albert, Tanganyika, George, Edward as well as some lakes in Eastern and Western Africa. It is also found in much smaller drainages and in Yarkon River found in the Middle East (Trewavas, 1983).

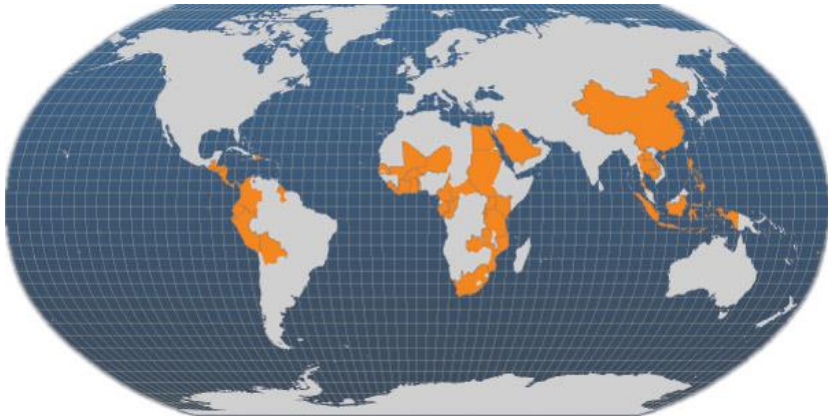

Figure 2.1: Main Producer Countries of Nile Tilapia (*Oreochromis niloticus*).Source: FAO, 2006.

2.2 Nile Tilapia Production and Consumption in Kenya

Nile tilapia represents about 90 % of fish farmed in Kenya (FAO, 2017; KNBS Economic Survey, 2017). Intensive, semi-intensive and extensive are the three major aquaculture systems practiced. Extensive aquaculture systems are the least managed with minimal or zero input in production. Normally production of fish is done in earthen ponds, floating cages and tanks. The fish are left to fend for themselves. The systems

depend on physical conditions and natural productivity water. They produce low yields due to low stocking densities (FAO, 2018). Floating cages are put in dams, rivers and lakes. The fish feed on organic material flowing through the cages thus depending on the natural resources water. *Cyprinus carpio*, *Oreochromis niloticus* and *Clarias gariepinus* are the most cultured. The system yields fish ranges from 500 to 1 500 kg/ha/year and this contributes to 10% in aqua farming industry (FAO, 2018).

Semi-intensive aquaculture is the major production of Nile tilapia in Kenya with an estimate of 3 tonnes/ha representing more than 70% in aqua farming production. This system is widely used in Kenya compared to other two contributing to bulk production in aquaculture. Fish is put in cages and earthen ponds. Feeds such as cereal bran are used as supplements in ponds. Organic and chemical fertilizers are used in varying proportions to fertilize ponds so as to enrich fish production. The fish yield in these culture systems ranges from 1000 to 2500 kilogram/ha/year (FAO, 2018).

Intensive aqua farming mainly uses raceway ponds. This system supports industries like tourism since the fish are cultured as a luxury by supplying to hotels serving mainly tourists. Fish are cultured in floating cages and tanks. Fish production is enhanced by aeration, bio-filtration and adding feeds. Productivity of fish in this aquatic systemis between 10 000 to 80 000 kg/ha/year and this highly depends on management level. Currently, hyper-intensive tilapia culture is being done by use of cages. The system is expected to represent 90 % of all cultured fish in Kenya in terms of value and volume (FAO, 2005).

2.3 Fish Contamination with Pathogenic Bacteria

Increase in human population has increased the dependence on aqua farming in supplying secure, reliable and affordable food supply. Even though food production is fundamental for a healthy reproductive community, antimicrobial resistance has become a threat to aquaculture as well as to human health. The spread of antibiotic resistant strains reduces treatment options at hand hence increasing deaths from infections that

were initially treatable (Watts *et al*., 2017). In a study carried out by Petronillah *et al*. (2014), *Pseudomonas*, *Staphylococcus*, *Escherichia coli*, *Salmonella* and *Shigella*, were the commonly found bacteria pathogens that were related with fish gotten from ponds linked to integrated fish cultivation. The pathogens were due to animal waste that contaminated the fish ponds (Abdelhamid *et al*., 2006). *Shigella, E.coli* and *Salmonella* were recovered from fish samples indicating that ponds were contaminated by animal wastes that originated from livestock manure, which was used as feed in ponds (Petronillah *et al*., 2014).

2.4 Antimicrobial Resistance Burden

Antimicrobials have majorly been used in aquatic environment because of intensive growth of fish farming hence causing serious health problems in aquaculture, human as well as in other animals (Miller & Harbottle, 2017). Farmers have used different types of antimicrobials in huge amounts, including non-biodegradable antimicrobials, which remain in the aquatic system for lengthy periods of time resulting in emerging of bacteria with resistance to antimicrobials in the aquatic systems, growth of antimicrobial resistance in pathogenic bacteria of fish, shifting of resistant genes to bacteria, terrestrial animals and ultimately becoming pathogenic to people along with altering bacterial flora in water column and sediments (Pathmalal, 2018).

Pathogenic bacteria transfer antimicrobial resistant genes to susceptible bacteria via horizontal gene transfer which allows genetic exchange within microbial populations. Aquaculture environment harbour huge numbers of different species of bacteria, which are as a result of present and previous use of prebiotics, probiotics and antibiotics. These aquatic environments are “genetic hotspots” for resistant gene transfer and this can propagate the development of future resistance profiles (Banquero *et al*., 2008).

Bacterial pathogens are among the priority microbial fishborne hazards, and the carriage of antimicrobial resistance (AMR) by these bacteria or other fish microbiota adds a further dimension to their importance. According to the World Health Organization

(WHO), antimicrobial resistance is among the top 10 global public health threats (WHO, 2021b). The development and spread of AMR is propagated by the use of antibiotics for growth promotion and prophylactic measures. Regular monitoring and surveillance of antibiotic-resistant bacteria that contaminate foods may help to track the cause of foodborne diseases and lead to appropriate safety policy for interventions, prevention and/or effective treatment of foodborne diseases. Bacteria species belonging to the Enterobacteriaceae family are included in AMR surveillance programmes worldwide. Previous studies have shown that foodborne pathogens isolated from fish could be resistant to various antibiotics, including methicillin (Sergelidis *et al*., 2014). Fish may potentially facilitate the spread of antimicrobial resistance determinants to other bacteria species through horizontal gene transfer, thus making them a threat to public health and safety (Walsh *et al*., 2008).

Multidrug resistance (MDR) has increased all over the world and is considered a public health threat (Catalano *et al*., 2022). Several recent investigations reported the emergence of multidrug-resistant bacterial pathogens from different origins, which increases the necessity of the proper use of antibiotics, routine applications of the antimicrobial susceptibility testing to detect the antibiotic of choice, and screening of emerging MDR strains (Abolghait *et al*., 2020; Algammal *et al*., 2020; Algammal *et al*., 2022). MDR in bacteria may be generated by several mechanisms. First, bacteria may accumulate multiple genes each coding for resistance to a single drug within a single cell, and this accumulation typically occurs on resistance (R) plasmids. Moreover, multidrug resistance may also occur due to the increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs (Catalano *et al*., 2022). Finally, MDR can be developed by enzymatic inactivation of the drugs through their degradation or by transfer of a chemical group to them (Reygaert, 2018).

Microbial genetic markers coding for drug resistance are molecules produced by pathogenic microorganisms and have the ability to evade their host defenses and cause disease. These molecules range from secreted products such as enzymes, toxins, and exopolysaccharides, to cell surface structures such as lipopolysaccharides, capsules,

lipoproteins, and glycoproteins. Some secreted molecules can manipulate the host cell machinery and hence cause infection (Jorge, 2020). Given the widespread consumption of *O. niloticus* in urban communities in Kenya, there is a need to conduct a surveillance study to determine the safety of *O. niloticus* consumed. The subject of multidrug resistance in aquatic environment has received little attention even though there's rapid drug resistance by different pathogens. Enterobacteriaceae are indicators of feacal contamination (Stange *et al*., 2016; Adegoke *et al*., 2020) hence the bacteria contaminated fish when handled or consumed will cause negative effect to human health which may result to treatment failures and in chronic cases may lead to deaths (Bueno *et al*., 2018).

Multidrug resistance in Enterobacteriaceae has become a major problem globally owing to the fact that they possess beta-lactamases such as extended-spectrum beta-lactamases (ESBLs) and carbapenemases which confer resistance to penicillins, first-, second- and third-generation cephalosporins, and aztreonam (Paterson & Bonomo, 2005).

Antibiotic resistance of *C. freundii* has increased worldwide, and some strains harbored extended-spectrum β-lactamase (ESBL) (Park *et al*., 2005; Moland *et al*., 2006; Choi *et al*., 2007) and plasmid-mediated quinolone resistance (PMQR) determinants (Shao *et al*., 2011). *Enterobacter* spp., possess beta-lactamases which is key to antimicrobial resistance. These beta lactamases are capable of hydrolyzing the beta-lactam ring found in cephalosporins and penicillin (Davin-RegliandPagès, 2015). *Klebsiella* spp., produce biofilm and harbour extended-spectrum β-lactamase (ESBL) (Ballén *et al*., 2021).

2.5 Antimicrobial Use and Antimicrobial Resistance in Aqua Farming

Over the years antibiotics have been used in aquaculture as prophylactic, feed additives or as therapeutic. Most farmers do not employ the supervision when using antimicrobials in aquafarming in terms of quantity and therefore information on the antimicrobials used is scarce in many countries (Smith, 2008). Local regulations monitor the use of antimicrobials in aquatic systems and these directives vary depending on the country. In

countries like Japan, Europe, and North America, there are strict laws on the use of antibotics in aqua farming. In developing nations, where 90% of the world's aqua farming production is practiced, they lack rules that enforce the use of antibiotics hence high variability in the use of antimicrobials (Chuah *et al*., 2016).

Mutation and horizontal gene transfer (HGT) are ways through which bacteria get antimicrobial resistance (AMR) in the environment. This occurs via natural transformation, conjugation or transduction (Chamier *et al*., 1993; Wright, 2007; Iwasaki & Takagi, 2009; Aminov, 2011). Use of animal wastes or human wastes as well as practising of integrated fish farming contaminate aquatic environment with antibiotics. This is evident in the increase of food-borne infections caused by pathogenic bacteria resistant to antibiotics (Subasinghe *et al*., 2005). Studies show that antibiotic resistant pathogens in animals and humans are due to the use of antibiotics in food animals (Van den *et al*., 2002; Teuber *et al*., 1999). Many farmers fertilize their ponds by use of animal's wastes (Sørum & Sunde, 2001). Therefore contact between human, animals and aquatic environment is responsible for transmission of antimicrobial resistant (AMR) bacteria to aquatic system leading to transfer of antibiotic resistant factors to fish (Cantas *et al*., 2013).

Bacteria that acquire antimicrobial resistant genes may remain in the aquatic system for long, even after selective force stops (Tamminen *et al*., 2011). The long-term use of antimicrobials in aquafarming enhances selective pressure on bacteria species, even at antibiotic concentrations lower than minimum inhibitory concentration of susceptible wild type population (Gullberg *et al*., 2011), also increasing the rate of HGT (horizontal gene transfer), including fish and human bacteria. Antimicrobials being non-biodegradable and stable, they can remain in commercialised fish consumption (Cabello, 2006; Santos & Ramos, 2016).

2.6 Antibiotic Resistance Genes

Overuse of antibiotics in aquaculture creates selection pressure on bacteria in fish to acquire and maintain antibiotic resistance genes. This results into an increase in the number of resistant strains. Various ARGs of bacteria can confer resistance to different types of antimicrobials. These resistance genes can be classified based on the class of antimicrobials they are resistant to for example; tetracyclines (tet), *str*A (streptomycin), sulfonamides (sul), cat (chloramphenicol) β-lactams (bla), dfrA (Trimethoprim), aminoglycosides (aad) (He *et al*., 2020).

2.7 Extended B-Lactamases

Extended β-lactamases (ESBL) are plasmid-mediated β–lactamase enzyme recognized for their remarkable ability to hydrolyze penicillin, 3rd and 4th generation cephalosporins and monobactams except for carbapenem and cephamycin (Hassuna *et al*., 2020). These enzymes emerged from *bla*TEM-1, *bla*TEM-2 and *bla*SHV as narrow-spectrum parents. Recently, *bla*CTX-M, a new class of ESBL genes, appeared to have gained global attention. The rates of CTX-M producing bacteria have increased worldwide and the situation is more complicated as these enzymes confer co-resistance to other commonly used antibiotic classes (Chande *et al*., 2011; Yu *et al.*, 2020). In aquaculture, a variety of these antibiotics are authorized for use, resulting in the emergence of ESBL-producing Gram-negative bacteria.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was done in five sub-Counties namely Kasarani, Makadara, Westlands, Embakasi, and Langata in Nairobi County (Figure 3.1). The study site was located in the following coordinates; Kasarani (latitude -1.227841 and longitude 36.905729), Makadara (latitude -1.296140 and longitude 36.871042), Westlands (latitude -1.2683 and longitude 36.8111), Embakasi (latitude -1.3000 and longitude 36.9167) and Lang'ata (latitude -1.366667 and longitude 36.733333).This study targeted retail markets in the selected five sub-Counties where the residents of Nairobi commonly purchase Nile tilapia fish. Fish sources were Lake Victoria in Kisumu County and five selected ponds in Nyeri County.

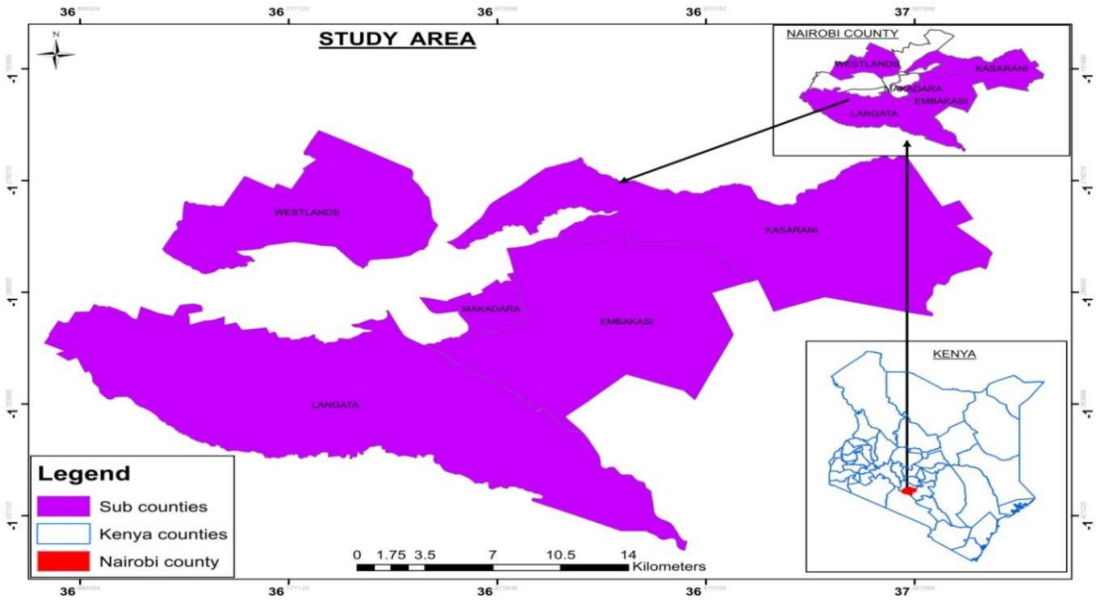

Figure 3.1: The Five Sub-Counties of Nairobi County Where Fish Samples for the Study Were Obtained.

3.2 Determination of Sample Size

The formula by Fisher *et al*. (1999) was used to calculate the sample size based on 25% prevalence of *Escherichia coli* (Shimaa *et al*., 2016).

$$\text{Sample size (n)} = \frac{Z^2 P (1-P)}{d^2}$$

n=Sample size

Z= Statistic corresponding to the level of confidence; 95% or 99%

P= Expected prevalence that can be obtained from same studies

d= Precision (corresponding to effect of size); is selected according to the amount of "P"

Overall 68 fish samples were collected during the one year three months study period.

3.3 Ethical Considerations

Ethical review and approval was not required for the non-live fish samples because it is not needed in accordance with the local legislation and institutional requirements. The fish samples were collected from fish vendors in markets upon obtaining their permission.

3.4 Sample Collection

Simple random sampling within a cross-sectional study design was employed and a total of 68 fish samples were collected. Samples were collected between January 2020 and March 2021.The samples collected from each of the sub-Counties were 14 each for Kasarani, Makadara, Westlands sub-Counties and 13 each for Embakasi and Langata.

The number of fish collected in every sub-County depended on the availability of fish vendors. The fish samples were collected in sterile, transparent, zip-lock polypropylene bags and transported in cool boxes with ice packs to the Microbiology section at the Department of Biochemistry, University of Nairobi, within 1 hour of purchase, and analysis was conducted within 3 hours of collection.

3.4.1 Inclusion Criteria

Only intact fish were sampled. Only flesh and gills were homogenized since they are the main edible parts of Nile tilapia fish.

3.4.2 Exclusion Criteria

No cut pieces of fish were selected. Non-flesh and non-gills were not homogenized since they are not edible.

3.5 Preparation of Fish Samples, Isolation and Identification of Pathogenic Bacteria

O. niloticus fish samples were aseptically dissected to obtain tissue samples (flesh and gills) and were prepared for bacteriological examination according to the ISO 6887-3:2003 standard (ISOstandard 6887, 2003).

3.5.1 *Salmonella* spp. and *E. coli* Analysis

The media used were xylose lysine deoxychocolate agar (HIMedia Laboratories Pvt. Mumbai, India), triple sugar iron (TSI) agar (HIMedia Laboratories Pvt. Mumbai, India) (for *Salmonella* spp.), brain-heart infusion (BHI) agar (HIMedia Laboratories Pvt. Mumbai, India) (for both *Salmonella* spp. and *E. coli*), and MacConkey Agar (HIMedia Laboratories Pvt. Mumbai, India) and Eosin Methylene Blue (EMB) agar (HIMedia Laboratories Pvt. Mumbai, India) (for *E. coli*). Fish were aseptically dissected to obtain 15 g of the sample (the gills and flesh of fish) which were added to 50 ml buffered

peptone water (HIMedia Laboratories Pvt. Mumbai, India) and homogenized together using a stomacher 400 circulator (Seward Ltd, England). Five milliliters of each tissue homogenate was analyzed for any enterobacteriaceae. The homogenate was inoculated on Brain-heart infusion agar. The inoculated plates were incubated at 37 °C for 24 to 48 hours. Sub-culturing was done on MacConkey, Eosin Methylene Blue (EMB) and Xylose Lysine Deoxycholate agar plates to obtain pure cultures of the respective bacteria isolates (Kar *et al*., 2017). The bacterial isolates were also confirmed by standard morphological characteristics. The microorganisms were further confirmed with biochemical tests as described by Kar *et al*. (2017).

3.5.2 *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus* Analysis

For analysis of *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus*, the fish samples were homogenized and subjected to various procedures as follows:

Vibrio spp.: The homogenate was inoculated in 6 ml of alkaline peptone water (APW), pH 8.6, for enrichment, and incubated at 37° C for 8 hours. Two loopfuls of APW from the surface and topmost portion of the broth were inoculated on CHROMagar™ Vibrio (CHROMagar, Paris, France). Characteristic green-blue to turquoise blue (*V. cholerae*) and mauve colonies (*V. parahaemolyticus*) were picked and purified on thiosulfate-citrate-bile-salts-sucrose agar (Oxoid, Thermo Fischer Scientific, Lenexa,United States) with incubation at 37°C for 2 hours. The suspected colonies were re-streaked on tryptone soy agar (Oxoid, ThermoFischer Scientific) supplemented with 3% sodium chloride (TSA +3% NaCl) and incubated at 37°C for 2 hours to obtain pure isolates. The suspected *V. parahaemolyticus* colonies were confirmed by API 32E and VITEK 2 Compact, and later by halo tolerance test with different concentrations of NaCl.

S. aureus: The homogenate was inoculated on Baird-Parker agar and incubated at 37°C for 48 hours. Staphylococcus isolates were detected using the ISO6888-3:2003 + AC: 2005 method (ISO Standard 6888, 2003). Characteristic gray-black colonies with a halo were picked and sub-cultured on mannitol salt agar plates to obtain pure cultures. The coagulase-positive colonies obtained were then confirmed using API Staph and VITEK 2 Compact for Gram-positive bacteria. *Proteus* spp.: A 1 ml aliquot of the homogenate was inoculated on blood agar (HiMedia, Mumbai, India) and incubated at 28°C for 24 hours. The colonies were inoculated into 5 ml tryptone soya broth (TSB; HiMedia, Mumbai, India) in falcon tubes and incubated at 27°C for 24 hours. The inocula were streaked on xylose lysine deoxycholate Agar (XLD) and MacConkey agar (HiMedia, Mumbai, India) plates. The presumptive colonies of *Proteus* spp. with black colonies on XLD and pale or colorless colonies on MacConkey agar were selected.

P. aeruginosa: A 1 ml homogenate was inoculated in nutrient broth for enrichment and incubated at 37°C for 24 hours followed by culturing on pseudomonas cetrimide agar (PCA) (Labobasi, Mendrisio, Switzerland) plates (as selective media culture) and incubated at 37°C for 24 hours. Green-blue-pigmented colonies were considered colonies suspected of containing the *Pseudomonas* genus. Biochemical tests, including fermentation of lactose, indole, citrate, and oxidase, and hemolysis in blood, were performed to confirm *P. aeruginosa*. Colonies containing lactose-negative, citrate-positive, indole-negative, oxidase-positive and hemolytic bacteria were identified as *P. aeruginosa*.

3.5.3 *Citrobacter freundii, Klebsiella* spp., and *Enterobacter* spp. Analysis

Citrobacter freundii, Klebsiella spp., and *Enterobacter* spp.: One ml of each homogenate was inoculated on nutrient agar. The inoculated plates were incubated at 37 °C for 24 hours. Pure cultures were obtained by sub-culturing on MacConkey (Oxoid, UK), XLD and Eosin-Methylene blue (Oxoid, UK), Cystine Lactose Electrolyte Deficient (CLED) agar plates for isolation of *C. freundii, Klebsiella* spp., and *Enterobacter* spp., (Murray *et al*., 2017). The bacterial isolates from the fish were

identified by Gram staining (Petersen & MCLaughlin, 2016) and biochemical tests that included motility, catalase, Oxidase, Indole, Methyl Red, Nitrate reduction and urease tests (Hemraj *et al*., 2013). The suspected *C. freundii* colonies were confirmed by API 20E.

3.6 Enumeration of Total Bacterial Load

The homogenized samples were serially diluted (10^{-1} to 10^{-3}) and cultured onto their respective media (Slaby *et al*., 1981). The plates were inverted and incubated at 37 °C for 18 to 24 hrs. The bacterial load was determined by counting the number of discrete colonies using the viable plate count method (Collins *et al*., 1984), (Appendix I).

3.7 Antimicrobial Resistance Testing

The antimicrobial resistance test was performed using the Kirby–Bauer disk diffusion method (Mohamed *et al*., 2019) according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2018). Antibiotic discs (HiMedia, India) used were penicillin (10 µg), vancomycin (30 µg), rifampicin (5 µg), ampicillin (10 µg), cefepime (30 µg), cefpodoxime (10 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), ceftazidime (30 µg), meropenem (10 µg), and streptomycin (10 µg). These antimicrobials were selected based on the availability and upon the recommendation of World Health Organization and World Organization for Animal Health (OIE) for use in both human and food-producing animals (OIE, 2015; WHO, 2018). Sterile glass rods were used to streak the entire surface of Mueller–Hinton agar plates and antibiotic discs were applied aseptically on the Mueller–Hinton (MHA) (Oxoid Basingstoke, England) using an antibiotic dispenser (Mast Diagnostics, UK) and incubated for 18-24 hours at 37°C. The antibiotic discs were employed to determine the isolates' resistance patterns against 11 selected antibiotics (Table 3.1). Inhibition zone diameters around the discs were measured to the nearest millimeters and classified as resistant (R), intermediate (I), or susceptible (S) by measurement of inhibition zone diameters as per the criteria of Clinical Laboratory

Standards Institute 2021 (CLSI, 2021). The tested isolates were classified as MDR and XDR as described by Magiorakos *et al*. (2012).

Table 3.1: List of Antibiotics Used to Determine the Antibiotic Resistance Patterns of the Bacterial Isolates

Categories		**Class of antibiotics**	**Sub-classes of antibiotics**	**Antibiotics**
Cell wall inhibiting and disrupting membrane antibiotics	β-lactams	Penicillins	Natural penicillins	Penicillin-G (PEN, 10 µg)
			Aminopenicillins	Ampicillin/Cloxacillin (AX, 10 µg)
		Cephalosporins	3rd generation	Ceftazidime (CAZ, 30 µg),
				Cefpodoxime (CPD, 10 µg),
			4th generation	Cefepime (CPM, 30 µg),
		Carbapenems		Meropenem (MRP, 10 µg),
		Glycopeptides		Vancomycin (VAN, 30 µg)
Nucleic acids inhibiting antibiotics	Inhibiting RNA synthesis Antibiotics	Rifamycins		Rifampicin (RIF, 5 µg)
	DNA inhibitors antibiotics	Nitrofurans		Nitrofurantoin (NIT, 300 µg)
Protein synthesis inhibiting antibiotics	30S subunit inhibitors	Aminoglycosides		Streptomycin (STR, 10 µg)
		Phenolic derivatives		Chloramphenicol (CHL, 50 µg)

3.8 Phenotypic Detection of ESBL Production

Phenotypic testing of ESBL production was tested by the Double Disc Synergy Test (DDST) (Kaur *et al*., 2013) by using a disc of amoxicillin with clavulanate (20/10 µg) along with cefotaxime (30 µg) and ceftazidime (30 µg).

A standard inoculum (0.5 McF) of the *Salmonella* spp. and *E. coli* isolates were swapped on the surface of MuellerHinton II (MH II) agar plates (Biolife, IT). An amoxicillin with clavulanate (20/10 µg) disc was placed at the center of MH II agar plates while discs of cefotaxime (30 µg) and ceftazidime (30 µg) were placed in close proximity of 15 mm distance. Any distortion or increase in the inhibition zone of cephalosporin antibiotics towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production.

3.9 Molecular Identification and Characterization of Multidrug Resistant (MDR) Bacteria

3.9.1 Genomic Deoxyribonucleic Acid Extraction, Quantification and Quality Check

Genomic DNA was extracted by phenol/chloroform method. Bacterial culture was grown in LB medium at 36.5 °C overnight (O/N) with shaker at 200 rpm. About 1.5 ml of the overnight bacterial culture was poured into 2 ml Eppendorf tube and centrifuged at 12000 rpm for 60 seconds to get a pellet. The supernatant was discarded. Then 600 µl lysis buffer [9.35 ml Tris-EDTA buffer, 600 µl of 10% Sodium Dodecyl Sulfate and 60 µl of Proteinase K (20 mg ml^{-1})] was used to resuspend the cell pellet. This was vortexed to form a suspension then incubated 1 h at 37 °C. Equivalent amount of chloroform/phenol was added and the tube shaken slowly upside down, a homogenous mixture was seen. The mixture was centrifuged at 12000 revolutions per minute for 5 minutes at room temperature. The upper aqueous phase was poured into a new Eppendorf tube by using 1 ml pipette. Equivalent amount of chloroform was added to

the upper layer (to remove phenol) and mixed by inverting the tubes. The mixture was centrifuged at 12000 revolutions per minute for 5 minutes at 25 °C. The upper layer was transferred into a clean Eppendorf tube. DNA was precipitated by adding 3 volumes of cold absolute ethanol and mixed gently. The mixture was centrifuged at 12000 revolutions per minute for 10 min to form a DNA pellet. The supernatant was discarded and the DNA pellet washed two times with 70% ethanol. The ethanol was removed by 1 ml pipette and the tubes were inverted on a piece of paper towel to completely remove the DNA droplets from the tubes. The tubes were air dried for 10 to 20 minutes. DNA pellet was suspended in 50 µl TE buffer [10 mM Tris-HCl (pH 8.0), 1mM EDTA]. The pellet was allowed to dissolve and stored at –20 °C till use.

The quantity of DNA was determined by measuring the absorbance using spectrophotometer (Nanodrop 2000c spectrophotometer, Thermoscientific, USA). The optical density (OD) at wavelengths of 260 and 280 nanometres was employed assessing DNA's purity. Optical Density between 1.8 and 2.0 denoted an absorption in the UV range by nucleic acids, those below 1.8 indicated presence of proteins and/ or other UV absorbance, while those higher than 2.0 indicated possible contaminations with chloroform or phenol. The DNA integrity was assessed and validated using 0.8% agarose gel electrophoresis, (Appendix II).

3.9.2 Polymerase Chain Reaction Amplification Using 16S rRNA Primers

The extracted DNA was used as a template for PCR amplification of the 16S rRNA gene using 27F 5′-GAGTTTGATCCTGGCTCA-3′ and 1492R 5′-TACGGCTACCTTGTTACGACTT-3′ oligonucleotide primers (Fontana *et al*., 2005). A total of 50 ml reaction mixture was prepared with 2 ml of template DNA (100 ng/ml), 0.5 ml each of forward and reverse primer pair, 25 ml of GoTaq Green Master Mix (Promega, Madison, United States), and nuclease-free water up to 50 ml. Recycling conditions and time of the primers during PCR are shown in Table 3.2.

The PCR products were resolved by gel electrophoresis on 1.5% (w/v) agarose gel (Qiagen, Hombrechtikon, Switzerland). The gels were viewed under a gel imager (Bio-Rad Gel Doc XR System, United States).

Table 3.2:16S rRNA, Genetic Markers and Antibiotic Resistance Genes Primer Sequences, Expected Amplicons Sizes and PCR Cycling Conditions

Target microorganism	Target gene	Primer sequence (5′ → 3′)	Amplicon size (bp)	PCR cycling condition	Reference
All bacteria	16SrRNA	F: GAG TTT GAT CCT GGC TCA R: TAC GGC TAC CTT GTT ACG ACT T	1500	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 40 s and final extension at 72 °C for 7 min	(Fontana *et al*., 2005)
Salmonella	*InvA* (Genetic marker)	F: ACA GTG CTC GTT TAC GAC CTG AAT R: AGA CGA CTG GTA CTG ATC GAT AAT	244	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Bhatta *et al*., 2007)
Salmonella	*hilA* (Genetic marker)	F:CGTGAAGGGATTATCGCAGT R: TCCGGGAATACATCTGAGC	600	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Nora *et al*., 2002)
E. coli	*uidA* (Genetic marker)	F: AAA ACG GCA AGAAAA AGC AG R: ACG CGT GGT TAACAG TCT TGC G	147	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 40 s and final extension at 72 °C for 7 min	(Tsai *et al*., 1993)
Antibiotic resistance genes	^{bla}TEM-1	F: TTG GGT GCA CGA GTGGGT R: TAA TTG TTG CCG GGA AGC	500	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Fang *et al*., 2008)
	^{bla}CMY-2	F: ATA ACC ACC CAG TCA CGC R: CAG TAG CGA GAC TGC GCA	600	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Fang *et al*., 2008)
	^{bla}CTX-M	F: CGC TTT GCG ATG TGC AG R: ACC GCG ATA TCG TTG GT	590	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Edelstein *et al*., 2003)

Target microorganism	Target gene	Primer sequence (5′ → 3′)	Amplicon size (bp)	PCR cycling condition	Reference
	^{bla}Z	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	490	94 °C for 5 min followed by 30 cycles of denaturation 94 °C for 30 s, annealing 60 °C for 30 s, extension 72 °C for 90 s and final incubation at 72 °C for 5 min	(Baddour *et al*., 2007)
	catI	F: AGTTGCTCAATGTACCTATAACC R: TTGTAATTCATTAAGCATTCTGCC	280	5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min and final incubation at 72 °C for 5 min.	(Maynard *et al*., 2004)
	sul2	F: CGG CAT CGT CAA CAT AAC C R: GTG TGC GGA TGA AGT CAG	720	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Ribeiro *et al*., 2011)
	tetA	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	210	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Ng *et al*., 2001)
	tetC	F: CTT GAG AGC CTT CAA CCC AG R: ATG GTC GTC ATC TAC CTG CC	418	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Ng *et al*., 2001)
	dfrA7	F: GGT AAT GGC CCT GAT ATC CC R: TGT AGA TTT GAC CGC CAC C	280	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Grape *et al*., 2007)
	strA	F: CTT GGT GAT AAC GGC AAT TC R: CCA ATC GCA GAT AGA AGG C	548	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min	(Velusamy *et al*., 2007)
	aadA	F: GTG GAT GGC GGC CTG AAG CC R: AAT GCC CAG TCG GCA GCG	525	5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s and final extension at 72 °C for 7 min	(Velusamy *et al*., 2007)

3.9.3 Purification of Amplified Products and Sequencing

A QIAquick kit (Qiagen, Hilden, Germany) was used to purify amplicons according to the manufacturer's instructions (Appendix III), and they were sequenced using the Sanger sequencing (Dideoxy sequencing of DNA) (Macrogen, Netherlands).

3.9.4 Phylogenetic Analysis

The obtained sequences were edited manually using BioEdit v7.0.5.3 (Hall, 1999) to remove gaps and minimize insertions and aligned in MUSCLE (Edgar, 2004). BLASTn searches were done using target sequences from the study isolates and compared with those obtained from the GenBank database (Altschul *et al*., 1997) for identification of bacteria. The homologous sequences to the queries were selected based on the Expectation value (E value) as well as query coverage and percent identity. Phylogenetic trees were constructed with closely related GenBank sequences using the Bayesian inference method by MrBayes software v3.2.7 (https://nbisweden.github.io/MrBayes/).The resulting phylogenetic trees constructed by MrBayes were visualized on FigTree software v1.4.4 (http://tree.bio.ed.ac.uk/ software/figtree/).

3.10 Molecular Detection of the Antimicrobial Resistance and Genetic Markers Coding for Drug Resistance

Genomic DNA of MDR bacterial isolates were used for PCR amplification of the antibiotic resistance and genetic markers. The PCR reaction mixture consisted of 12.5 µl GoTaq Green Master Mix (Promega, United States), 1 µl DNA extract (50 ng/µl), and 0.5 µl each of the forward and reverse primer pair, and was topped up with nuclease-free water to 25 µl. PCR cycling conditions and primer sequences are shown in Table 3.2. The reaction was done using a ProFlexPCRsystem (Applied Biosystems™, United States). The primers used were b-lactamase-encoding genes (blaTEM-1 and blaCMY-2), tetracycline-resistant genes (tetA and tetC), sulfonamide-resistantgenes (sul2), trimethoprim-resistant genes (dfrA7), and aminoglycoside-resistant genes (strA and aadA) for antibiotics (Table 3.2).The

genetic markers were tested in *Salmonella* spp. (invA and hilA) and *E. coli* (uidA) isolates (Table 3.2). The PCR products were resolved by electrophoresis on a 1.5% (w/v) agarose gel with 1× Tris-HCl-EDTA (TE) buffer and then allowed to run for 1 hour at 100 V. The gels were viewed and photographed under a gel imager (Bio-Rad Gel Doc XR System, United States).

3.11 Determination of Multiple Antibiotic Resistance (MAR) among Isolated Bacteria

Multiple antibiotic resistance (MAR) index was analyzed as described by Krumperman (1983). MAR index was calculated by dividing the number of antibiotics to which the test isolate depicted resistance to the number of antibiotics to which the test isolate was evaluated for susceptibility;

$$\text{MAR} = \frac{\text{Number of antibiotics to which isolates are resistant to}}{\text{Total number of antibiotics used}}$$

Multiple antibiotic-resistant phenotypes (MARPs) for each sampling site were generated for isolates that showed resistance to more than three antibiotics following the method described by Wose *et al*. (2010). The antibiotic resistance pattern, number of antibiotics to which the isolates were resistant, frequencies and percentages were obtained from Kirby Bauer tests.

3.12 Relationship between Phenotypic Resistance Pattern to the Antibiotics for MDR Bacteria and the Presence of Antibiotic Resistance Genes

The correlation coefficient (r) was assessed among various tested antibiotics and the detected antibiotic resistance genes in bacterial isolates, using Pearson correlation coefficient analysis in MS Excel 2016 (Microsoft Corporation) and confirmed in Minitab version 17.1.

3.13 Data Analysis

Statistical analysis was performed using Minitab v17.1 statistical software (Minitab, LLC). In order to compare bacterial counts in five sampling sites, chi-square (X^2) was used. Correlations were established using Pearson's correlation coefficient (r2) in bivariate linear correlations ($P< 0.05$). P-value was regarded significant if it was ≤ 0.05. A Microsoft Excel 2016 spreadsheet software package (Microsoft Corporation) was also used to generate graphs.

CHAPTER FOUR

RESULTS

4.1 Microbial Load by Total Viable Count (TVC) in Retail Nile Tilapia

A total of 106 bacteria isolates were obtained from 68 fish samples collected. The average total viable count for the 68 fish samples was 3.78 log CFU/ml. The bacteria isolates at all sites ranged between 3.70 log CFU/ml in Westlands and 3.87 log CFU/ml in Lang'ata (Figure 4.1). Different species of bacteria from respective sampling sites showed significant levels of bacterial load (Figure 4.2).

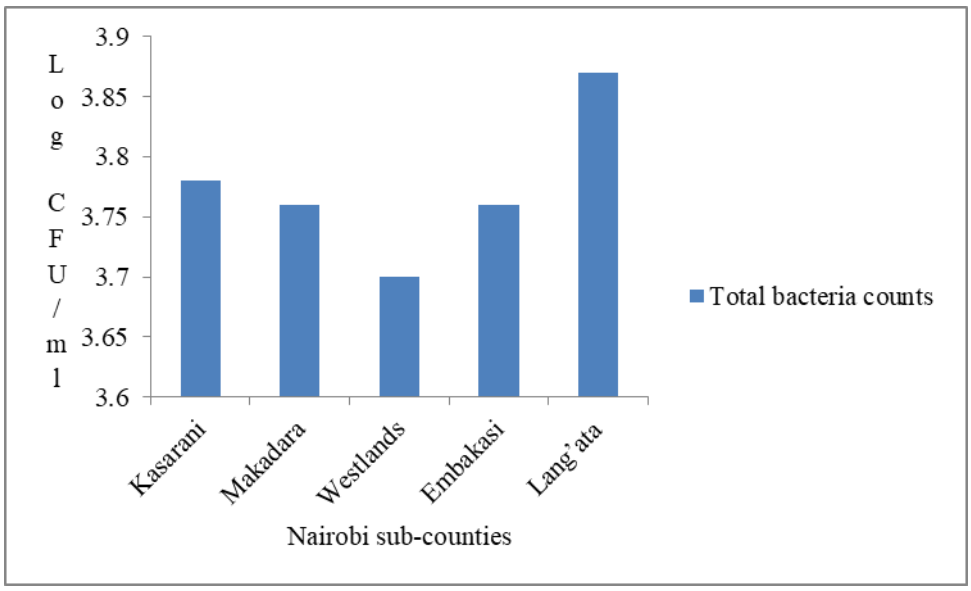

Figure 4.1: Comparative Counts of Bacteria Isolates in Five Sampling Sites of Nairobi County

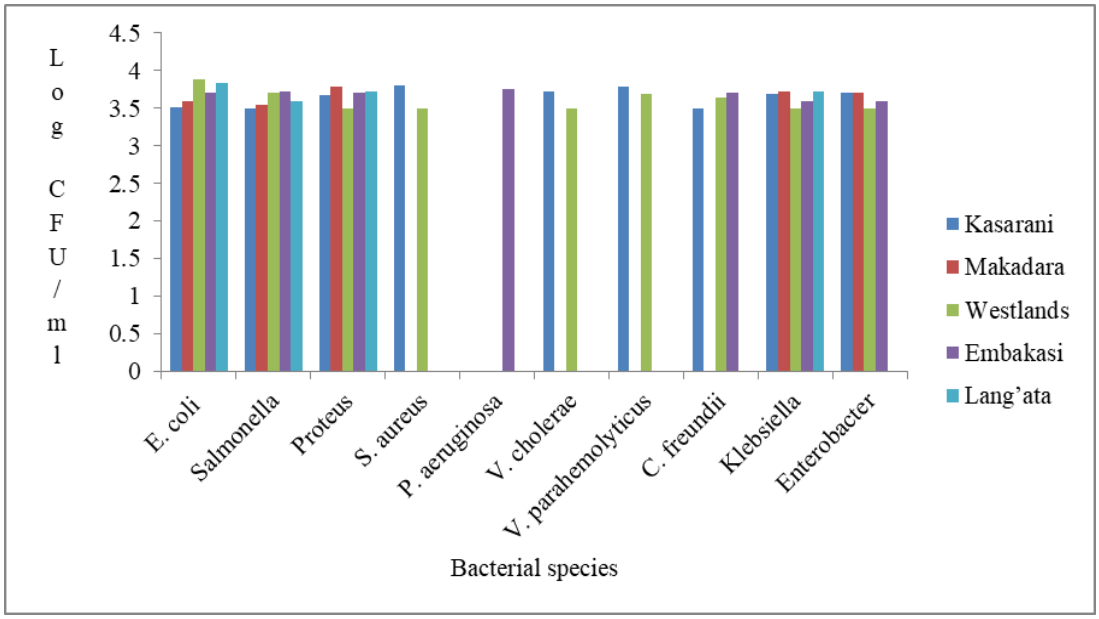

Figure 4.2: Bacterial Counts of Different Species in Five Sampling Sites of Nairobi County

4.2 Phenotypic Identification of Isolated Bacteria

Salmonella spp. colonies on xylose lysine deoxycholate (XLD) agar appeared red with black centres. *Salmonella* spp. on triple sugar iron (TSI) agar had a characteristic appearance of red slant surface, yellow butt, produced hydrogen sulphide (black) and gas (cracks in the media). *E. coli* colonies on MacConkey agar appeared pink and on EMB agar appeared as green metallic sheen with a dark center.*Vibrio cholerae* on thiosulfate-citrate-bile-salts-sucrose agar were large yellow colonies and *Vibrio parahaemolyticus* colonies had blue to green centres. *S. aureus* on mannitol salt agar appeared yellow colonies. *Proteus* spp. colonies appeared black on XLD and on triple sugar iron (TSI) agar had a characteristic appearance of red slant surface, yellow butt, produced hydrogen sulphide (black) and no gas production. *P. aeruginosa* on pseudomonas cetrimide agar had green-blue-pigmented colonies.*Citrobacter* spp. on MacConkey agar appeared as pink colonies, on XLD it appeared as yellow colonies and on EMB they were brown in colour. *Klebsiella* spp., on EMB agar produced large mucoid, brown to pink colonies with no metallic green sheen and on CLED Agar appeared yellow. *Enterobacter* spp., colonies on EMB agar appeared as mucoid, brown to pink.

4.3 Prevalence of Bacterial Isolates in Fresh Nile Tilapia Samples from Retail Markets

The occurrence and contamination rates of *Salmonella* species ranged from 16.7% to 22.2% and for *E. coli* from 16.7% to 20.8%, respectively (Fig. 4.3). The number of *Salmonella* isolates for each of five markets were 4 (22.2%), 3 (16.7%), 3 (16.7%), 4 (22.2%), and 4 (22.2%), for Kasarani, Makadara, Westlands, Embakasi and Lang'ata, respectively. For *E. coli*, the number of isolates were 5 (20.8%) for Kasarani, Makadara, Westlands and Embakasi and 4 (16.7%) for Lang'ata.

The prevalence and contamination rates of *Proteus* spp. ranged from 38.46% to 50% and *S. aureus* ranged from 0% to 28.57% for the five different sub-counties under study. *P. aeruginosa* was detected in fish samples from only Embakasi sub-county, with a prevalence of 23.08%. With regards to *V. cholerae* and *V. parahaemolyticus*, prevalence of 7.14% was obtained from fish samples collected from two sub-counties, Kasarani and Westlands. The prevalence of *C. freundii* ranged from 0 to 7.69%, *Klebsiella* spp. ranged from 7.69 to 28.57% and *Enterobacter* spp. ranged from 0 to 21.43% (Table 4.1). Overall, the prevalence of contamination of Nile tilapia fish with *Salmonella* species, *E. coli*, *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, *V. parahaemolyticus*, *C. freundii*, *Klebsiella* spp., and *Enterobacter* spp. was 26.47% (18/68), 35.29% (24/68), 44.12% (30/68), 10.29% (7/68), 4.41% (3/68), 2.94% (2/68), 2.94% (2/68), 4.41% (3/68), 16.17% (11/68), 8.82% (6/68) respectively (Table 4.1).

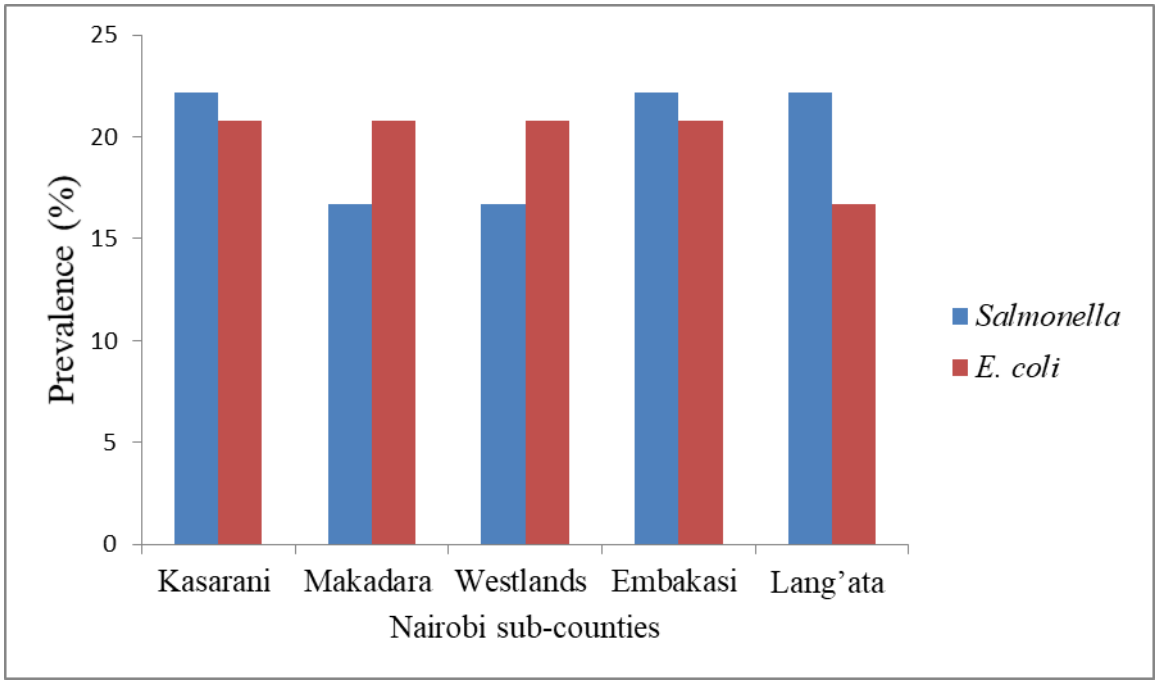

Figure 4.3: Prevalence of *Salmonella Species* and *E. coli* Isolated from Nile Tilapia Collected from Five Locations in Nairobi, Kenya. For *Salmonella* isolates, n = 4, 3, 3, 4, and 4 for Kasarani, Makadara, Westlands, Embakasi and Lang'ata, respectively. For *E. coli*, n = 5, 5, 5, 5 and 4 for Kasarani, Makadara, Westlands, Embakasi and Lang'ata, respectively.

Table 4.1: Bacterial Pathogen Prevalence among Fresh O. niloticus Fish Samples from Different Markets of Five Sub-Counties in Nairobi.

Sub-County	N collected samples	Positive for bacterial pathogens, N (%)									
		***Salmonella* spp.**	***E. coli***	***Proteus* spp.**	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Vibrio cholerae***	***Vibrio parahemolyticus***	***C. freundii***	***Klebsiella* spp.**	***Enterobacter* spp.**
Kasarani	14	4 (28.57)	5 (35.71)	7 (50)	3 (21.43)	0 (0)	1 (7.14)	1 (7.14)	1 (7.14)	2 (14.29)	3 (21.43)
Makadara	14	3 (21.43)	5 (35.71)	6 (42.86)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (28.57)	1 (7.14)
Westlands	14	3 (21.43)	5 (35.71)	6 (42.86)	4 (28.57)	0 (0)	1 (7.14)	1 (7.14)	1 (7.14)	2 (14.29)	1 (7.14)
Embakasi	13	4 (30.77)	5 (38.46)	5 (38.46)	0 (0)	3 (23.08)	0 (0)	0 (0)	1 (7.69)	1 (7.69)	1 (7.69)
Lang'ata	13	4 (30.77)	4 (30.77)	6 (46.15)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (15.38)	0 (0)
Total	**68**	**18 (26.47)**	**24 (35.29)**	**30 (44.12)**	**7 (10.29)**	**3 (4.41)**	**2 (2.94)**	**2 (2.94)**	**3 (4.41)**	**11 (16.17)**	**6 (8.82)**

4.4 Antibiotic Susceptibility Testing According to the Species of Isolated Bacteria

4.4.1 Occurrence of Antimicrobial Resistant *Salmonella* Species and *E. coli* Isolates

The distribution of antimicrobial susceptibility profile of *Salmonella* species and *E. coli* is presented in Table 4.2. *Salmonella* isolates showed resistance to all the antibiotics tested except to cefepime. The highest resistance of *Salmonella* species isolates from samples collected from all the locations were to penicillin (22.2%) followed by vancomycin (16.7%). Resistance to ampicillin, cefpodoxime and rifampicin was 11.1%, whereas resistance to ceftazidime, chloramphenicol, meropenem, nitrofurantoin and streptomycin was 5.5%. None of the *Salmonella* isolates was resistant to cefepime. Intermediates were common in all the antibiotics tested with the range of 11.1% to 61.1% (Table 4.2).

The percentage of resistance to the antibiotics differed among the *E. coli* isolates, of which 25% were resistant to rifampicin. Resistant to penicillin-G, vancomycin, and meropenem was 20.8%, 16.7% and 12.5%, respectively (Table 4.2). None of the *E. coli* isolates were resistant to cefepime, nitrofurantoin and streptomycin (Table 4.2). A higher percentage of intermediate isolates 58.3% was observed in meropenem (Table 4.2). For both *Salmonella* and *E. coli* isolates, the new generation cephalosporins such as cefepime (fourth generation) were completely effective against all the isolates.

Correlation coefficient was determined among various tested antibiotics and significant positive correlations were observed in a number of antibiotics including CAZ and CPD (r = 1), C and CAZ (r = 1), S and C (r = 1), S and P (r = 1), and VA and RIF (r = 1) (Appendix IV).

Table 4.2: Antibiotic Resistance Patterns of *Salmonella Species* and *E. coli* Isolates

Sub-County/ Location	Microorganisms & Isolate ID	Phenotype*													
		AX	CPM	CPD	CAZ	C	MRP	NIT	P	RIF	S	VA	Resistance (%)	Intermediate (%)	Susceptible (%)
Makadara	MAK-22	R	S	S	I	S	I	R	R	R	S	R	45.5	18.2	36.4
	MAK-01	S	S	S	I	S	I	S	S	S	S	S	0	18.2	81.8
	MAK-02	I	S	S	I	S	I	S	I	I	S	I	0	54.5	45.5
Embakasi	EMB-32	R	S	S	R	R	R	I	S	I	I	R	45.5	27.3	27.3
	EMB-02	I	S	S	S	I	S	S	S	I	S	S	0	27.3	72.7
	EMB-03	S	S	S	I	S	I	I	I	I	I	I	0	63.6	36.4
	EMB-07	S	S	I	S	I	S	I	S	S	I	S	0	36.4	63.6
Langata	LAN-16	S	S	S	I	S	S	S	I	I	S	S	0	27.3	72.7
	LAN-28	I	I	S	S	S	I	I	R	S	I	I	9.1	54.5	36.4
	LAN-15	I	S	I	I	S	S	I	I	S	I	I	0	63.6	36.4
	LAN-20	S	S	I	I	S	I	I	R	S	S	S	9.1	36.4	54.5
Westlands	WES-09	I	S	R	S	S	S	I	S	R	R	R	36.4	18.2	45.5
	WES-01	S	S	S	I	S	I	I	S	S	S	S	0	27.3	72.7
	WES-02	I	S	S	I	S	I	S	I	I	I	I	9.1	63.6	36.4
Kasarani	KAS-01	S	I	R	S	S	S	S	I	I	S	I	9.1	36.4	54.5
	KAS-05	S	S	I	I	S	S	I	R	S	I	S	9.1	36.4	54.5
	KAS-06	S	S	S	I	S	S	S	S	I	S	S	0	18.2	81.8
	KAS-07	I	S	S	S	I	S	I	I	I	S	I	0	54.5	45.5
Resistance (%)		11.1	0	11.1	5.5	5.5	5.5	5.5	22.2	11.1	5.5	16.7			
Intermediate (%)		38.9	11.1	22.2	61.1	16.7	44.4	55.6	38.9	50	38.9	38.9			
Susceptible (%)		50	88.9	66.7	33.3	77.8	50	38.9	38.9	38.9	55.6	44.4			
	E. coli														

Sub-County/Location		AX	CPM	CPD	CAZ	C	MRP	NIT	P	RIF	S	VA	Resistance (%)	Intermediate (%)	Susceptible (%)
Makadara	MAK-26	R	S	S	S	S	R	S	S	R	S	R	36.4	0	63.6
	MAK-01	I	S	S	S	S	I	S	I	I	I	I	0	54.5	45.5
	MAK-12	I	S	I	S	S	I	S	S	I	I	R	9.1	45.5	45.5
	MAK-13	I	S	S	S	S	I	S	R	I	S	I	9.1	36.4	54.5
	MAK-15	S	I	I	S	S	I	S	S	R	S	I	9.1	36.4	54.5
Embakasi	EMB-01	S	I	I	S	S	R	S	I	I	S	S	9.1	36.4	54.5
	EMB-12	I	I	I	S	S	I	S	S	S	I	I	0	54.5	45.5
	EMB-11	S	S	I	I	S	I	S	I	I	S	S	0	45.5	54.5
	EMB-10	I	S	R	S	S	I	S	I	S	S	S	9.1	27.3	63.6
	EMB-15	S	S	S	S	S	I	S	S	S	S	S	0	9.1	90.9
Langata	LAN-35	R	S	S	I	S	R	S	S	R	S	R	36.4	9.1	54.5
	LAN-20	I	I	S	I	S	S	S	R	S	S	I	9.1	36.4	54.5
	LAN-23	I	I	I	S	S	I	S	R	S	I	R	18.2	45.5	36.4
	LAN-24	I	I	R	S	S	S	S	I	S	I	I	9.1	45.5	45.5
Westlands	WES-01	I	S	S	I	S	S	S	R	I	I	S	9.1	36.4	54.5
	WES-02	S	S	I	S	R	I	S	S	S	I	I	9.1	36.4	54.5
	WES-03	I	S	I	R	S	I	S	I	I	I	I	9.1	63.6	27.3
	WES-04	I	I	I	S	S	I	S	I	S	S	I	0	54.5	45.5
	WES-05	I	S	S	S	S	I	S	R	S	S	S	9.1	18.2	72.7
Kasarani	KAS-11	I	S	I	S	S	S	S	I	R	S	S	9.1	27.3	63.6
	KAS-12	S	S	I	S	S	S	S	I	S	I	S	0	27.3	72.7
	KAS-13	S	S	S	S	S	I	S	I	S	S	S	0	18.2	81.8
	KAS-14	S	I	S	S	S	S	S	I	R	I	I	9.1	36.4	54.5
	KAS-15	S	S	S	I	S	S	S	S	R	I	S	9.1	18.2	72.7
Resistance(%)		8.3	0	8.3	4.2	4.2	12.5	0	20.8	25	0	16.7			
Intermediate (%)		54.2	33.3	45.8	20.8	0	58.3	0	45.8	29.2	45.8	41.7			
Susceptible (%)		37.5	66.7	45.8	75	95.8	29.2	100	33.3	45.8	54.2	41.7			

AX = Ampicillin/Cloxacillin; CPM = Cefepime; CPD = Cefpodoxime; CAZ = Ceftazidime; C = Chloramphenicol; MRP = Meropenem; NIT = Nitrofurantoin; P = Penicillin-G; RIF = Rifampicin; S = Streptomycin; VA = Vancomycin.

The classes of antibiotics are:

Carbapenems (MRP), Cephalosporin (third generation; CAZ), Penicillin (P-G), Beta-lactam (AX), Cephalosporin (third generation; CPD), Phenicol (C), Cephalosporin (fourth generation, CPM), Nitrofurans (NIT), Glycopeptides (VA), Ansamycin (RIF), and Aminoglycosides (S)**.**

The results are depicted by: susceptible (S), Intermediate (I), or resistant (R) to each antibiotic.

ESBL production in MDR *Salmonella* spp. and *E. coli* isolates All MDR *Salmonella* spp. and *E. coli* isolates were ESBL producers (Table 4.3), as determined by the Double Disc Synergy Test method.

Table 4.3: Resistance and ESBL Production Test Results of MDR *Salmonella* spp. and *E. coli* Isolates

Microorganism	Isolate ID code	Antibiotic resistance	ESBL production
Salmonella spp.	WES-09	CPD + RIF + S +VA	Positive
	MAK-22	AX + NIT + P + RIF +VA	Positive
	EMB-32	AX + CAZ + C + MRP +VA	Positive
E. coli	MAK-26	AX + MRP + RIF +VA	Positive
	LAN-35	AX + MRP + RIF +VA	Positive

AX =Ampicillin/Cloxacillin; CPD =Cefpodoxime; CAZ= Ceftazidime; C=Chloramphenicol; MRP=Meropenem; NIT=Nitrofurantoin; P=Penicillin-G; RIF =Rifampicin; S =Streptomycin; VA=Vancomycin. The classes of antibiotics are: Carbapenems (MRP), Cephalosporin (third generation; CAZ), Penicillin (P-G), Beta-lactam (AX), Cephalosporin (third generation; CPD), Phenicol (C), Nitrofurans (NIT), Glycopeptides (VA), Ansamycin (RIF), and Aminoglycosides (S). ESBL, Extended-spectrum β-lactamase. Positive means that the bacteria produce extended-spectrum β-lactamases, which make them resistant to beta lactamase antibiotics.

4.4.2 Occurrence of Antimicrobial Resistant *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae* and *V. parahaemolyticus* Isolates

The distribution of antimicrobial resistance pattern of *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae* and *V. parahaemolyticus* is presented in Table 4.4. None of the bacterial isolates from the 5 different bacterial pathogens were resistant to cefepime (fourth-generation cephalosporin). The antibiotic resistance profile among the *Proteus* species isolates demonstrated varying levels of resistance against the 11 antibiotics. The highest level of resistance was observed for rifampicin (73.3%) followed by vancomycin and ampicillin/cloxacillin (70%), streptomycin (60%), and cefpodoxime (50%). For *S. aureus*, the antibiotics that showed the highest resistance were rifampicin, ampicillin/cloxacillin, and meropenem (100%) followed by cefpodoxime (85.7%), vancomycin and ceftazidime (71.4%), and penicillin-G (57.1%). In *P. aeruginosa*, all the isolates were resistant to penicillin-G and vancomycin (100%) followed by ceftazidime, meropenem, and streptomycin (66.7%), and cefpodoxime (33.3%), but were not resistant to any of the remaining antibiotics (cefepime, chloramphenicol, and nitrofurantoin). For *V. cholerae*, 100% were resistant to penicillin-G, vancomycin, ampicillin/cloxacillin, cefpodoxime, and streptomycin. For *V. parahaemolyticus*, 100% were resistant to penicillin-G, vancomycin, and ampicillin/cloxacillin. Intermediate percentages ranged from 6.7% to 100% (Table 4.4). No resistance to chloramphenicol was noted for any of the bacterial pathogens except *Proteus* species and *Vibrio cholerae* isolates.

Correlation coefficient was also determined among various tested antibiotics and significant positive correlations were observed in various antibiotics e.g.VAN and PEN ($r = 0.97$), NIT and CPD ($r = 0.82$), NITand CHL ($r = 0.95$), and MRP and CAZ ($r = 0.89$) (Appendix V).

Table 4.4: Phenotypic Resistance Pattern of *Proteus Spp., Staphylococcus Aureus, Pseudomonas Aeruginosa, Vibrio Cholerae, and Vibrio Parahemolyticus* to 11 Antimicrobial Agents

Antimicrobial class	Antibiotics	Bacteria isolates													
		Proteus spp.(n = 30)			*S. aureus* (n = 7)			*P. aeruginosa* (n = 3)			*V. cholerae*(n = 2)			*V. parahemolyticus* (n = 2)		
		R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Penicillin	PEN	33.3	40	26.7	57.1	42.9	0	100	0	0	100	0	0	100	0	0
Glycopeptides	VAN	70	6.7	23.3	71.4	28.6	0	100	0	0	100	0	0	100	0	0
Rifamycins	RIF	73.3	16.7	10	100	0	0	66.7	33.3	0	0	100	0	50	50	0
Beta-lactam	AX	70	26.7	3.3	100	0	0	66.7	33.3	0	100	0	0	100	0	0
Cephalosporin (4^{th})	CPM	0	6.7	93.3	0	28.6	71.4	0	66.7	33.3	0	0	100	0	0	100
Cephalosporin (3^{rd})	CPD	50	20	30	85.7	14.3	0	33.3	66.7	0	100	0	0	0	100	0
Phenicol	CHL	10	10	80	0	14.3	85.7	0	0	100	50	50	0	0	0	100
Nitrofuran	NIT	6.7	33.3	60	14.3	14.3	71.4	0	33.3	66.7	50	50	0	0	0	100
Cephalosporin (3^{rd})	CAZ	13.3	16.7	70	71.4	28.6	0	100	0	0	0	100	0	0	0	100
Carbapenems	MRP	36.7	0	63.3	100	0	0	100	0	0	0	0	100	50	0	50
Aminoglycosides	STR	60	20	20	28.6	42.9	28.6	100	0	0	100	0	0	50	50	0
	Mean	38.5	17.9	43.6	57.1	19.5	23.4	60.6	21.2	18.2	54.5	27.3	18.2	40.9	18.2	40.9

PEN, Penicillin-G (10 µg); VAN = Vancomycin (30 µg); RIF, Rifampicin (5 µg); AX, Ampicillin/Cloxacillin (10 µg); CPM, Cefepime (30 µg); CPD, Cefpodoxime (10 µg); CHL, Chloramphenicol (50 µg); NIT, Nitrofurantoin (300 µg); CAZ, Ceftazidime (30 µg); MRP, Meropenem (10 µg); STR, Streptomycin (10 µg); R, Resistant; I, Intermediate; S, Sensitive.

4.4.3 Antibiotic Resistant Phenotypes of *Citrobacter freundii, Klebsiella* spp. and *Enterobacter* spp.

Antibiotic susceptibility test revealed 3, 2 and 1 isolates belonging to *C. freundii*, *Klebsiella* spp. and *Enterobacter* spp. were resistant to at least three different classes of antibiotics.All bacteria isolates were sensitive (100%) to Cefepime (4^{th} generation Cephalosporin), Chloramphenicol (phenicol), Nitrofurantoin (nitrofuran). Resistance to β-lactams (MRP, CAZ, PEN, AX, CPD and CMP) as well as the four non- β-lactams (NIT, VA, C and RIF) tested ranged from 0% to 100% for *C. freundii*, 0% to 36.4% for *Klebsiella* spp., and 0% to 50% for *Enterobacter* spp. In *C. freundii*, the highest level of resistance was observed for vancomycin, rifampicin and meropenem (100%) followed by Ampicillin/Cloxacillin and cefpodoxime (66.7%). In *Klebsiella* spp., 36.4% were resistant to Ampicillin/Cloxacillin and meropenem followed by 27.3% resistant to penicillin-G and vancomycin. In *Enterobacter* spp., 50% of the bacterial isolates were resistant to rifampicin followed by penicillin-G and meropenem (33.3%) and vancomycin (16.7%) but all the isolates were 100% susceptible to Ampicillin/Cloxacillin, Cefepime, Cefpodoxime, Chloramphenicol, Nitrofurantoin and Ceftazidime (Table 4.5).

Table 4.5: Antimicrobial Susceptibility Profiles of *Citrobacter freundii, Klebsiella* spp., and *Enterobacter* spp.

Antimicrobial class	Antibiotics	Bacteria isolates								
		Citrobacter freundii (n=3)			*Klebsiella* spp. (n=11)			*Enterobacter* spp. (n=6)		
		R	I	S	R	I	S	R	I	S
Penicillin	PEN	0 (0)	3 (100)	0 (0)	3 (27.3)	6 (54.5)	2 (18.2)	2 (33.3)	2 (33.3)	2 (33.3)
Glycopeptides	VAN	3 (100)	0 (0)	0 (0)	3 (27.3)	4 (36.4)	4 (36.4)	1 (16.7)	5 (83.3)	0 (0)
Ansamycin	RIF	3 (100)	0 (0)	0 (0)	2 (18.2)	4 (36.4)	5 (45.5)	3 (50)	1 (16.7)	2 (33.3)
Beta-lactam	AX	2 (66.7)	1 (33.3)	0 (0)	4 (36.4)	5 (45.5)	2 (18.2)	0 (0)	3 (50)	3 (50)
Cephalosporin (4^{th})	CPM	0 (0)	0 (0)	3 (100)	0 (0)	3 (27.3)	8 (72.7)	0 (0)	0 (0)	6 (100)
Cephalosporin (3^{rd})	CPD	2 (66.7)	1 (33.3)	0 (0)	0 (0)	3 (27.3)	8 (72.7)	0 (0)	3 (50)	3 (50)
Phenicol	CHL	0 (0)	0 (0)	3 (100)	0 (0)	2 (18.2)	9 (81.8)	0 (0)	1 (16.7)	5 (83.3)
Nitrofuran	NIT	0 (0)	1 (33.3)	2 (66.7)	0 (0)	4 (36.4)	7 (63.6)	0 (0)	0 (0)	6 (100)
Cephalosporin (3^{rd})	CAZ	0 (0)	2 (66.7)	1 (33.3)	2 (18.2)	8 (72.7)	1 (9.1)	0 (0)	2 (33.3)	4 (66.7)
Carbapenems	MRP	3 (100)	0 (0)	0 (0)	4 (36.4)	3 (27.3)	4 (36.4)	2 (33.3)	4 (66.7)	0 (0)
Aminoglycosides	STR	0 (0)	3 (100)	0 (0)	2 (18.2)	3 (27.3)	6 (54.5)	0 (0)	4 (66.7)	2 (33.3)

PEN, Penicillin-G (10 µg); VAN, Vancomycin (30 µg); RIF, Rifampicin (5 µg); AX, Ampicillin/Cloxacillin (10 µg); CPM, Cefepime (30 µg); CPD, Cefpodoxime (10 µg); CHL, Chloramphenicol (50 µg); NIT,Nitrofurantoin (300 µg); CAZ,Ceftazidime (30 µg); MRP, Meropenem (10 µg); STR, Streptomycin (10 µg); R, Resistant; I, Intermediate; S, Sensitive.

4.5 Molecular Identification and Characterization of MDR Bacterial Isolates

4.5.1 Molecular Identification of MDR *Salmonella Species* and *E. coli* Isolates

For the molecular identification of the recovered MDR isolates, DNA were extracted and 1500 bp size of 16S rRNA genes were amplified and sequenced. The sequences of 16S rRNA gene of five MDR isolates were deposited in the NCBI database under accession numbers OP293362.1, OP293363.1 and OP293364.1 for *Salmonella typhimurium* and OP293365.1 and OP293366.1 for *Escherichia coli*. BLASTn results revealed that the three isolates WES-09, MAK-22 and EMB-32 were closely related to *Salmonella typhimurium*NR_074910.1with similarity of 93%, 91% and 93%. The two isolates MAK-26 and LAN-35 were closely related to *Escherichia coli* NR_114042.1 with identity percentage of 90% and 93%, respectively (Table 4.6).

Table 4.6: Similarity of 16S Rrna Sequences of Antibiotic Resistant *E. coli* and *S.Typhimurium* Isolates from Nile Tilapia, Compared with Accessions from the Genbank Database.

Isolate ID Code	Sub-County/ Location	16S rRNA Accession	Closest Match in Blast	Similarity (%)	GenBankAccession No.
WES-09	Westland	OP293362.1	*Salmonella typhimurium*	93	NR_074910.1
MAK-22	Makadara	OP293363.1	*Salmonella typhimurium*	91	NR_074910.1
EMB-32	Embakasi	OP293364.1	*Salmonella typhimurium*	93	NR_074910.1
MAK-26	Makadara	OP293365.1	*Escherichia coli*	90	NR_114042.1
LAN-35	Lang'ata	OP293366.1	*Escherichia coli*	93	NR_114042.1

16S rRNA Phylogenetic Analysis:

1. *Salmonella typhimurium*

The phylogenetic analysis (Figure 4.4) of 16S rRNA sequence of the three MDR *Salmonella* isolates (OP293362.1, OP293363.1 and OP293364.1) showed distinct clustering and had the same node showing that they both evolved from the same ancestor. The three isolates clustered together in the same cladograph and had 100% homology.

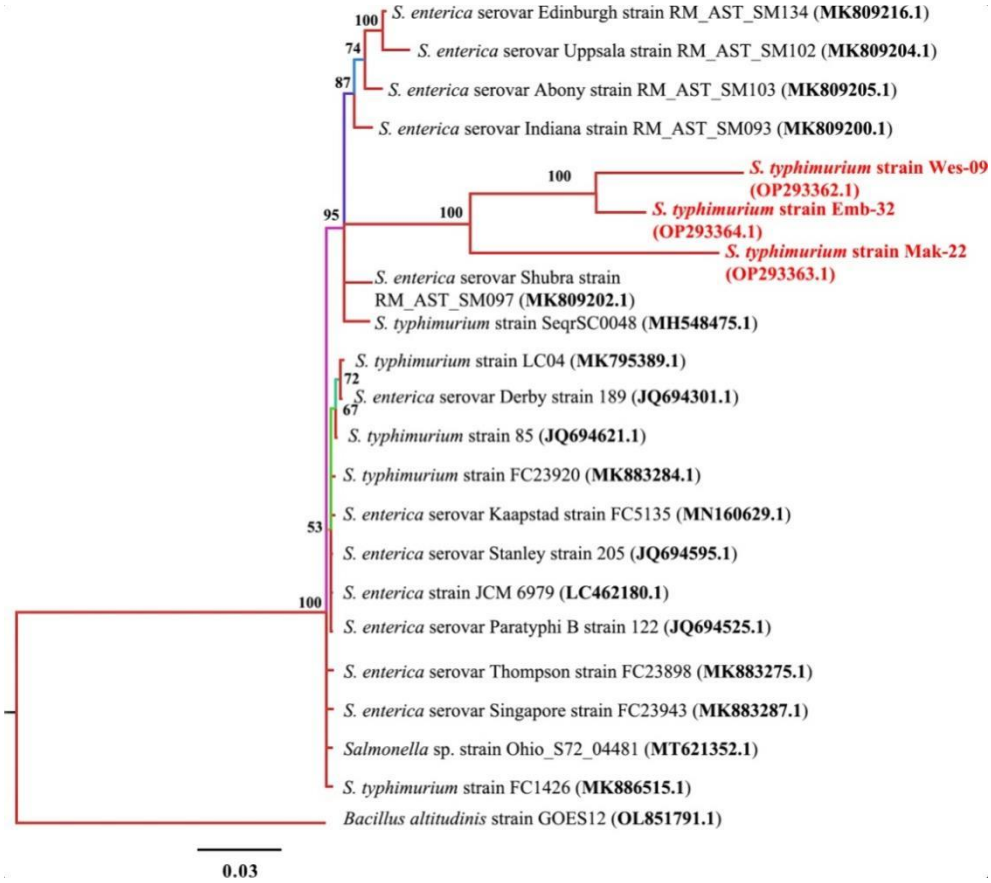

Figure 4.4: Phylogenetic Tree Built Using Eighteen16s rRNA Sequences of *Salmonella* Species.

New isolates *S. typhimurium* strains WES-09, MAK-22 and EMB-31 are shown in red. Numbers indicated on the nodes are percent posterior probabilities showing statistical support for each node. Branches are coloured based on percent posterior probabilities. The

scale bar below the tree indicates the number of expected changes (or substitutions) per site. The outgroup *B. altitudinis* strain GOES12 (OL851791.1) was used in rooting the tree.

2. *Escherichia coli*

The phylogenetic analysis (Figure 4.5) of 16S rRNA sequence of the two MDR *E. coli* isolates (OP293365.1 and OP293366.1) showed distinct clustering and had the same node showing that they both evolved from the same ancestor.

Figure 4.5: Phylogenetic Treebuilt Using 16S rRNA Sequences of *Escherichia coli* Species. New isolates of *E. coli* strains MAK-26 and LAN-35 are shown in red. Numbers indicated on the nodes are percent posterior probabilities showing

statistical support for each node. Branches are coloured based on percent posterior probabilities. The scale bar below the tree indicates the number of expected changes (or substitutions) per site. The outgroup *B. altitudinis* strain GOES12 (OL851791.1) was used in rooting the tree.

4.5.2 Identification of MDR *Proteus* spp., *Staphylococcus aureus, Pseudomonas aeruginosa, Vibrio cholerae,* and *Vibrio parahemolyticus* Using BLASTn Analysis

The sequences of 16S rRNA genes of MDR isolates of bacterial pathogens were compared with strains in the GenBank to determine the degree of similarity between them and closely related genotypes. BLASTn results revealed the percentage of similarity between MDR isolates and closely related bacteria in the GenBank (Table 4.7). The 16S rRNA 1465 bp amplicon sequences were registered in GenBank and the accession numbers are provided in Table 4.7. From the 16S rRNA sequences, BLASTn analysis showed the four MDR Vibrio isolates belonged to *V. cholerae* (accession numbers OP293360.1 and OP293361.1) and *V. parahaemolyticus* (accession numbers OP293358.1 and OP293359.1). Using BLASTn analysis and the globally published NCBI database, three MDR isolates of *Pseudomonas* were confirmed as *P. aeruginosa*, seven MDR isolates of *Staphylococcus* were confirmed as *S. aureus*, and three *Proteus* spp. were confirmed as *P. mirabilis* and other *Proteus* spp. (Table 4.7).

Table 4.7: Similarity of 16S Rrna Sequences of MDR Isolates of Different Bacteria Pathogens from Nile Tilapia, Compared with that of Accessions in the Genbank Database.

No.	Isolate ID Code	Sub-County/ Location of the isolate	16S rRNA Accession	Closest Match in Blast of the isolate	Similarity (%)	Accession of the closest match
1	MAK-38	Makadara	OP293367.1	*Proteus mirabilis* strain P13	98.32	MT276300.1
2	EMB-43	Embakasi	OP293368.1	*Proteus mirabilis* strain BLPS5	98.25	ON460264.1
3	LAN-46	Lang'ata	OP293369.1	*Proteus mirabilis* strain P13	98.87	MT276300.1
4	KS-19	Kasarani	OP047935.1	*Proteus penneri* strain A254	94.22	KX692873.1
5	KS-20	Kasarani	OP047936.1	*Proteus vulgaris* strain CQC01	94.89	MN517893.1
6	KS-23	Kasarani	OP047937.1	*Proteus penneri* strain njp2	96.89	KU992679.1
7	MK-24	Makadara	OP047938.1	*Proteus faecis* strain An Lec 78	96.58	ON688699.1
8	MK-25	Makadara	OP047939.1	*Proteus faecis* strain CA120921	96.23	MG269475.1
9	MK-27	Makadara	OP047940.1	*Proteus faecis* strain 08MAS2231	96.30	MG269472.1
10	MK-28	Makadara	OP047941.1	*Proteus alimentorum* strain NA-32	95.04	MN882658.1
11	EM30	Embakasi	OP047943.1	*Proteus alimentorum* strain NA-32	94.43	MN882658.1
12	EM33	Embakasi	OP047945.1	*Proteus penneri* strain njp2	98.51	KU992679.1
13	L-34	Lang'ata	OP047946.1	*Proteus faecis* strain 08MAS2231	96.97	MG269472.1
14	L-36	Lang'ata	OP047947.1	*Proteus penneri* strain njp2	96.80	KU992679.1
15	WT1	Westlands	OP047928.1	*Proteus faecis* strain TJ1636	96.75	MG269469.1
16	WT2	Westlands	OP047929.1	*Proteus* sp. SBP10	96.27	GU812899.1
17	WT7	Westlands	OP047932.1	*Proteus faecis* strain 08MAS1603	95.57	MG269471.1
18	WT8	Westlands	OP047933.1	*Proteus alimentorum* strain NA-32	96.42	MN882658.1
19	WES-03	Westlands	OP293351.1	*S. aureus* strain SA1	96.65	OP364883.1
20	WES-10	Westlands	OP293352.1	*S. aureus* strain SA1	96.17	OP364883.1
21	WES-11	Westlands	OP293353.1	*S. aureus* strain MRSA-4	97.17	OP824648.1
22	WES-13	Westlands	OP293354.1	*S. aureus* strain S1245	94.51	KX447585.1
23	KAS-15	Kasarani	OP293355.1	*S. aureus* strain EB12	96.99	MT509600.1
24	KAS-17	Kasarani	OP293356.1	*S. aureus* strain S1245	94.06	KX447585.1
25	KAS-18	Kasarani	OP293357.1	*S. aureus* strain RM_AST_SA001	94.05	MK809238.1
26	EMB-40	Embakasi	OP293370.1	*P. aeruginosa* strainBCr3	97.65	KP717554.1
27	EMB-41	Embakasi	OP293371.1	*P. aeruginosa* strain PB3A	98.40	KF029593.1
28	EMB-42	Embakasi	OP293372.1	*P. aeruginosa* strain B13	97.59	DQ350823.1
29	WES-14	Westlands	OP293360.1	*V. cholera* strain W2-13	99.33	KY496305.1
30	KAS-21	Kasarani	OP293361.1	*V. cholera* strainCTI2	98.76	KM362726.1
31	WES-06	Westlands	OP293358.1	*V. parahemolyticus* strain TV18	92.62	MT549167.1
32	KAS-16	Kasarani	OP293359.1	*V. parahemolyticus* strainSR3	93.07	KT006932.1

Note: The isolates L-39, L-44, WT-48, WT-49, KS-45 and KS-47 which were presumptive *Proteus* species were not included in the table because their sequences showed no similarity with any isolate from GenBank database during BLASTn searches.

1. *Proteus* species

Based on 16S rRNA sequencing and subsequent BLAST analysis, all strains shared more than 96% sequence homology with different types of strains of the genus *Proteus*. Out of the 18 *Proteus* spp. isolates, three (accession numbers OP293367.1, OP293368.1, and OP293369.1) shared more than 98% similarity with *Proteus mirabilis* 16S rRNA gene sequences obtained on the NCBI (Figure 4.6). Other *Proteus* spp. (accession numbers OP047928.1 and OP047929.1) shared 84% similarity with *Proteus penneri*. The isolates EM33 (OP047945.1) and L-36 (OP047947.1) had similar sequence identity although the isolates were obtained from different locations.

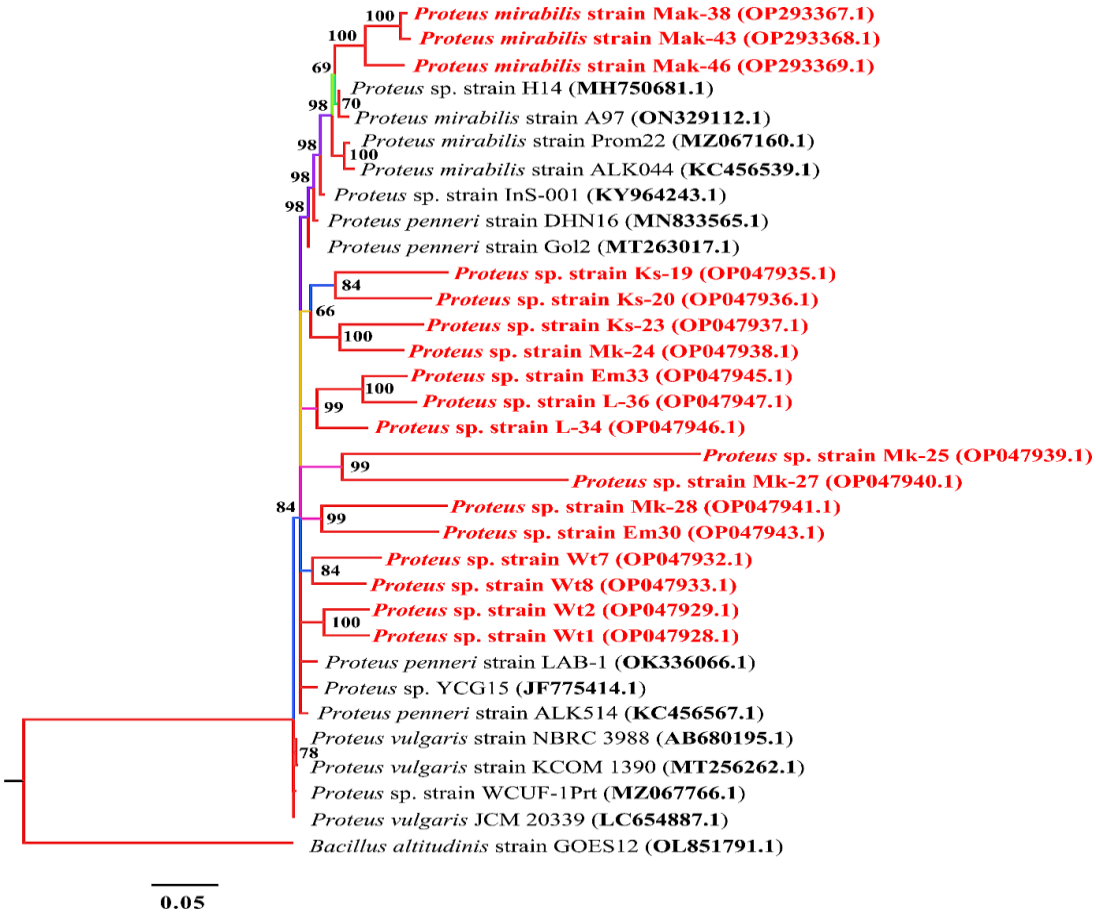

Figure 4.6: Phylogenetic Tree Built by MrBayes v3.2.7 Using 14 16S rRNA sequences of the Genus *Proteus*.

New isolates *P. mirabilis* strains, Mak-38, Emb-43, and Lan-46, and *Proteus* spp. strains Ks-19, Ks-20, Ks-23, Mk-24, Mk-25, Mk-27, Mk-28, Em30, Em33, L-34, L-36, Wt1, Wt2, Wt7, and Wt8 are shown in red. Numbers indicated on the nodes are percent posterior probabilities showing statistical support for each node. Branches are colored based on percent posterior probabilities. The scale bar below the tree indicates the number of expected changes (or substitutions) per site. The *B. altitudinis* strain GOES12 (OL851791.1) was used as an outgroup in the phylogenetic tree.

2. *Staphylococcus aureus*

In the neighbour-joining phylogenetic trees based on 16S rRNA gene sequences, the MDR isolates formed clades with the related strains from the database. All seven isolates shared nodes, with bootstrap values ranging from 87% to 100% (Figure 4.7). All seven isolates were closely related to *S. aureus* strains in the databases and thus were identified as *S. aureus*.

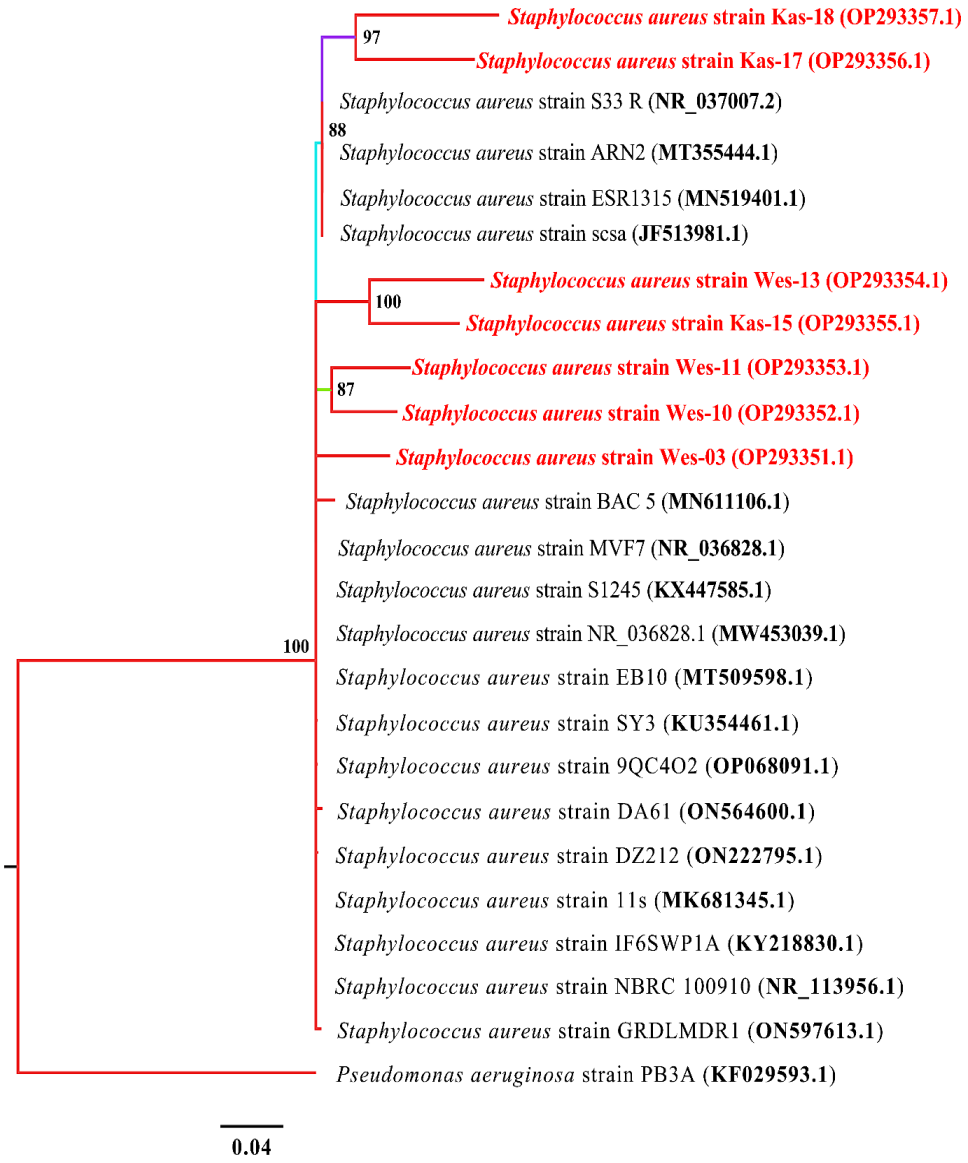

Figure 4.7: Phylogenetic Tree Built by MrBayes v3.2.7 using 17 16S rRNA Sequences of *S. aureus* Species.

New isolates *S. aureus* strains Wes-03, Wes-10, Wes-11, Wes-13, Kas-15, Kas-17, and Kas-18 are shown in red. Numbers indicated on the nodes are percent posterior probabilities showing statistical support for each node. Branches are colored based on percent posterior probabilities. The scale bar below the tree indicates the number of expected changes (or substitutions) per site. The *P. aeruginosa* strain PB3A (KF029593.1) was used as an outgroup in the phylogenetic tree.

3. *Pseudomonas aeruginosa*

Based on the phylogenetic analysis using 16S rRNA, the three MDR isolates of *P. aeruginosa* exhibited a high degree of similaritywith one another, with similarity percentages between 98.5% and 100%. *P. aeruginosa* strains Emb-40 and Emb-41 had similar identity although they were obtained from different markets at the same location (Figure 4.8).

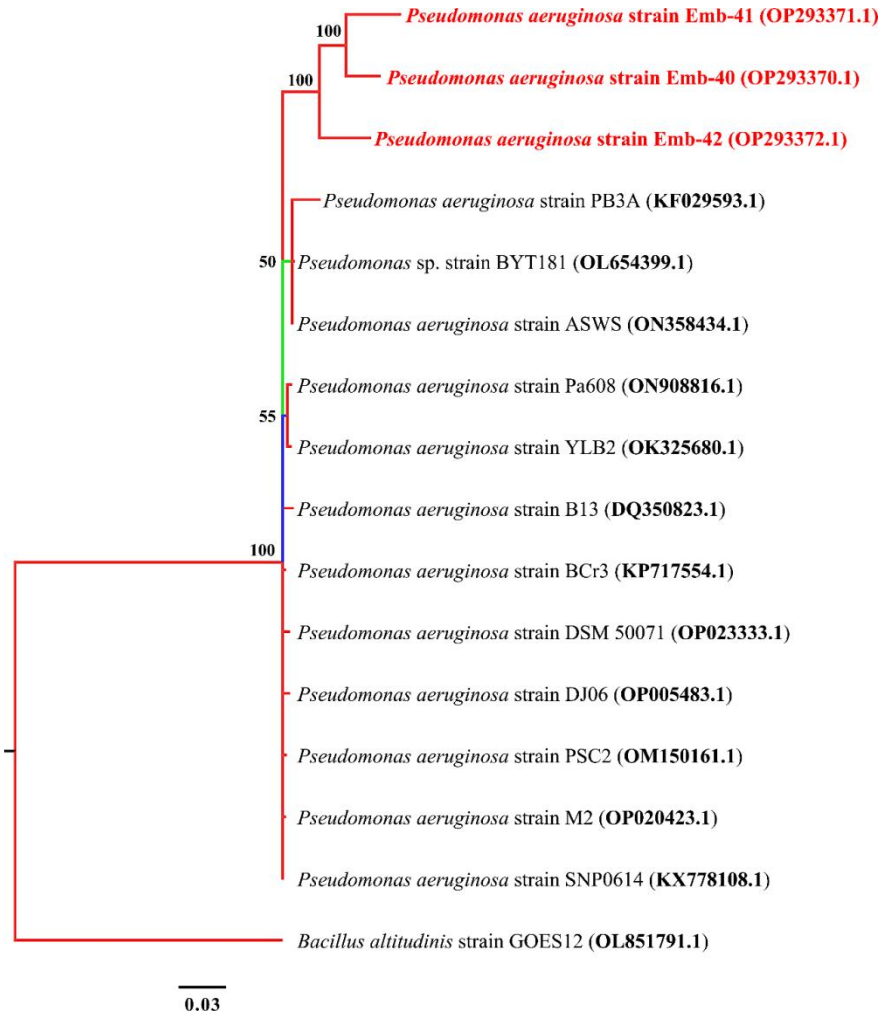

Figure 4.8: Phylogenetic tree built by MrBayes v3.2.7 using 21 16S rRNA sequences of *P. aeruginosa* species. New isolates *P. aeruginosa* strains Emb-40, Emb-41, and Emb-42 are shown in red. Numbers indicated on the nodes are percent posterior probabilities showing statistical support for each node. Branches are colored based on percent posterior probabilities. The scale bar below the tree indicates the number of expected changes (or substitutions) per site.

4. *Vibrio* species

A phylogenetic tree of 16S rRNA sequences showed that all the tested samples were grouped into two main clusters. Each of the two species identified, *V. cholerae* and *V. parahaemolyticus*, clustered separately (Figure 4.9).

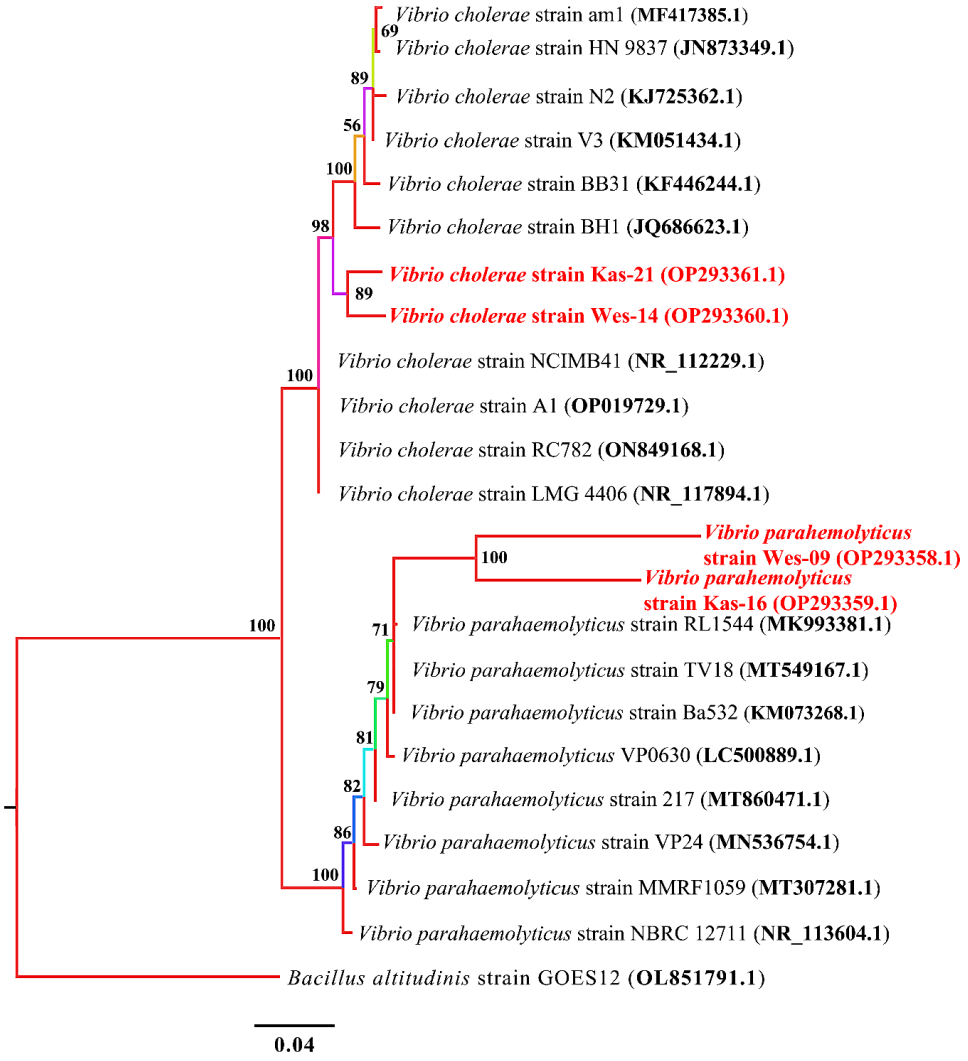

Figure 4.9: Phylogenetic tree built by MrBayes v3.2.7 using 10 and 8 16S rRNA sequences of *V. cholerae* and *V. parahaemolyticus*, respectively. New isolates *V. cholerae* strains Wes-14 and Kas-21, and *V. parahaemolyticus* strains Wes-06 and Kas-16 are shown in red. Numbers indicated on the nodes arepercent posterior probabilities showing statistical support for each node. Branches are colored based on percent posterior probabilities. The scale bar below the tree indicates the number of expected changes (or

substitutions) per site. The *B. altitudinis* strain GOES12 (OL851791.1) was used as an outgroup in the phylogenetic tree.

4.5.3 Identification of MDR *Citrobacter freundii, Klebsiella* spp., and *Enterobacter* spp. using BLASTn analysis

BLASTn results revealed the percentage of similarity between MDR isolates and closely related bacteria in the GenBank (Table 4.8). From the 16S rRNA sequences, BLASTn analysis showed the three MDR *C. freundii* (accession numbers OP047930.1, OP047934.1and OP047944.1) Using BLASTn analysis and the globally published NCBI database, two MDR isolates of *Klebsiella* spp., were confirmed as *K. michiganensis*, One MDR isolate of *Enterobacter* was confirmed as *Enterobacter* sp. strain 2B1C (Table 4.8).

Table 4.8: Similarity of 16S rRNA Sequences of Antibiotic Resistant Bacteria Isolates from Nile Tilapia, Compared with that of Accessions in the Genbank Database.

No.	Isolate ID Code	Sub-County/ Location	16S rRNA Accession	Closest Match in Blast	Similarity (%)	Accession
1	*Citrobacter freundii* strain Wt4	Westlands	OP047930.1	*C.freundi i*strain bright	95.53	OM538425.1
2	*Citrobacter freundii* strain Ks-12	Kasarani	OP047934.1	*C.freundii* strain haritD11	95.39	KC344791.1
3	*Citrobacter freundii* strain Em31	Embakasi	OP047944.1	*C.freundii* strain R2A5	94.53	KF938666.1
4	*Klebsiella* spp. strain Em29	Embakasi	OP047942.1	*K.michiganensis* strain B3	93.94	MF083086.1
5	*Klebsiella* spp. strain L-37	Lang'ata	OP047948.1	*K.michiganensis* strain B3	92.08	MF083086.1
6	*Enterobacter* spp. strain Wt5	Westlands	OP047931.1	*Enterobacter* sp. strain 2B1C	91.69	EU693561.1

1. Phylogenetic analysis of *C. freundii, Klebsiella* spp., and *Enterobacter* spp.

Phylogenetic analysis (Figure 4.10) revealed close relationship among isolates' respective genera, shown by the sharing of bootstrap values and clades with their representatives from NCBI. *Bacillus altitudinis* strain GOES12 (OL851791.1) was used as an out-group.

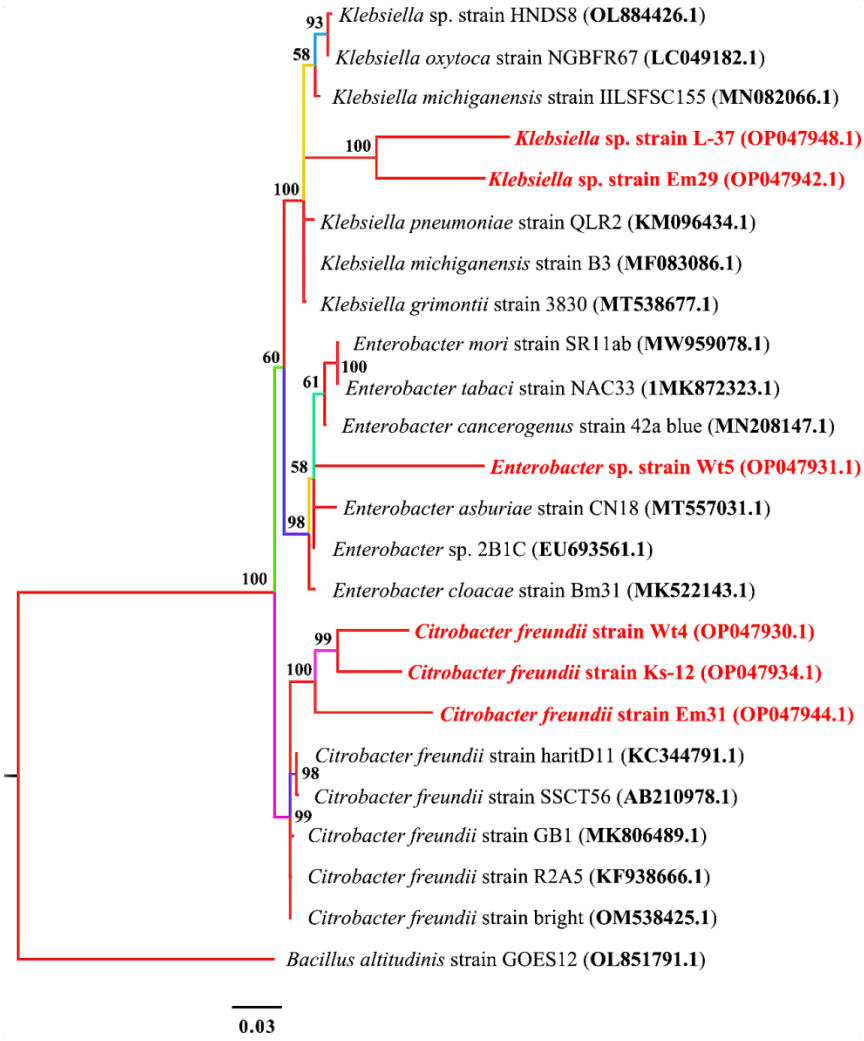

Figure 4.10: Phylogenetic Tree Built by MrBayes v3.2.7 Using Twenty-Three 16S rRNA Sequences of *C. freundii, Enterobacter,* and *Klebsiella* spp.

New isolates *C. freundii* strainWt4, Ks-12, and Em31; *Enterobacter* sp., strain Wt5, and *Klebsiella* sp., strain Em29 and L-37 are shown in red.Numbers indicated on the nodes are percent posterior probabilities showing statistical support for each node. Branches are coloured based on percent posterior probabilities. The scale bar below the tree indicates the

number of expected changes (or substitutions) per site. The outgroup *B. altitudinis* strain GOES12 (OL851791.1) was used in rerooting the tree.

4.6 Molecular detection of the antimicrobial resistance

4.6.1 Detection of antimicrobial resistance genes in *Salmonella typhimurium* and *E. coli*

The distribution of antibiotic-resistant elements among the multidrug resistant (MDR) *S. typhimurium* and *E. coli* are presented in Table 4.9. Among the *S. typhimurium* screened for resistant genes blaTEM-1, blaCMY-2, blaCTX-M, and blaZ (beta-lactamase resistance genes), catI (chloramphenicol resistant genes) in EMB-32 only, *sul*2 (sulphonamide resistant gene), *str*A (streptomycin inactivating gene), *aad*A (aminoglycoside resistant gene) and *tet*C (tetracycline resistance gene) were present. No amplification of *tet*A and *dfr*A7 was observed in *S. typhimurium* WES-09 and *S. typhimurium* EMB-32 isolates, respectively.

The two *E. coli* isolates tested positive for antimicrobial resistant genes blaTEM-1, blaCMY-2, blaCTX-M, blaZ(beta–lactamase resistance gene), *sul*2 (sulphonamide resistant gene), *str*A (streptomycin inactivating gene), *aad*A (aminoglycoside resistant gene), *tet*C (tetracycline resistance gene) and *dfr*A7 (trimethoprim resistant gene). One *E. coli* isolate (LAN-35) showed no amplification for *tet*A gene. Figure 4.12 shows a representative agarose gel of the amplification of tested antibiotic resistant genes in MDR *S. typhimurium* and *E. coli*.

Table 4.9: Multidrug Resistance Patterns, Genetic Markers, and Drug Resistance-Associated Genes of MDR *Salmonella Typhimurium* and *E. coli* Isolates

Microorganism	Isolate ID	Antibiotic resistant pattern	No. of antibiotics (Classes*)	Gentic markers for drug resistance			Antibiotic resistant associated genes										
Salmonella				InvA	HilA	uidA	tetA	tetC	blaTEM-1	blaCMY-2	blaCTX-M	blaZ	sul2	CatI	dfrA7	strA	aadA
	WES-09	CPD, RIF, S, VA	4 (4)	+	+	NT	-	+	+	+	+	+	+	-	+	+	+
	MAK-22	AX, NIT, P-G, RIF, VA	5 (5)	+	+	NT	+	+	+	+	+	+	+	-	+	+	+
	EMB-32	AX, CAZ, C, MRP, VA	5 (5)	+	+	NT	+	+	+	+	+	+	+	+	-	+	+
Escherichia coli	MAK-26	AX, MRP, RIF, VA	4 (4)	NT	NT	+	+	+	+	+	+	+	+	-	+	+	+
	LAN-35	AX, MRP, RIF, VA	4 (4)	NT	NT	+	-	+	+	+	+	+	+	-	+	+	+

AX = Ampicillin/Cloxacillin; CPM = Cefepime; CPD = Cefpodoxime; CAZ = Ceftazidime; C = Chloramphenicol; MRP = Meropenem; NIT = Nitrofurantoin; P = Penicillin-G; RIF = Rifampicin; S = Streptomycin; VA = Vancomycin. NT = Not Tested.

TetA and tetC = Tetracycline resistant genes, ^{bla}TEM-1, ^{bla}CMY-2, ^{bla}CTX-M, ^{bla}Z= Beta lactamases-encoding genes, catI = chloramphenicol resistant gene, sul2 = sulphonamide resistant gene, dfrA7 = Trimethoprim resistant gene, strA = streptomycin inactivating gene and aadA = Aminoglycoside resistant genes.

+ indicates the presence of the resistance genes following amplification by PCR; − indicates the absence of the target resistance genes following amplification by PCR.

*Classes of antibiotics are: Carbapenems (MRP), Cephalosporin (third generation; CAZ), Penicillin (P-G), Beta-lactam (AX), Cephalosporin (third generation; CPD), Phenicol (C), Cephalosporin (fourth generation, CPM), Nitrofurans (NIT), Glycopeptides (VA), Ansamycin (RIF), and Aminoglycosides (S)**.**

4.6.2 Detection of antimicrobial resistance genes by PCR in *Proteus* spp., *Staphylococcus aureus, Pseudomonas aeruginosa, Vibrio cholerae,* and *Vibrio parahemolyticus*

PCR was used to determine the drug resistance genes of MDR isolates of the different bacterial pathogens. The distribution of antimicrobial-resistant genes among multidrug-resistant (MDR) *Proteus spp*., *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus* are presented in Table 4.10. All the MDR isolates of the different bacteria species tested positive for blaTEM-1, blaCMY-2, sul2, strA, aadA, tetA, tetC, and dfrA7. However, sul2 gene was not amplified in two *S. aureus* isolates (accession numbers OP293352.1 and OP293356.1). Figure 4.11 shows a representative agarose gel of the amplification of *Proteus* spp., *S. aureus, P. aeruginosa, V. cholerae* and *V. parahaemolyticus* tested antibiotic-resistant genes.

Table 4.10: Distribution of Antimicrobial Resistant Genes in MDR Isolates of the Different Bacteria Pathogens

MDR isolates	**Antibiotic resistant associated genes (%)**							
	***tet*A**	***tet*C**	**^{bla}TEM-1**	**^{bla}CMY-2**	***sul*2**	***dfr*A7**	***str*A**	***Aad*A**
Proteus spp. (n = 24)	24 (100)	24(100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)
Staphylococcus aureus (n = 7)	7 (100)	7 (100)	7 (100)	7 (100)	5 (71.4)	7 (100)	7 (100)	7 (100)
Pseudomonas aeruginosa (n = 3)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3(100)	3 (100)
Vibrio cholera (n = 2)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
Vibrio parahemolyticus (n = 2)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
Total (n = 38)	38 (100)	38 (100)	38 (100)	38 (100)	36 (94.7)	38 (100)	38 (100)	38 (100)

*tet*A and *tet*C = Tetracycline resistant genes, ^{bla}TEM-1 and ^{bla}CMY-2 = beta lactamases-encoding genes, *sul*2 = sulphonamide resistant gene, *dfr*A7 = Trimethoprim resistant gene, *str*A = streptomycin resistant gene and *aad*A = Aminoglycoside resistant genes.

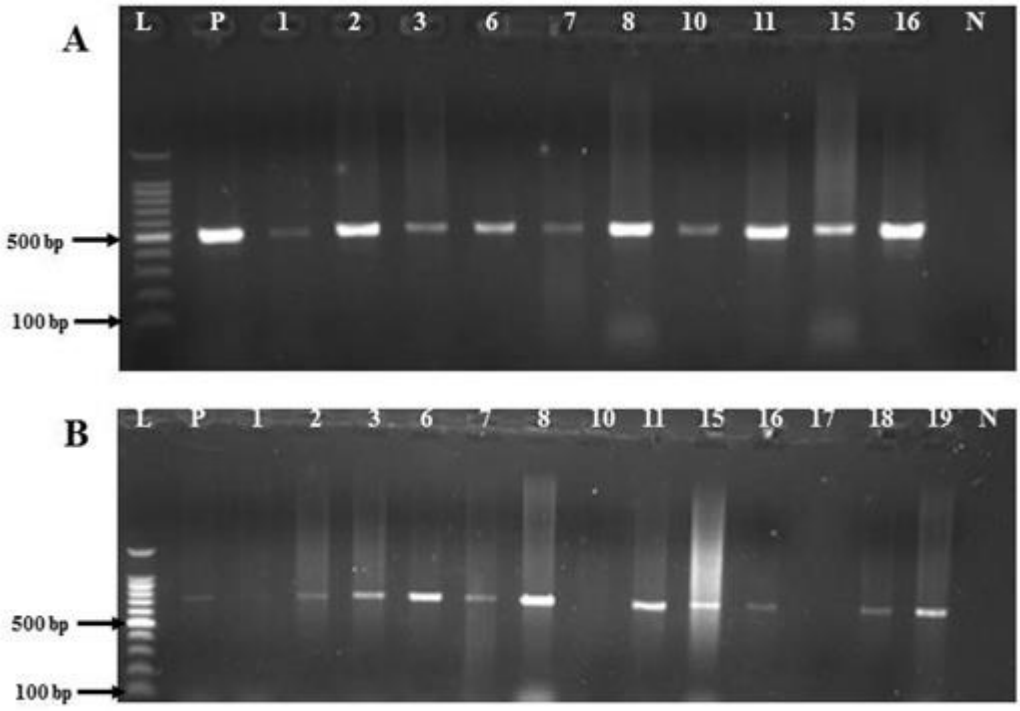

Figure 4.11: Agarose Gel Images Showing Amplification of Antibiotic Resistance Genes (A) Blatem-1 and (B) Sul2 for MDR Isolates of Different Bacterial Pathogens. Lane L: Molecular weight marker (100 bp), Lanes P and N represent positive and negative controls, respectively. Lane1 = *Proteus* sp. strain Wt1, 2 = *Proteus* sp. strain Wt2, 3 = *S. aureus* strain Wes-03, 6 = *V. parahaemolyticus* strain Wes-06, 7 = *Proteus* sp. strain Wt7, 8 = *Proteus* sp. strain Wt8, 10 = *S. aureus* strain Wes-10, 11 = *S. aureus* strain Wes-11, 15 = *S. aureus* strain Kas-15, 16 = *V. parahaemolyticus* strain Kas-16, 17 =*S. aureus* strain Kas-17, 18 = *S. aureus* strain Kas-18, 19 = *Proteus* sp. strain Ks-19.

4.6.3 Detection of Antimicrobial Resistance Genes by PCR in *C. Freundii, Klebsiella* and *Enterobacter*

This study detected antibiotic resistance genes (tetA, tetC, ^{bla}TEM-1, ^{bla}CMY-2, Sul2, dfrA7, strA, aadA) in all MDR isolates of *C. freundii*, *Klebsiella* and *Enterobacter* by conventional PCR. All the MDR bacteria harboured ^{bla}CMY-2 (Figure 4.12), as well as tetA, tetC, ^{bla}TEM-1, sul2, dfrA7, strA, aadA genes.

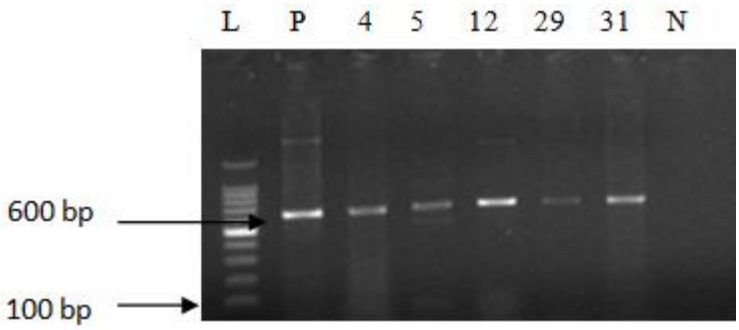

Figure 4.12: Gel Image Showing Antibiotic Resistance Gene (Blacmy-2) for MDR Bacteria: Lane L: Molecular weight marker (100 bp), Lanes P and N represent positive and negative controls, respectively. Lane 4 = *Citrobacter freundii* strain Wt4, 5 = *Enterobacter* spp. strain Wt5, 12 = *C. freundii* strain Ks-12, 29 = *Klebsiella* spp. strain Em29, 31 = *C. freundii* strain Em31. The positive confirmed Isolates have a molecular size of 600bp.

4.7 Detection of Genetic Markers Coding for Drug Resistance in *Salmonella typhimurium* and *E. coli*

The distribution of genetic markers that code for drug resistance among the multidrug resistant (MDR) *S. typhimurium* and *E. coli* are presented in Table 4.9. All the three MDR *S. typhimurium* harbored *invA* (*Salmonella* invasion gene) and *hilA*, whereas the two MDR *E. coli* isolates contained *uidA* (Figure 4.13).

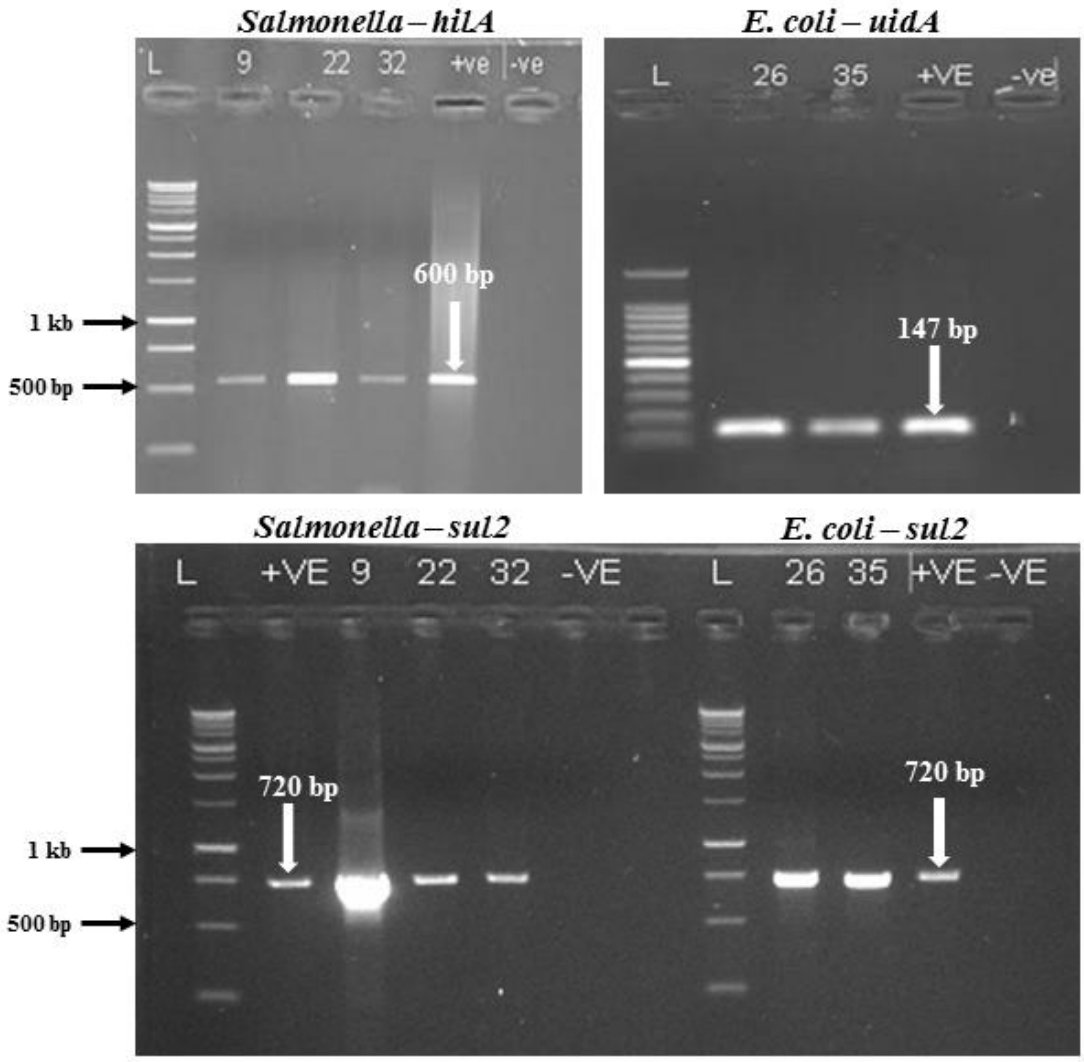

Figure 4.13: Agarose Gel Image Showing Genetic Markers hilA and uidA for *S. typhimurium* and *E. coli,* Respectively and Antibiotic Resistance Gene (sul2) for *S.typhimurium* and *E. coli*. Lane L: Molecular weight marker, Lane +ve: positive control, Lane -ve: negative control, Lanes 9, 22 and 32: MDR *Salmonella* isolates, Lanes 26 and 35: MDR *E. coli* isolates.

4.8 Determination of Multiple Antibiotic Resistance (MAR) among Isolated Bacteria

4.8.1 Multidrug Resistant Patterns of the *Salmonella typhimurium* and *E. coli* Isolates

The different *Salmonella* and *E. coli* isolates exhibited a diverse pattern of resistance to a minimum of one class and a maximum of 5 classes of antimicrobials, among the 9 classes tested. The distribution of MDR and MAR index of *S. typhimurium* and *E. coli* isolates is presented in Table 4.11. Isolates were classified as multi-drug resistant if they were resistant to at least three different classes of antibiotics. Based on this classification, 5 isolates for both *S. typhimurium* and *E. coli* were MDR. In this study we found that 3 out of 18 (16.7%) *S. typhimurium* isolates from fresh Nile tilapia fish were resistant to at least three different classes of antibiotics and were considered to be MDR isolates (Table 4.11). Out of the three *S. typhimurium* isolates, two were resistant to five antibiotics (AX + NIT + P + RIF + VA and AX + CAZ + C + MRP + VA) which belonged to 5 different classes of antibiotics with a MAR index of 0.45. One *S. typhimurium* isolates was resistant to four antibiotics (CPD + RIF + S + VA) which belonged to 4 different classes of antibiotics with a MAR index of 0.36. Two *E. coli* isolates were resistant to five antibiotics (AX + MRP + RIF + VA) which belonged to four different classes of antibiotics with a MAR index of 0.36 (Table 4.11)

Table 4.11: Distribution of Multiple Antibiotic Resistant Characterizations of the *Salmonella* species and *E. coli* Isolated from Fresh Nile Tilapia Sold in Retail Markets in Nairobi.

Microorganism	Antibiotic resistant pattern	No. of antibiotics (Classes)	No. of resistant species (%)	MDR pattern	MAR index
Salmonella	CPD	1 (1)	1(5.6)	No	0.09
(n = 18)	CAZ	1 (1)	1(5.6)	No	0.09
	P	1 (1)	3(16.7)	No	0.09
	CPD + RIF + S + VA	4 (4)	1(5.6)	Yes	0.36
	AX + NIT + P + RIF + VA	5 (5)	1(5.6)	Yes	0.45
	AX + CAZ + C + MRP + VA	5 (5)	1(5.6)	Yes	0.45
Escherichia coli	C	1 (1)	1(4.2)	No	0.09
(n = 24)	VA	1 (1)	1(4.2)	No	0.09
	MRP	1 (1)	1(4.2)	No	0.09
	P	1 (1)	4(16.7)	No	0.09
	RIF	1 (1)	4(16.7)	No	0.09
	P + VA	2 (2)	1(4.2)	No	0.18
	AX + MRP + RIF + VA	4 (4)	2(8.3)	Yes	0.36

AX = Ampicillin/Cloxacillin; CPM = Cefepime; CPD = Cefpodoxime; CAZ = Ceftazidime; C = Chloramphenicol; MRP = Meropenem; NIT = Nitrofurantoin; P = Penicillin-G; RIF = Rifampicin; S = Streptomycin; VA = Vancomycin. MAR = Multiple antibiotic resistance. The classes of antibiotics are: Carbapenems (MRP), Cephalosporin (third generation; CAZ), Penicillin (P-G), Beta-lactam (AX), Cephalosporin (third generation; CPD), Phenicol (C), Cephalosporin (fourth generation, CPM), Nitrofurans (NIT), Glycopeptides (VA), Ansamycin (RIF), and Aminoglycosides (S)

4.8.2 Multidrug Resistant Patterns of *Prote*us spp., *S. Aureus, P. Aeruginosa, V. Cholerae and V. Parahaemolyticus*

The MDR, XDR, and MAR index distribution of bacterial isolates presented in Table 4.12. Multidrug resistance was present among all five bacterial pathogens (Table 4.12). As revealed by the antibiogram typing, *Proteus* spp. (56.7%), *S. aureus* (71.4%), *P.*

aeruginosa (33.3%), *V. cholerae* (100%) and *V. parahaemolyticus* (100%) were multidrug resistant. XDR was expressed only in *Proteus* spp. (23.3%), *S. aureus* (28.6%), and *P. aeruginosa* (66.7%). No PDR was expressed by the tested bacterial isolates. Among the antibiogram types, PEN-VAN-RIF-AX-CPD-MRP-STR showed the highest prevalence (23.3%, seven isolates) in *Proteus* spp., with MAR of 0.64. A total of 6.7% of *Proteus* spp. were resistant to three antibiotics (VAN, RIF, AX) which belong to three different groups of antimicrobials with a MAR index of 0.27. A total of 1/7 (14.3%) *S. aureus* were resistant to four antibiotics (RIF, AX, CPD, MRP) which belong to four different groups of antimicrobials with a MAR index of 0.36. Furthermore, 2/7 (28.6%) *S. aureus* were resistant to seven antibiotics (PEN, VAN, RIF, AX, CPD, CAZ, MRP) which belong to six different groups of antimicrobials with a MAR index of 0.64. A total of 1/3 (33.3%) *P. aeruginosa* were resistant to six antibiotics (PEN, VAN, CPD, CAZ, MRP, STR) which belong to five different groups of antimicrobials with a MAR index of 0.55. Furthermore, 2/3 (66.7%) of *P. aeruginosa* were resistant to seven antibiotics (PEN, VAN, RIF, AX, CAZ, MRP, STR) which belong to seven different groups of antimicrobials with a MAR index of 0.64. *V. cholerae* showed a different MDR pattern (PEN, VAN, AX, CPD, CHL, STR and PEN, VAN, AX, CPD, NIT, STR) but with similar MAR index (0.55). A total of 1/2 (50%) *V. parahaemolyticus* were resistant to four antibiotics (PEN, VAN, RIF, AX) which belong to four different groups of antimicrobials with a MAR index of 0.36. Furthermore, 1/2 (50%) *V. parahaemolyticus* were resistant to five antibiotics (PEN, VAN, AX, MRP, STR) which belong to four different groups of antimicrobials with a MAR index of 0.45 (Table 4.12). Overall, diverse patterns of resistance to different classes of antibiotics were observed among *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus* isolates (Table 4.12).

Table 4.12: Distribution of Multiple Antibiotic Resistances in *Proteus* spp., *S. aureus, P.aeruginosa, V.cholerae* and *V. parahemolyticus*

Microorganism	Number of antimicrobial class	Number of antibiotics	Resistance phenotypes	MDR prevalence (%)	XDR prevalence (%)	MAR index
Proteus spp. (n = 30)						
	3	3	VAN^R, RIF^R, AX^R	2 (6.7)	-	0.27
	4	4	VAN^R, AX^R, CHL^R, STR^R	2 (6.7)	-	0.36
	4	4	RIF^R, AX^R, CHL^R, STR^R	1 (3.3)	-	0.36
	4	4	PEN^R, VAN^R, RIF^R, CPD^R	1 (3.3)	-	0.36
	4	4	VAN^R, RIF^R, AX^R, CPD^R	1 (3.3)	-	0.36
	4	4	RIF^R, CPD^R, NIT^R, STR^R	1 (3.3)	-	0.36
	4	4	RIF^R, AX^R, MRP^R, STR^R	1 (3.3)	-	0.36
	5	5	VAN^R, RIF^R, CPD^R, NIT^R, STR^R	1 (3.3)	-	0.45
	5	5	VAN^R, RIF^R, AX^R, MRP^R, STR^R	3 (10)	-	0.45
	4	5	VAN^R, RIF^R, AX^R, CPD^R, CAZ^R,	2 (6.7)	-	0.45
	7	7	PEN^R, VAN^R, RIF^R, AX^R, CPD^R, MRP^R, STR^R	-	7 (23.3)	0.64
	6	7	PEN^R, VAN^R, RIF^R, AX^R, CPD^R, CAZ^R, STR^R	2 (6.7)		0.64
				17 (56.7)	**7 (23.3)**	
S. aureus (n = 7)						
	4	4	RIF^R, AX^R, CPD^R, MRP^R	1 (14.3)		0.36
	5	6	PEN^R, RIF^R, AX^R, CPD^R, CAZ^R, MRP^R	1 (14.3)		0.55
	5	6	VAN^R, RIF^R, AX^R, CPD^R, CAZ^R, MRP^R	1 (14.3)		0.55
	7	7	PEN^R, VAN^R, RIF^R, AX^R, CPD^R, MRP^R, STR^R		1 (14.3)	0.64
	7	7	VAN^R, RIF^R, AX^R, NIT^R, CAZ^R, MRP^R, STR^R		1 (14.3)	0.64
	6	7	PEN^R, VAN^R, RIF^R, AX^R, CPD^R, CAZ^R, MRP^R	2 (28.6)		0.64
				5 (71.4)	**2 (28.6)**	
P. aeruginosa (n = 3)						
	5	6	PEN^R, VAN^R, CPD^R, CAZ^R, MRP^R, STR^R	1 (33.3)		0.55
	7	7	PEN^R, VAN^R, RIF^R, AX^R, CAZ^R, MRP^R, STR^R		2 (66.7)	0.64
				1 (33.3)	**2 (66.7)**	
V. cholera (n = 2)						
	6	6	PEN^R, VAN^R, AX^R, CPD^R, CHL^R, STR^R	1 (50)		0.55
	6	6	PEN^R, VAN^R, AX^R, CPD^R, NIT^R, STR^R	1 (50)		0.55
				2 (100)		
V. parahemolyticus(n = 2)						
	4	4	PEN^R, VAN^R, RIF^R, AX^R	1 (50)		0.36
	4	5	PEN^R, VAN^R, AX^R, MRP^R, STR^R	1 (50)		0.45
				2 (100)		

PEN, Penicillin-G (10 µg); VAN, Vancomycin (30 µg); RIF, Rifampicin (5 µg); AX, Ampicillin/Cloxacillin (10 µg); CPM, Cefepime (30 µg); CPD, Cefpodoxime (10 µg); CHL, Chloramphenicol (50 µg); NIT, Nitrofurantoin (300 µg); CAZ, Ceftazidime (30 µg); MRP, Meropenem (10 µg); STR, Streptomycin (10 µg). MDR = Multidrug resistance, XDR = Extensively drug resistance, and MAR = Multiple antibiotic resistance index. MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories. XDR: non-susceptible to ≥1 agent in all but ≤2 categories.

4.8.3 Multiple Antibiotic Resistance Phenotypes (MARPs) and Multiple Antibiotic Resistance (MAR) Index of *Citrobacter freundii, Klebsiella* and *Enterobacter* spp.

Varied MARP and MAR index distribution pattern of bacterial isolates in Table 4.13 illustrate that the potential pathogens harboured antimicrobial resistance to at least three different classes of antibiotics. All the *C. freundii* (100%) were multidrug resistant. *Klebsiella* spp., and *Enterobacter* spp., showed 18.2% and 16.7% multidrug resistance respectively.

For *C. freundii*, the VAN^R-RIF^R-AX^R-MRP^R, VAN^R-RIF^R-CPD^R-MRP^R and VAN^R-RIF^R-AX^R-CPD^R-CAZ^R-MRP^R MAR-phenotypes were detected each in one isolate with MAR-index of 0.36, 0.36 and 0.45 respectively. *Klebsiella* spp., showed MAR-phenotypes VAN^R-AX^R-CAZ^R-MRP^R and PEN^R-VAN^R-RIF^R-AX^R-CAZ^R-MRP^R each in one isolate with MAR index of 0.36 and 0.55 respectively. *Enterobacter* spp., showed MAR-phenotype P-VA-RIF-MRP with MAR-index of 0.36.

Table 4.13: Distribution of multiple antibiotic resistances in *C. freundii, Klebsiella* spp., and *Enterobacter* spp.

Microorganism	Number of antimicrobial class	Number of antibiotics	Resistance phenotypes	MDR prevalence (%)	MAR index
C. freundii (n=3)					
	4	4	VAN^R, RIF^R, AX^R, MRP^R	1(33.3)	0.36
	4	4	VAN^R, RIF^R, CPD^R, MRP^R	1(33.3)	0.36
	5	6	VAN^R, RIF^R, AX^R, CPD^R, CAZ^R, MRP^R	1(33.3)	0.55
				3 (100)	
Klebsiella spp. (n=11)					
	2	2	VAN^R, STR^R	1(9.1)	0.18
	2	2	PEN^R, AX^R	1(9.1)	0.18
	2	2	AX^R, STR^R	1(9.1)	0.18
	2	2	PEN^R, MRP^R	1(9.1)	0.18
	2	2	RIF^R, MRP^R	1(9.1)	0.18
	4	4	VAN^R, AX^R, CAZ^R, MRP^R	1(9.1)	0.36
	6	6	PEN^R, VAN^R, RIF^R, AX^R, CAZ^R, MRP^R	1(9.1)	0.55
				2 (18.2)	
Enterobacter spp. (n=6)					
	2	2	P, RIF	1 (16.7)	0.18
	2	2	RIF, MRP	1 (16.7)	0.18
	4	4	P, VA, RIF, MRP	1 (16.7)	0.36
				1 (16.7)	

PEN, Penicillin-G (10 µg); VAN, Vancomycin (30 µg); RIF, Rifampicin (5 µg); AX, Ampicillin/Cloxacillin (10 µg); CPM, Cefepime (30 µg); CPD, Cefpodoxime (10 µg); CHL, Chloramphenicol (50 µg); NIT, Nitrofurantoin (300 µg); CAZ, Ceftazidime (30 µg); MRP, Meropenem (10 µg); STR, Streptomycin (10 µg). MDR = Multidrug resistance, and MAR = Multiple antibiotic resistance index.

4.9 Correlation Coefficient between Phenotypic Resistance Pattern to the Antibiotics for MDR Bacteria and the Presence of Antibiotic Resistance Genes

4.9.1 Pearson Correlation Coefficient Among Various Tested Antibiotics and the Detected Antibiotic Resistance Genes in *S. typhimurium* and *E. coli* Isolates

The correlation coefficient (r) was assessed among various tested antibiotics and the detected antibiotic resistance genes in bacterial isolates. The results revealed significant positive correlations between MRP and *aad*A gene (r=1), NIT and *str*A gene (r=1), P and *str*A gene (r=1), RIF and *sul*2 (r=1), RIF and *dfr*A7 (r=1), S and *bla*CMY-2 gene (r=1), S and *bla*CTX-M gene (r=1), VA and *bla*TEM-1 (r=1) (Figure 4.14).

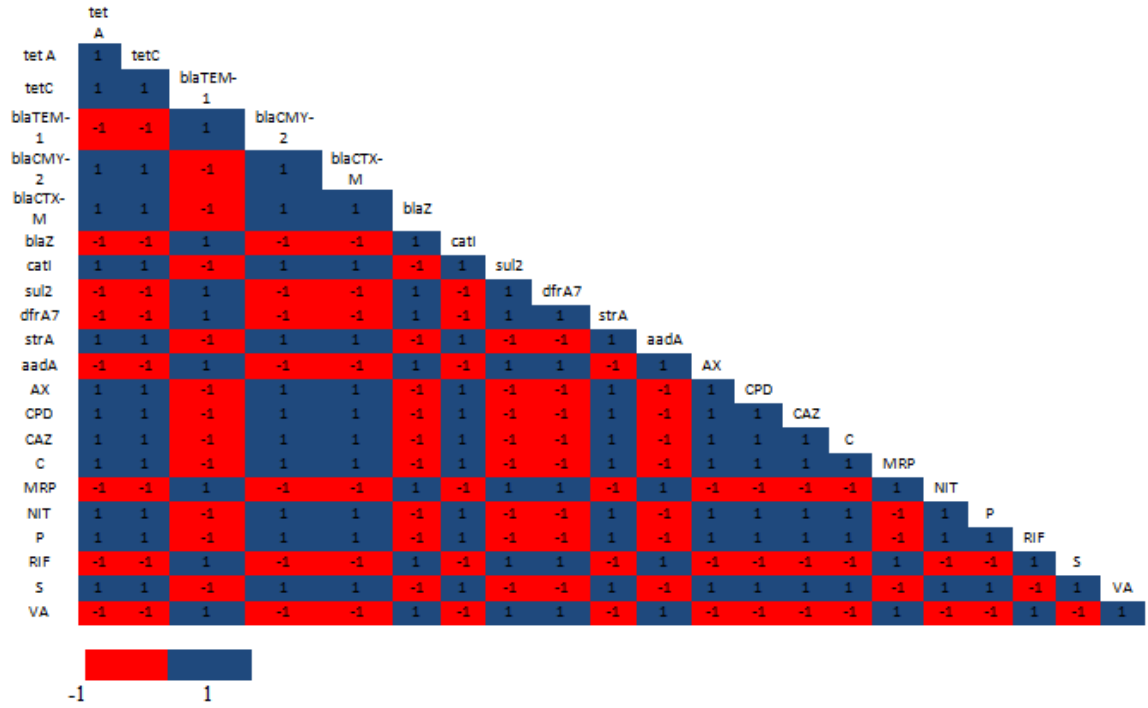

Figure 4.14: Heat-Map Showing the Correlation Coefficient (R) among the Tested Antibiotics and Antibiotic Resistance Genes Detected in the MDR Bacterial Isolates. The intensity of colors indicates the numerical value of the correlation coefficient (r), Red and blue colors refer to the negative and positive correlations, respectively.

4.9.2 Pearson Correlation Coefficient among Various Tested Antibiotics and the Detected Antibiotic Resistance Genes in *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae* and *V. parahemolyticus*

The correlation coefficient (r) was assessed between phenotypic and genotypic multidrug resistance in bacterial isolates. The results showed significant positive correlations between: AX and *str*A gene (r = 0.93), PEN and *bla*CMY-2 gene (r = 0.91), RIF and *dfr*A7 (r = 0.81), CAZ and *aad*A gene (r= 0.81), VAN and *bla*CMY-2 gene (r = 0.78), and CAZ and *dfr*A7 (r= 0.73) (Figure 4.15).

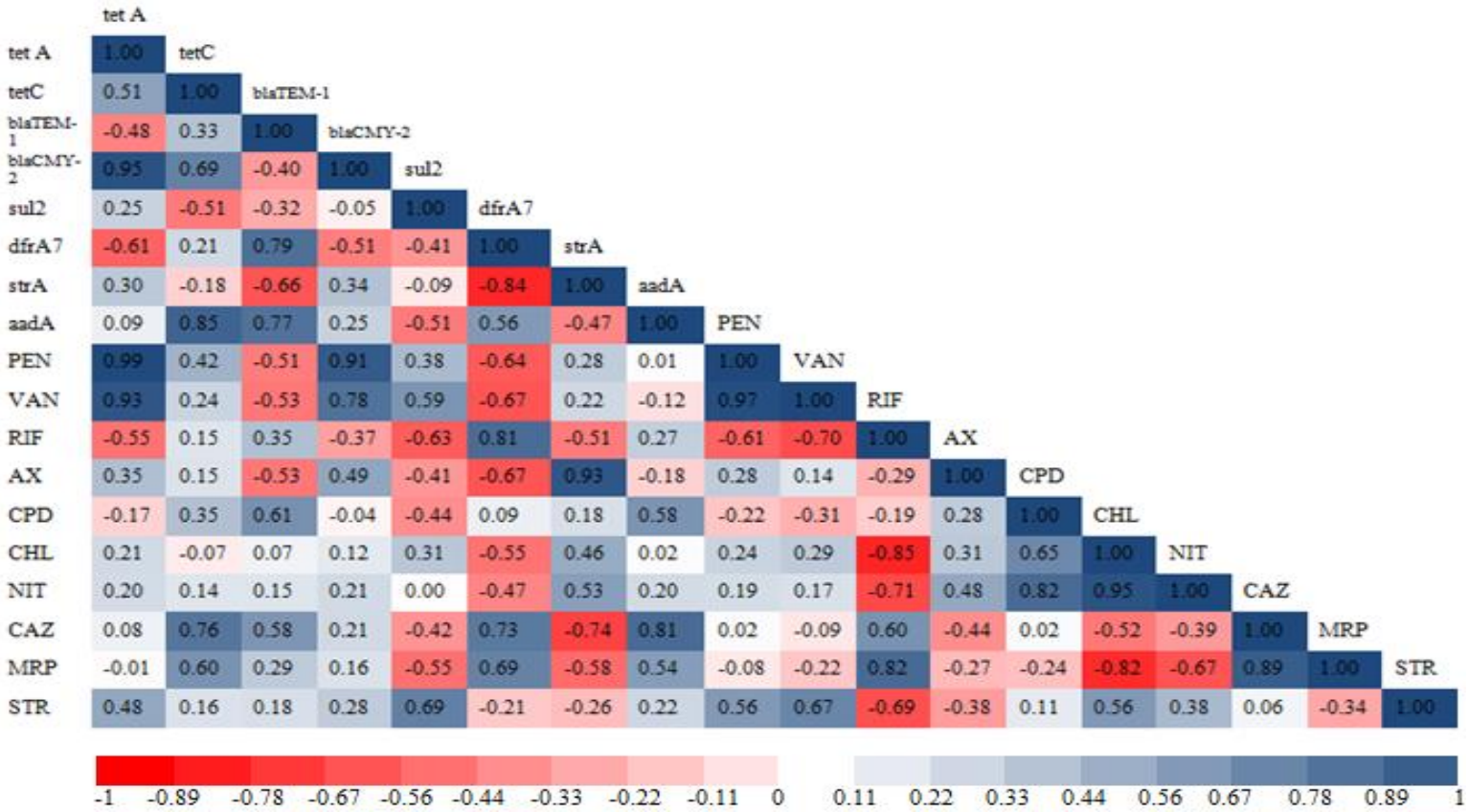

Figure 4.15: The Heat-Map Shows the Correlation Coefficient (R) among the Tested Antibiotics and Antibiotic Resistance Genes Detected in the Recovered Bacterial Isolates. The intensity of colors indicates the numerical value of the correlation coefficient (r), Red and blue colors refer to the negative and positive correlations respectively.

4.9.3 Pearson Correlation Coefficient among Various Tested Antibiotics and the Detected Antibiotic Resistance Genes in *C. freundii, Klebsiella* spp., and *Enterobacter* spp.

The correlation coefficient (r) was also assessed between phenotypic and genotypic multidrug resistance in *C. freundii*, *Klebsiella* spp., and *Enterobacter* spp. The results exhibited significant positive correlations between: PEN and *bla*CMY-2 (r = 1), PEN and *dfr*A7 (r = 0.99), RIF and *str*A gene (r= 0.99), CPD and *str*A gene (r = 0.97), MRP and *str*A (r= 0.96), PEN and *sul*2 gene (r = 0.94), VAN and *str*A (r = 0.94), and PEN and *bla*TEM=1 gene (r = 0.82) (Figure 4.16).

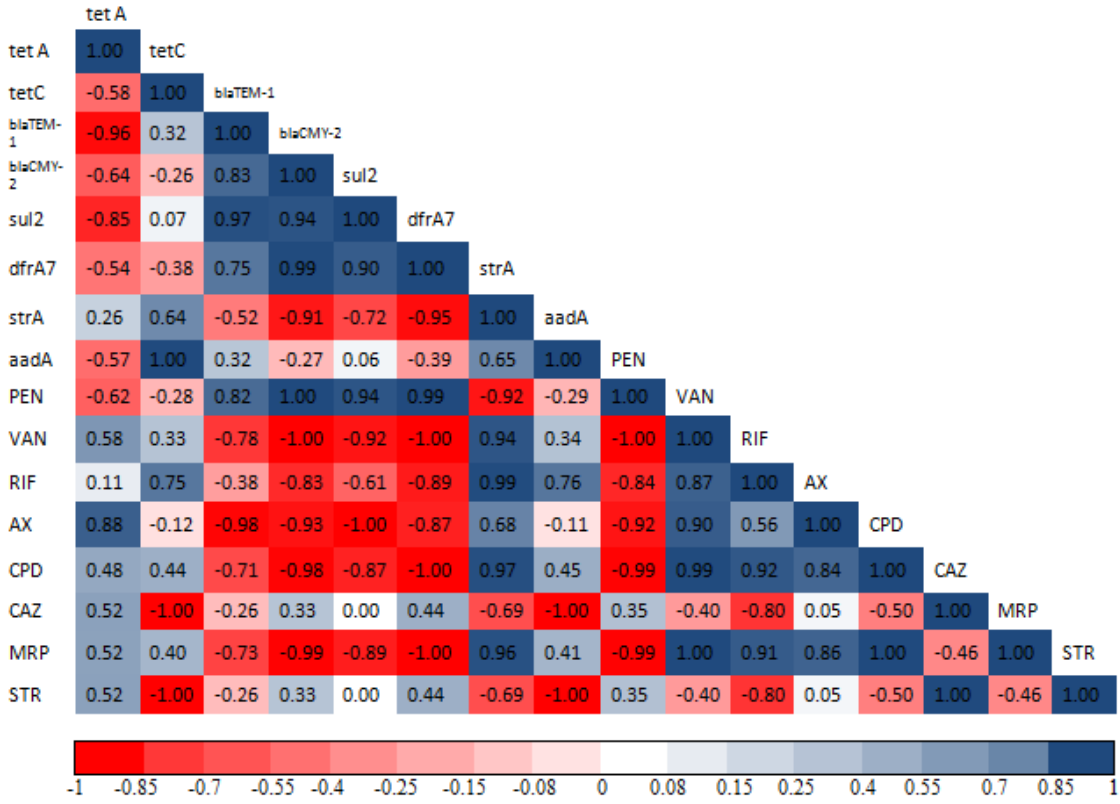

Figure 4.16: The Heat-Map Shows the Correlation Coefficient (R) among the Tested Antibiotics and Antibiotic Resistance Genes Detected in the Recovered Bacterial Isolates. The intensity of colors indicates the numerical value of the correlation coefficient (r), Red and blue colors refer to the negative and positive correlations respectively.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Antimicrobial Resistance Patterns of Bacteria Found in Raw Fish.

5.1.1 Antimicrobial Resistance Patterns of *Salmonella* Spp., and *Pathogenic E. coli*

Salmonella spp. and pathogenic *E. coli* are the most frequent causes of foodborne illnesses, and the increasing occurrence of MDR is an additional cause of concern. The emergence of MDR and extensively drug-resistant *Salmonella* species and *E. coli* isolates is a critical health issue suggesting that the treatment of foodborne diseases caused by these pathogens may become practically challenging in the near future especially in developing countries. Antibiotics are frequently used in the treatment of infectious diseases in both animals and humans (Granowitz and Brown 2008). However, the over-use, misuse and abuse of antibiotics in food-animals have become a great public health concern. Contrarily, because withdrawal periods before harvesting or marketing food-animals products have been ignored, antibiotic residues are now another rising concern to public health (Algammal *et al*., 2020).

The main consequence of the antibiotic residues in animal-derived foods is the enhancement of development of antimicrobial resistance (AMR). The presence of antibiotic-resistant pathogens in animal-derived foods may lead to gastrointestinal disorders in humans (Hossain *et al*., 2022). On the other hand, antibiotic-resistant pathogens may transfer the resistant genes to other co-colonized microorganisms through horizontal or vertical transmission (Granowitz and Brown 2008), and this may result in the spread of antimicrobial resistant pathogens. Several studies have shown the emergence of MDR bacterial pathogens from a wide variety of sources in the food chain, increasing the need for proper use of antibiotics in both the veterinary and human health sectors (Algammal *et al*., 2020; Enany *et al*., 2019). MDR pathogens may cause difficult-to-treat diseases, increase mortality and financial burden. Furthermore, infections caused by MDR pathogens are considered a major global public health crisis by the World Health Organization (WHO) as the discovery

of effective antibiotics has not kept pace with the increase in the bacterial antibiotic resistance (Saraiva *et al*., 2021). The demand for high-value fish protein has escarleted because of the rapid urbanization and change in food habits of nations (Broglia and Kapel, 2011). Food borne pathogens make their way into the food cycle during marketing, production or processing. People get antibiotic-resistant bacterial infections related to fish and fish products through consumption of raw, undercooked or insufficiently heat-treated fish and cross contamination during handling. Due to the increased popularity of fish consumption, it has become necessary to study sea-food associated pathogens and their impact on public health.

Foodborne pathogens transmitted from fish, fish and seafood products can lead to serious infections or even death. It is estimated that more than 12% of food-borne outbreaks related to the consumption of fish are caused by bacteria pathogens (Huss *et al*., 2000; Aberoumand, 2010).Total bacteria count in fish is an important parameter in assessing the level of contamination, quality and public health concern. Several indicator bacteria are not pathogenic themselves but their abundance represents potential risks of contamination (Nabeel *et al*., 2016). In this study, high counts of *Salmonella* and *E. coli* were obtained from the fish samples collected from all the five different locations/sites in Nairobi County, and this confirms previous reports that these are important foodborne pathogens of animal-derived foods. The findings from this study are similar to the high total bacterial counts in fish observed in other studies (Nabeel *et al*., 2016; Marijani, 2022).The presence of *Salmonella* and *E. coli* in fish is due to the fact that aquatic environment is tremendously vulnerable to pollution and run-off from anthropogenic sources which contaminate fish products representing a potential hazard to humans (Sichewo *et al*., 2013).

E. coli is among the major pathogenic microorganisms reaching animal-derived foods and its presence indicates faecal contamination from warm-blooded animals (Chao *et al*., 2003). They are commensal bacteria and its pathotypes can cause zoonotic disease that poses a public health risk. The presence of *E. coli* in the fish samples sold in open-air markets could be due to poor handling of fish by traders as well as unhygienic handling during transportation and storage. The use of contaminated water for cleaning and processing of fish in the markets may also

contribute to secondary contamination. The lack of proper drainage facilities and heavy fly infestation in these fish markets also promotes tertiary contamination to a great extent (Marijani, 2022).

The examination of *Salmonella* and *E. coli* isolated from fish for resistance to 11 antibiotics from 9 different classes of antibiotics revealed the existence of antibiotic resistant phenotypes. The isolated *Salmonella* and *E. coli* showed resistance to ampicillin/Cloxacillin, ceftazidime, chloramphenicol, nitrofurantoin, rifampicin, streptomycin, penicillin-G, rifampicin, cefpodoxime, meropenem and vancomycin which is in agreement with a study by Sifuna *et al*. (2008).This antibiotic resistant profile may be as a result of the frequent use of antimicrobials in fish for growth promotion and therapeutic (Alderman andHastings, 1998; Cabello, 2006). The presence of antibiotic-resistant *Salmonella* spp. and *E. coli* in fresh Nile tilapia fish indicates the role of these fish as spreaders of resistant microorganisms in aquatic environments. The low resistance observed in chloramphenicol by *Salmonella* and *E. coli* could be as a result of the ban on its usage, since it inhibits protein translation causing aplastic anaemia in some patients. Of specific concern is the high rate of resistance seen to streptomycin, as this is one of the watch group antibiotics in the WHO Access, Watch and Reserve (AWaRe) classification of antibiotics for the evaluation and monitoring of use (WHO, 2021c). Resistance to carbapenems (meropenem) may be due to the transmission of bacteria from human sources, especially since carbapenems are not approved for use in food-animals (Poirel *et al*., 2014). According to the WHO, carbapenem-resistant *Salmonella* spp. and *E. coli* are considered to be among the most critical pathogens (Tacconelli *et al*., 2018). The detection of carbapenem-resistant *Salmonella* spp. and *E. coli* in fish has to be treated as an urgent public health problem. Additionally, Vancomycin is an antibiotic of the last resort in bacterial infections, so presence of vancomycin resistant *Salmonella* and *E. coli* isolates in this study is a concern for consumer health.

The morphological characteristics observed in this study support the previous observation contained in the WHO Global Salm-Surv as described by Henderiksen *et al*. (2003) for *Salmonella* and also in the manual for identification of medical bacteria as described by Phillips (Phillips *et al*., 1993) for *E. coli*.

The emergence and spread of ESBL *Salmonella* spp. and *E. coli* have become a public health concern because of their association with morbidity and mortality and reduced treatment options. These results showed that all the MDR *S. typhimurium* isolates showed resistance to antibiotic classes important in human medicine such as beta-lactamases. Therefore, double disc synergy test (DDST) for ESBL phenotype production was conducted and the experiments indicated that all the MDR isolates had an ESBL phenotype (European Food Safety Authority, 2021). The *bla*CTX-M genes encode for ESBLs frequently identified in Gram-negative pathogens. These types of enzymes are active against cephalosporins and monobactams (but not cephamycins or carbapenems), and are of great epidemiological and clinical interest (Cantón and Coque, 2006).

5.1.2 Antimicrobial Resistance Patterns of *Proteus* Spp., *S. Aureus, P. Aeruginosa, V. Cholerae,* and *V. Parahaemolyticus*

The current study revealed thepresence of *Proteus* spp., *S. aureus, P. aeruginosa*, *V. cholerae,* and *V. parahaemolyticus* in fresh *O. niloticus* fish samples obtained from retail markets in Nairobi, Kenya. The presence of the bacterial contaminants is a reflection of the wide range of infections to which consumers of *O. niloticus* fish are exposed to, especially if the fish is undercooked prior to consumption. The presence in fish of some of these bacteria (e.g., *Proteus* spp. and *S. aureus*), have been reported to cause outbreaks of food poisoning.

The prevalence of *Proteus spp.* and *S. aureus* in the present study was 44.12% and 10.29%, respectively, which is within the range reported by previous studies from different countries (Omoe *et al*., 2002; Saito *et al*., 2011; Bujjamma and Padmavathi, 2015). The current prevalence of *Proteus* spp. and *S. aureus* represents a major public health and economic burden for the country. Their source in *O. niloticus* fish could be human contamination, as they are not part of the known bacterial flora of fish and in retail markets could be due to unhygienic handling during processing, transportation, and storage (Titilawo *et al*., 2015). Thus, it is necessary for regulatory agencies to increase the robustness with which they monitor and enforce the microbial safety of fish and other fish products, and for vendors to strictly adhere to

proper handling practices. It is also necessary to sensitize consumers on the need to ensure proper cooking of fish for complete removal of bacterial contaminants before consumption.

P. aeruginosa is naturally found in aquatic environments. In the current study, the prevalence of *P. aeruginosa* (6.8%) was higher than in other studies reported for *O. niloticus* (5.1%) in Uganda (Wamala *et al*., 2018), and for salmon (1.1%), shrimp (0.9%), and *O. niloticus* (2.3%) (Tate *et al*., 2022). Marijani (2022) reported prevalence rate of *Pseudomonas* spp. at 1% in marine and freshwater fish inTanzania. *Vibrio* spp., are found in fish and fish environments and may be harmful to both wild and cultured fish (Gauthier, 2015).The findings of this study showed that *Vibrio* were the least prevalent, at 4.5%, which is lower than in studies by Wamala *et al*. (2018), where *V. cholerae* (12.8%) was reported in *O. niloticus*; *V. parahaemolyticus* (11.22%) in ready-to-eat foods, shrimp and fish in China (Xie *et al*., 2020). The presence of *Vibrio* in aquatic environments indicates contamination by human waste. *V. parahaemolyticus* is associated with human vibriosis and occurs mainly due to the ingestion of undercooked fish or fish products (Iwamoto *et al*., 2010; Callol *et al*., 2015).

The rates of resistance of the various classes of antimicrobial agents used among the bacterial pathogens recovered in this study ranged from 0%–100%, with high-level resistance to ampicillin/cloxacillin, vancomycin and streptomycin. The high-level resistance to ampicillin, a derivative of penicillin, could be due to ease of its access and its frequent use in aquaculture. Such resistant phenotypes indicate antibiotic failure, should these members of antibiotics be used in the treatment of any disease implicated by any of the characterized members of organisms. All bacterial isolates were sensitive to cefepime, which is a fourth-generation cephalosporin. Cefepime has higher activity against both Gram-positive and Gram-negative bacteria than third-generation cephalosporins and, because it is new to the market, it is possible that there is zero to low use in aquaculture farming in Kenya (Mahon *et al*., 2014; Titilawo *et al*., 2015).

Gram-negative bacteria use antibacterial resistance mechanisms such as drug efflux, inactivating drugs, limiting uptake of drugs, and modifying the drug target, unlike Gram-positive bacteria, which lack lipopolysaccharide in the outer membrane and hence cannot pump out drugs from their cell wall (Chancey *et al*., 2012; Mahon *et al*., 2014). The current study revealed that *S. aureus* was resistant to vancomycin, which is in agreement with a study by Reygaert (2018); however, the mechanism is yet to be explained. Previous studies indicate that the bacteria produce a thickened cell wall; hence, the drug cannot enter the cell and as a result it provides an intermediate resistance to vancomycin (Lambert, 2002; Miller *et al*., 2014).

Formation of biofilm by pathogenic bacteria, e.g., *P. aeruginosa*, protects the bacteria from antimicrobial agents and the host immune system. Horizontal gene transfer is enabled by the proximity of the bacterial cells in biofilms and hence the transfer of antimicrobial resistance genes (Mah, 2012; Van Acker *et al*., 2014). The prevalence of antibiotic resistance for *P. aeruginosa* was highest in penicillin (100%), vancomycin (100%), Ceftazidime (100%), meropenem (100%), and streptomycin (100%). The reported resistance is due to restricted outer membrane permeability of the antimicrobials, efflux systems that pump antimicrobials out of the cell, and synthesis of antibiotic-inactivating factors such as b-lactamases. The resistance of *P. aeruginosa* to the b-lactam antibiotics, including penicillin (1^{st}, $2^{nd}$and 3^{rd}generations) and cephalosporin (such as cefotaxime), is mainly attributed to the extended spectrum b-lactamases (ESBLs). blaCTX-M and blaTEM are the main extended spectrum b-lactamase genes that induce such type of resistance (Peymani *et al*., 2017). In addition, *P. aeruginosa* are capable of producing carbapenemase enzymes, which makes them resistant (Breidenstein *et al*., 2011). Furthermore, *P. aeruginosa* produces phenazine compounds, which are biologically active substances involved in bacterial competitiveness and virulence in both human and animal hosts (Mavrodi *et al*., 2001). The outermembrane proteins (L-lipoproteins) of *P. aeruginosa* are associated with bacterial resistance to antiseptics and antibiotics (Nikbin *et al*., 2012).

The prevalence of antibiotic-resistant *V. parahaemolyticus* is an important concern for public human health and veterinary medicine (de Melo *et al*., 2011; Sudha *et al*.,

2014). High level of resistance of *V. parahaemolyticus* isolates to penicillin (100%), vancomycin (100%), ampicillin/Cloxacillin (100%), meropenem (50%), and streptomycin (50%) was reported in this study; however, the isolates were sensitive to 3rd- and 4th-generation cephalosporins, such as cefepime, cefpodoxime, nitrofurantoin, and ceftazidime. These findings are similar to reports from Korea and Malaysia that revealed high levels of resistance of *V. parahaemolyticus* to penicillin (92.5%), Vancomycin (98%), ampicillin/cloxacillin (82.1%), Meropenem (55%), and streptomycin (50%) (Jun *et al*., 2012; Kang *et al*., 2017; Tan *et al*., 2017). *V. cholerae* also showed high resistance to penicillin (100%), vancomycin (100%), ampicillin/cloxacillin (100%), and streptomycin (100%), and this is similar to previous studies by Das *et al*. (2020). Penicillins, including ampicillin, are the most commonly used antibiotic agents in aquaculture and therefore the results suggest that penicillins should not be used for clinical treatment of *V. parahaemolyticus* infections, whereas 3rd- and 4th-generation cephalosporins are still useful for treatment. Other types of treatment that would play a role in reducing the prevalence of *Vibrio* spp., include low-temperature treatment, use of saline, and ultrasound (Zhou *et al*., 2002). The use of sugar, lemon juice, citric acid, or vinegar has also been linked with decreased contamination with *Vibrio* spp. in fish and shellfish (Borazjani *et al*., 2003; Ibrahim *et al*., 2018). Marinating fish prior to consumption is an additional method that can be used to reduce contamination with *Vibrio* spp.

S. aureus is widespread in the environment and in the human body and its infections, especially the one caused by methicillin-resistant *S. aureus* strains (MRSA), are a threat to public health due to the emergence of multidrug-resistant strains (Kaplan *et al*., 2005). Most of the isolates in this study showed resistance to penicillin, vancomycin, rifampicin, cefpodoxime, ceftazidime, ampicillin/cloxacillin, and meropenem, whereas levels of resistance to nitrofurantoin and streptomycin were lower. Moreover, none of the isolates were resistant to cefepime and chloramphenicol. Currently, vancomycin is the drug of choice for the treatment of *S. aureus* infections. In the current study 71.4% of *S. aureus* isolates were resistant to vancomycin, and this could be an alert for the emergence of multidrug-resistant *S. aureus* infections, especially after the consumption of undercooked fish.

5.1.3 Antimicrobial Resistance Patterns of *Klebsiella, Enterobacter* and *C. Freundii*

These bacteria are collectively known as coliforms. They belong to Enterobacteriaceae family and are capable of causing GIT infection in human beings. Coliforms are indicators of faecal contamination however other coliforms such as *K. pneumonia* and *E. aerogenes* grow in non-animal environments such as plant surfaces, and soil (Halkman and Halkman 2014*),* hence soils could be primary sources of these bacteria when wind blows into ponds and lakes where aquaculture is practised.

The prevalence of *C. freundii*, *Klebsiella* spp., and *Enterobacter* spp., in the current study was 4.41%, 16.7% and 8.82% respectively, which lies within the same range (4%-28%) reported by other studies (Claudious *et al*., 2019; Marijani, 2022). The presence of these bacterial contaminants in fish is an indication of the range of infections to which consumers of fish predispose themselves, especially if those fish are not well cooked prior to consumption.These bacteria can cause diarrhoea, pneumonia and other related respiratory infections. Coliforms such *C. freundii*, *Klebsiella* spp., and *Enterobacter* spp., are not normal bacteria in fish and therefore may have originated from fecal matter or environmental sources hence unreliable indicators of fecal contamination (Leclerc *et al*., 2001). The three bacteria species are collectively referred to as thermophilic and ubiquitous coliforms (Leclerc *et al*., 2001), meaning they can be found in faecal matter (either from humans and or from animals) or natural environment. The presence of these bacteria in Nile tilapia could also be as a result of mishandling or unhygienic handling of fish during processing, transportation and storage by fish handlers and traders. Therefore, it would be necessary for regulatory bodies to increase the robustness with which they monitor and enforce the microbial safety of fish and other fish products as well as strict adherence of handlers and traders to proper hygiene during handling. It would also be necessary to sensitize consumers on the need to ensure that they cook their fish well to allow for removal of contaminating bacteria before they consume them.

The study of antimicrobial resistance in pathogenic bacteria from fish is vital, since it may indicate the extent of alteration of aquatic system by anthropogenic activities. Bacteria in water could be indigenous to aquatic environments, or exogenous, transiently and occasionally present in water as a result of shedding from animal, vegetation or soil surfaces blown into aquatic system. Antibiotic resistance could be due to the heavy use of non-biodegradable compounds in aquaculture, hence increases antibiotic selective pressure in water, enabling the transfer of antibiotic-resistant factors between aquatic bacteria, including human pathogens and fish and allowing the presence of residual antibiotics in commercialized fish (Alonso *et al*., 2001; Alanis, 2005; Seiler & Berendonk, 2012).

This study reports in vitro antimicrobial susceptibility rates for penicillin-G, vancomycin, rifampicin, ampicillin/cloxacillin, cefepime, cefpodoxime, chloramphenicol, nitrofurantoin, ceftazidime, Meropenem, Streptomycin antibiotics to bacterial isolates of *C. freundii, Klebsiella* spp. and *Enterobacter* spp. from retail markets of Nairobi County, Kenya. The findings of this study are similar to the susceptibility rates observed for ceftazidime/avibactam in *Enterobacterales* isolates in Europe in the INFORM study conducted between 2012 and 2016. In their study, ceftazidime/avibactam was most effective against all *Enterobacterales* (98% susceptibility) (Ramalheira & Stone, 2019). Same findings by Kaye and Pogue, (2015) that studied the *in vitro* activity of ceftazidime/avibactam against *Enterobacterales* isolates from Israel and Central Europe. Even though the current study did not have the combination of ceftazidime/avibactam, the susceptibility rate of *Enterobacter* to ceftazidime alone was 100%. In the analysis, 16.7% of *Enterobacter* isolates were identified as multidrug resistant isolates. This could be attributed to the fact that *Enterobacter* spp., are intrinsically resistant to ampicillin, amoxicillin, amoxicillin-clavulanate, first-generation cephalosporins, and cefoxitin which is due to the production of constitutive AmpC beta-lactamase.

C. freundii, Klebsiella and *Enterobacter* registered resistance against vancomycin (16.7%-100%), rifampicin (16.7%-100%), and ampicillin/cloxacillin (16.7%-66.7%) with *C. freundii* registering the highest resistance in vancomycin and rifampicin (100%), this trend is similar for *Klebsiella* in previous studies (European Centre for

Disease Prevention and Control (ECDC), 2018; Ballén *et al*., 2021).The resistance of bacterial isolates to common antibiotics like penicillin-G, vancomycin, rifampicin and ampicillin/cloxacillin could be due to the frequent use or misuse of these antibiotics in aquaculture as well as affordability and availability across the counter (Shakya *et al*., 2013). The 100% susceptibility rate to cefepime, chloramphenicol and nitrofurantoin could be attributed to antimicrobial resistance burden as well as slowed rate of evolution by individual bacterial isolates in the fish sources. It could also mean that the use of these antibiotics in aquaculture and its environs is between zero to minimal amounts. Cefepime is a 4^{th} -generation cephalosporin which is still very effective because it is new in the market.

The antimicrobial susceptibility profile of bacteria isolates (Table 4.5) showed that 45% (9/20) of the bacteria isolates were resistant to meropenem, 40% (8/20) were resistant to rifampicin, 35% (7/20) were resistant to vancomycin and 30% (6/20) of the isolates were resistant to Ampicillin/Cloxacillin etc. These findings are consistent with previous studies that reported a similar pattern of resistance to ampicillin in aquatic fish and water samples (Hatha *et al*., 2005). The observation of high ampicillin resistance in the present study points towards widespread use in diverse field such as human medicine, veterinary and aquaculture. The findings from this study imply the severe resistance developed in the isolates against the glycopeptide class of antibiotics. This kind of resistance by the bacterial phenotypes implies antimicrobial failure, should these antibiotics be used in the treatment of any disease caused by these bacteria, hence the need for effective alternative antibiotics (Hope & Bright, 2022).

Klebsiella spp. has often been reported in fish and fish products as a contaminant from water, containers and feaces of animal or human origin (Preena *et al*., 2020). It was noted that the *Klebsiella* spp. isolates showed high-level resistance to vancomycin, rifampicin and meropenem at 100% and ampicillin/cloxacillin and cefpodoxine at 66.7%. The antibiotic susceptibility rate of *Klebsiella* spp. to cefpodoxime of 72.7% with 0% resistance was observed in the current study. Cefpodoxime is a third generation cephalosporin and it was more effective than the first and second generation cephalosporins even though 18.2% of *Klebsiella* isolates

were resistant to ceftazidime, which is also a third generation cephalosporin. Antibiotics, in and of themselves, do not cause resistance, but frequent and high exposure of antibiotics to bacteria creates selection pressure that triggers bacterial resistance mechanisms. This explains the variation in antibiotic resistance observed between cefpodoxime and ceftazidime in *Klebsiella* isolates obtained from Nile tilapia fish. Studies indicate that the selection pressures imposed by antibiotics and the evolutionary principle of survival of the fittest have led to many bacteria pathogens to evolve a variety of antibiotic evasion mechanisms (Deku *et al*., 2022). This study showed that out of the 11 antibiotics used, chloramphenicol, cefepime and cefpodoxime were the most effective against *Klebsiella* spp. isolated from raw Nile tilapia fish.

Citrobacter freundii is an anaerobic or facultative anaerobic Gram-negative bacterium in the genus *Citrobacter* of Enterobacteriaceae and it is widely distributed in the environment causing human-animal-fish coccurrence (Liu *et al*., 2020). Antibiotic resistance in fish pathogens such as *C. freundii*, the transfer of their genetic determinants and virulence factors to terrestrial animal and human bacteria pathogens and alterations in the bacterial microbiota of the aquatic environment constitute a threat to human and animal health. The results from the present study indicate that *C. freundii* is the enterobacteria with the highest frequency and multidrug resistance to meropenem, vancomycin and Rifampicin (Shakya *et al*., 2013). The resistance of bacterial isolates to common antibiotics like penicillin-G, vancomycin, rifampicin and ampicillin/Cloxacillin could be due to the frequent use of these antibiotics in aquaculture (Shakya *et al*., 2013). The emergency of bacteria resistant to multiple antibiotics is a growing threat to antibiotic therapy.

5.2 Molecular Diversity of Multidrug Resistant Bacteria in Raw Fish from Selected Retail Outlets in Nairobi County.

5.2.1 Molecular Diversity of Multidrug Resistant in *Salmonella* spp., and *E. coli* Isolates

The 16S rRNA sequencing further confirmed the *Salmonella* and *E. coli* isolates, and clustered them into closely related phylogenetic clades. The 16S rRNA gene is an

important landmark in the study of the evolution and classification of bacteria, and has served as base molecular identification tool for study of evolutionary relationships among groups of bacteria (Hoque *et al*., 2019). Based on the results from this study, all the MDR *Salmonella* spp. and *E. coli* were identified as *S. typhimurium* and *E. coli*, respectively. PCR is a robust and rapid detection method with increased specificity and sensitivity for detecting *Salmonella* in environmental, food and clinical samples (Toze, 1999).

The presence of MDR isolates from fresh Nile tilapia fish investigated indicate that consumers are exposed to disease-causing pathogens that make treatment challenging. This is significant to human health due to the zoonotic nature of these pathogens. To avoid the development of MDR, the use of antibiotics should be more strategic and selective. Given that fish harbour multiple bacterial communities living in close proximity to each other, antibiotic resistance in some of these bacteria could lead to easy transfer of resistant genes to others. This could result in increased spread of antibiotic resistance to humans. The spread of MDR bacteria could also be exacerbated by consumption of raw, undercooked or insufficiently heat-treated fish and fish products. Based on these results, there should be improvement in sanitary handling and processing of fish to reduce the risk of spread of bacterial pathogens capable of spreading antibiotic resistant genes to humans. This study highlights the serious issue of *S.typhimurium* and *E. coli* multidrug resistance in retail Nile tilapia fish which could result in their evolution into super bacteria and pose a risk to public health.

In the current study, PCR analysis revealed the presence of antibiotic-resistance genes belonging to β-lactamases, tetracycline-resistant, sulfonamide-resistant, trimethoprim-resistant and aminoglycosides-resistant genes. Genes like *aadA, dfrA7* and *sul2* detected in MDR *S.typhimurium* and *E. coli* often co-exist as part of gene cassettes on class 1 integrons (Sung *et al*., 2014). The class 1 resistance integrons is located on mobile elements like transposons and plasmids and is widely distributed among clinical and environmental isolates and plays an important role as reservoirs of antimicrobial resistance genes (Koczura *et al*., 2013; Koczura *et al*., 2014). Amplification of ^{bla}TEM-1, ^{bla}CMY-2, ^{bla}CTX-M, and ^{bla}Z is attributed to long term

exposure of β-lactam antibiotics in animal and fish farming and for treatment of Gram-negative infections (Zaniani *et al*., 2012). The presence of antibiotic resistant genes shared across the bacterial isolates reflects active horizontal gene transfer (HGF) among bacteria in aquaculture. Horizontal gene transfer allows bacteria to exchange their genetic materials including antibiotic resistance genes (ARGs) among different species (Le Roux & Blokesch, 2018; Titilawo *et al*., 2015), hence promoting multidrug resistance.

The use of antimicrobial agents in aquaculture for long periods of time have contributed to increase of antibacterial resistance in fish pathogens, emergence of antimicrobial resistant bacteria in aquatic environments, and also increasing the potential to transfer these resistant genes to pathogenic bacteria of terrestrial animals and humans (Miller & Harbottle, 2017).The use of antibiotics in aquatic culture in Kenya is not regulated and their indiscriminate use has led to the rise of antibiotic resistant bacteria hence the transfer of the resistance to human bacteria.

5.2.2 Molecular Diversity of Multidrug Resistant in *Proteus* Spp., *S. Aureus, P. Aeruginosa, V. Cholerae,* and *V. Parahaemolyticus*

Multidrug-resistant foodborne bacterial pathogens are a major public health and economic concern worldwide. The presence of MDR bacteria in fresh *O. niloticus* fish in this study is a cause for worry for several reasons. Undercooked fish could expose consumers to colonization and infection with these MDR bacteria. This has the potential for dissemination of the resistance genes from MDR bacterial pathogens to the microbiota with which they co-colonize the gut (Duedu *et al*., 2017). It is also possible that the resistance traits could be transferred to other microorganisms in circulation when colonized persons shed them in fecal matter. MDR bacteria could also spread from fish markets to hospital environments through cockroaches and other insects as well as other vehicles for transmission of foodborne pathogens (Tetteh-Quarcoo *et al*., 2013; Futagbi *et al*., 2017; Donkor, 2019; Obeng-Nkrumah *et al*., 2019). The risk of transmission could be pronounced in cases of close proximity between markets and healthcare facilities. These pathogens could be disseminated further in the hospital facilities and negatively impact disease outcomes of patients

and healthcare costs (Tetteh-Quarcoo *et al*., 2013; Obeng-Nkrumah *et al*., 2015;Tette *et al*., 2016; Donkor *et al*., 2018; Donkor & Kotey, 2020). Continued research on the antibiotic resistance of pathogenic bacteria that infect fish is needed because it is essential for controlling the occurrence of multidrug-resistant bacteria and for the selection of appropriate therapeutic agents. The percentage of MDR *S. aureus* (100%) in the current study is slightly higher than in previous studies conducted in South Africa (82%) (Fri *et al*., 2020) and Egypt (86.6%) (Badawy *et al*., 2022).

The spread of antibiotic-resistant bacteria and antibiotic resistance genes is considered one of the most serious emerging threats to public health. An important factor in bacterial resistance to antimicrobials is that they carry related resistance genes (Iwu & Okoh, 2019; Iwu *et al*., 2020). To ascertain the resistant phenotypes, antibiotic-resistant gene profiles were conducted using the PCR method, which revealed the presence of resistance genes belonging to extended-spectrum b-lactamase, aminoglycoside resistance genes, streptomycin resistance genes, sulphonamide genes, and other b-lactamase resistance genes.

5.2.3 Molecular Diversity of Multidrug Resistant In *Klebsiella, Enterobacter* and *C. Freundii*

The current study detected antimicrobial resistant genes; blaTEM-1, blaCMY-2 (β-lactamases-encoding genes), tetA, tetC (tetracycline resistant genes), sul2 (sulfonamide resistant genes), dfrA7 (Trimethoprim-resistant genes), strA, aadA (aminoglycosides resistant genes) across *C.freundii*, *Klebsiella* and *Enterobacter*. The presence of the aforementioned AMR genes in all the three genera could be as a result of wide spread of antimicrobial resistant bacteria and resistant factors in aquaculture. Horizontal gene transfer could be the active mode of sharing the resistant factors. The results of this study confer with other studies; Liu *et al*. (2017) identified $bla_{\text{TEM–1}}$ gene in *C. freundii* isolates, Iwu and Okoh. (2019); Iwu, *et al*. (2020) revealing the presence of resistance genes in enterobacteriaceae pathogens.

The heavy use of these antibiotics in aquaculture especially the non-biodegradable, increases the antibiotic selective pressure in water, enabling the transfer of ARGs (antibiotic resistance genes) between aquatic bacteria, fish and human pathogens

enabling the presence of residual antibiotics in fish (Alanis, 2005; Seiler & Berendonk, 2012).

5.3 Genetic Markers Coding for Drug Resistance in Antibiotic Resistant *Salmonella* Spp., and Pathogenic *E. coli*

Detection of genetic markers is a key step to identify the potential pathogenicity of the obtained bacteria isolates. Fish surface and tissue invasion by the bacterial pathogens is considered to be facilitated by the functioning of genetic markers (Sen & Lye, 2007). The *invA* gene has been the target for many PCR protocols, as it is found in almost all known serovars of *Salmonella* (Chiu &Ou, 1996). This gene encodes an inner membrane protein necessary for invasion of epithelial cells by *Salmonella*. The *hilA* gene encodes OmpR/ToxR family transcriptional regulator that activates expression of invasion genes. Pathogenic *E. coli* in this study harboured *uidA* gene known to hydrolyze glycosaminoglycans (Darwin & Miller, 1999). The occurrence of the MDR invasive *Salmonella* isolates among the fish samples suggests that consumers and other stakeholders within the food and value chain might be at a risk of *Salmonella*-borne infections.

5.4 Multiple Antibiotic Resistance (MAR) Indices of MDR Bacteria

5.4.1 MAR Indices of MDR *Salmonella* Spp., and Pathogenic *E. coli*

The MAR ranged from 0.09 to 0.36. The MAR of 0.36 in *Escherichia coli* having been sampled from fish in Makadara and Lang'ata retail markets, indicated that the bacteria are exposed to antibiotics. These results could as well mean that there's active horizontal gene transfer in aquaculture farms. This high MAR is enough to cause antibiotic resistance in aquaculture farming.

5.4.2 MAR Indices of MDR *Proteus* Spp., *S. Aureus*, *P. Aeruginosa*, *V. Cholerae*, and *V. Parahaemolyticus*

The MAR index ranged from 0.27–0.64, indicating that all the isolates have repeatedly been exposed to antibiotics. All the bacteria pathogens tested in the present study showed a MAR index of more than 0.2, indicating a high risk of

contamination that is potentially harmful to human health (Tanil *et al*., 2005). The dissemination of these resistant clones can pose serious public health problems. The MAR index results suggest that bacteria (*Proteus* spp., *S. aureus, P. aeruginosa*, *V. cholerae*, and *V. parahaemolyticus*) isolated from *O. niloticus* fish can contribute significantly to the spread of multidrug resistance and antibiotic resistance genes to consumers. The findings of the current study are in line with previous studies conducted in Nigeria (Chigor *et al*., 2020) and Malaysia (Noorlis *et al*., 2011; Saifedden *et al*., 2016).

5.4.3 MAR Indices of MDR *Klebsiella, Enterobacter* Spp., and *C. Freundii*

The antimicrobials used in the current study revealed a number of variant multiple antibiotic resistant phenotypes (MARPs) and multiple antibiotic resistant indices (MAR) ranging from 0.18 to 0.55 with antibiotics resistance numbers ranging from 2 to 6 out of 11 antibiotics used. An MAR higher than 0.2 implies that the bacteria are from a high-risk source where there's active use of antibiotics. The high MAR could also be as a result of faecal contamination in the sources or during unhygienic handling by the fish traders. Unregulated use of antibiotics may have contributed to antibiotic resistance (Claudious *et al*., 2019). These results are similar with studies conducted in South Africa (Fadare *et al*., 2020); Nigeria (Chigor *et al*., 2020).

The presence of MDR in the current study implies need for production of alternative effective antimicrobials to curb human pathogens.

5.5 Relationship between Phenotypic Resistance Pattern to the Antibiotics for MDR Bacteria and the Presence of Antibiotic Resistance Genes

There was a strong correlation between the phenotypic resistance pattern to the antibiotics for MDR bacteria and the presence of antibiotic resistance genes. However, only a few antibiotic resistance genes were selected for analysis in the study based on the antibiotic usage and previous reports from various regions of Kenya, although several types of antibiotics and their variants are used in the country. Therefore, the bacterial pathogens could contain other antibiotic resistance genes that were not included in this study. These results are in agreement with a

study done in Nigeria (Beshiru *et al*., 2019) and Cambodia, USA (Schwan *et al*., 2021)

5.6 Conclusions

i. The results in the current study show high prevalence of antimicrobial-resistant foodborne pathogens in fish purchased from retail markets and underscore the risk associated with improper handling of fish. The isolation and characterization of bacteria, especially those with multiple antimicrobial resistance, in Nile tilapia in retail markets is of public health concern.

ii. The occurrence of MDR isolates is of specific concern for human and domestic animal health.The potential ability of these MDR bacteria to enter into the food chain can expose humans to serious health risks. This calls for application of more hygienic practices during all stages of fish production and processing for selling. Further, it requires a wide microbiological surveillance and strict governing of the uncontrolled use of antimicrobials either for treatment or as growth promoters, not only in fish production but also in other livestock production systems.

iii. All antibiotic resistance genes used in this study were detected in all the MDR bacteria.

iv. The MAR index results suggest that the bacteria isolated from fish can contribute significantly to the spread of multidrug resistance and antibiotic resistance genes to consumers. Use of animal manure in aquaculture as feeds could also be a contributing factor to AMR burden leading to bacterial resistance.

v. There is significant positive correlation between phenotypic and genotypic multidrug resistance

5.7 Recommendations

1. Regular monitoring and surveillance of antibiotic resistance bacteria foodborne pathogens may help to track the cause of the food-borne diseases and lead to appropriate safety policy for interventions, prevention and/or effective treatment measures of food-borne diseases.

2. The need for routine surveillance/monitoring of potential pathogens in fish and fish products, including seafood in retail markets, remains a priority to ascertain the safety of these food products.
3. Government to enect laws on the use of antimicrobials in aquaculture farming.
4. Therefore, there is need for studies to understand the epidemiology of antibiotics in aquaculture in Kenya, as this will be an important step in solving the problem of antibiotic resistance in the aquaculture environment.
5. Measures to be put in place to ensure communities around lakes and ponds have proper sanitary facilities to avoid contamination of water bodies.

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APPENDICES

Appendix I: Procedure for Enumeration of Total Bacterial Load

1g of the fish sample was dissected out and homogenized using stomacher 400 circulator (Seward Ltd, England). It was aseptically transferred to a sterile tube containing 9mls of 0.1% sterile buffered peptone water. The tube was tightly closed and shaken for 15 minutes and allowed to stand for 20 minutes, after which a 10 fold serial dilution was done and viable bacterial counts were made in plate count agar after incubation at 37^{o}C for 18 to 24 hrs as described by (Slaby *et al*., 1981). The bacterial load was determined by counting the number of discrete colonies using the viable plate count method. A plate with an average between 30 and 300 colonies of the target bacteria is acceptable. Fewer than 30 colonies make the interpretation statistically unsound and more than 300 colonies indicate overlapping colonies (Collins *et al*., 1984).

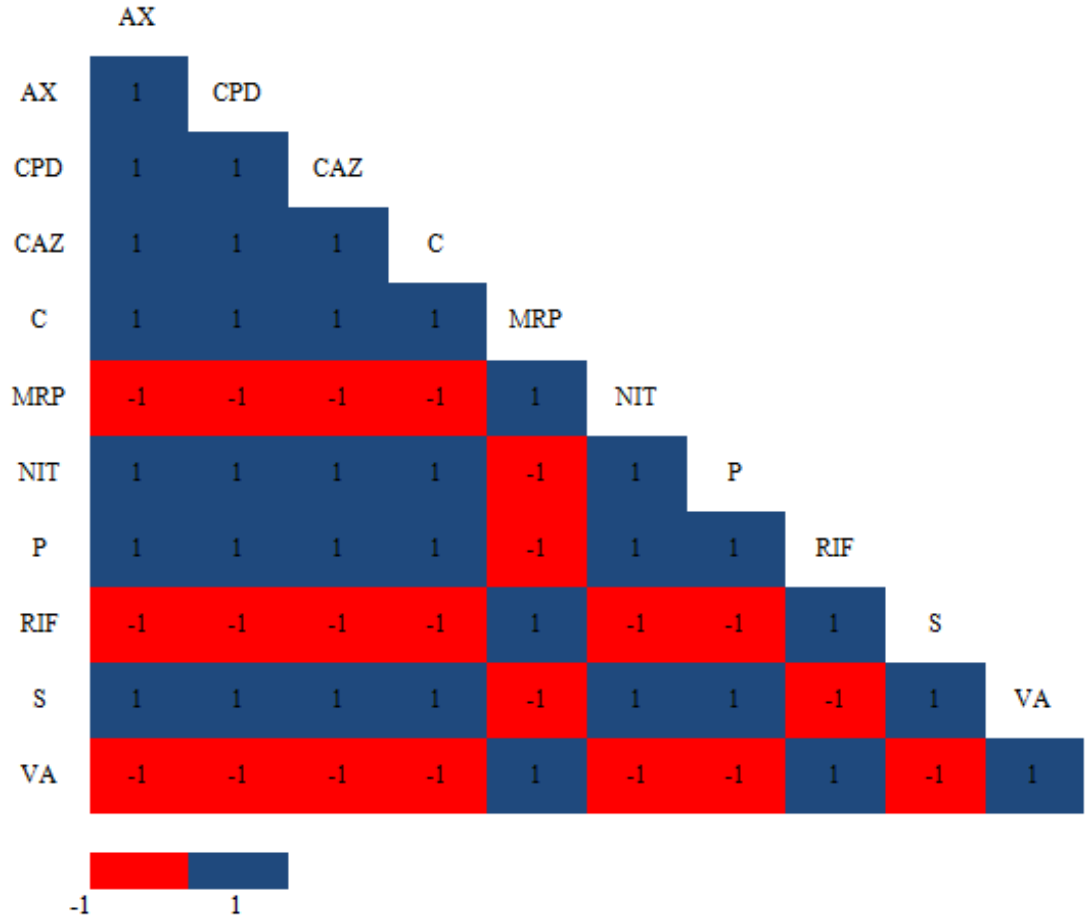
Appendix II: Procedure for Agarose Gel Electrophoresis for Genomic DNA

The denaturing 0.8% formaldehyde agarose gel electrophoresis (0.4g Agarose, 2ml 10× MOPS buffer, 18ml DH_2O, 280 µl 37% formaldehyde and 0.5µl ethidium bromide) was used to assess the integrity of genomic DNA. About 3 µl genomic DNA were mixed with 6× DNA loading dye; 5 µl of the mixture was be loaded on the gel alongside a 1Kb DNA ladder and run at 60 volts 80mA for 1 hour.

Appendix III: Purification of PCR Amplicons

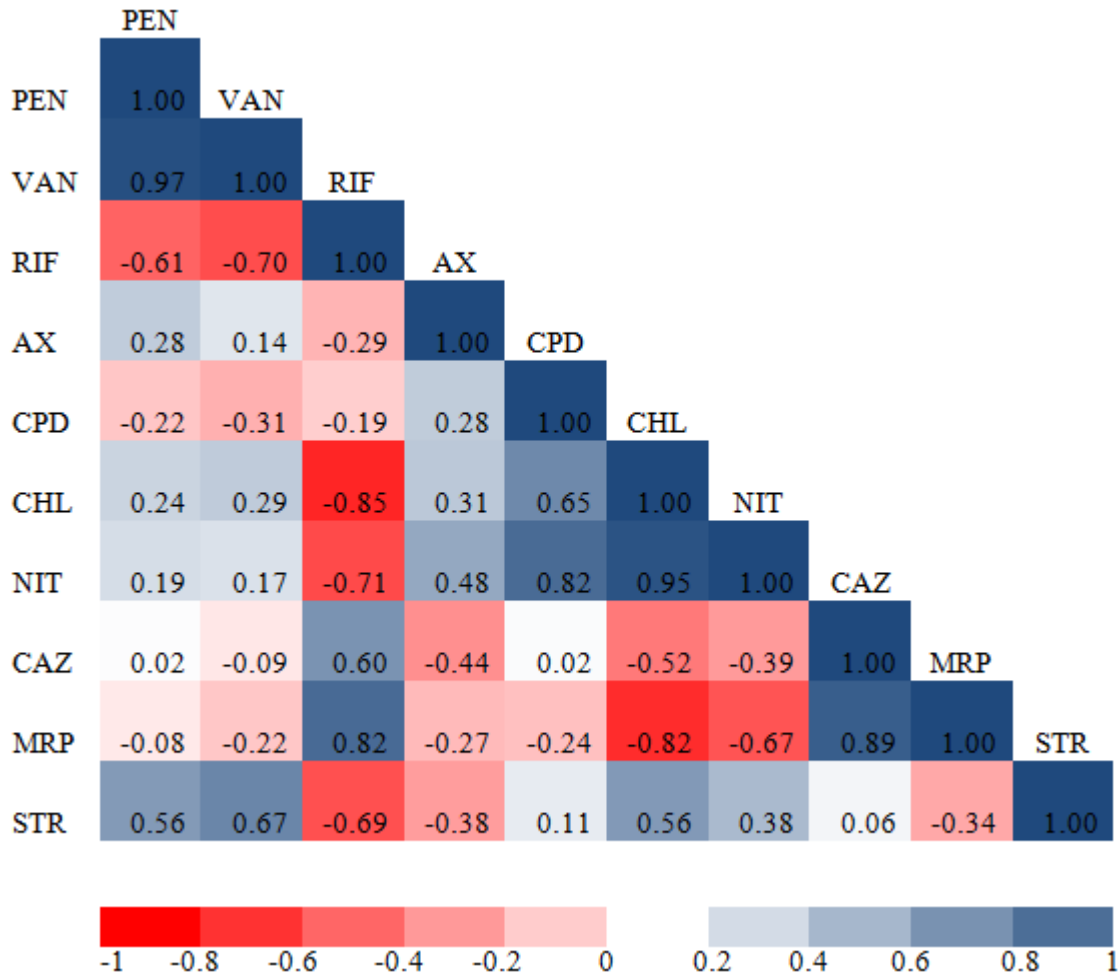
Add an equal volume of Membrane Binding Solution to the PCR amplification. Insert SV Minicolumn into Collection Tube. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute. Centrifuge at 16,000 ×g for 1 minute. Discard flow through and reinsert Minicolumn into Collection Tube. Add 700µl Membrane Wash Solution. Centrifuge at 16,000 ×g for 1 minute. Discard flow through and re-insert Minicolumn into Collection Tube. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 ×g for 5 minutes. Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 ×g for 1 minute. Discard Minicolumn and store DNA at 4°C or –20°C.

Appendix IV: Correlation Coefficient (r) Analysis among Various Tested Antibiotics in *Salmonella* spp., and *E. coli* Isolates



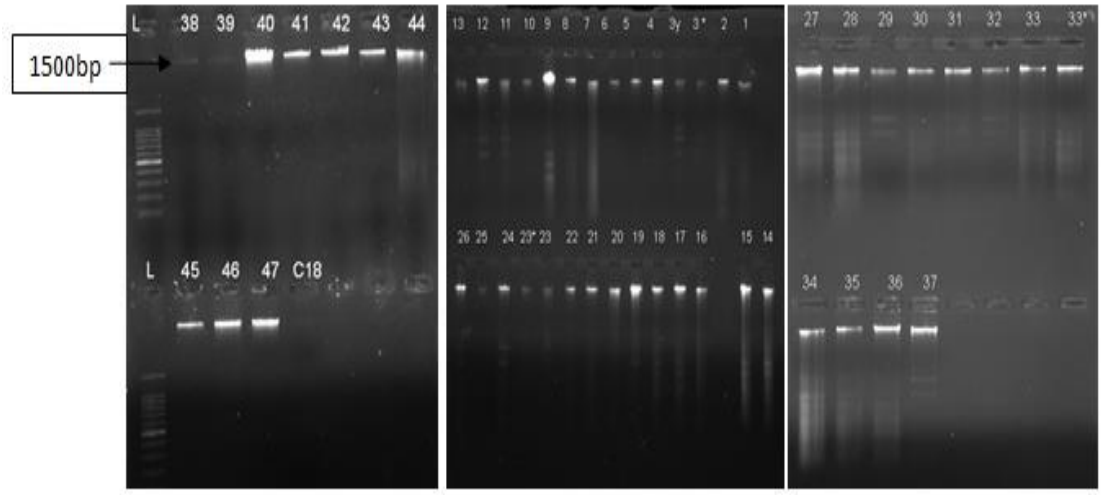
Appendix IV: The heat-map shows the correlation coefficient (r) among various tested antibiotics in the current study. The intensity of colors indicates the numerical value of the correlation coefficient (r), Red and blue colors refer to the negative and positive correlations, respectively.

Appendix V: Correlation Coefficient (r) Analysis among Various Tested Antibiotics in *Proteus* spp., *S. aureus*, *P. aeruginosa*, *V. cholerae* and *V. parahemolyticus*



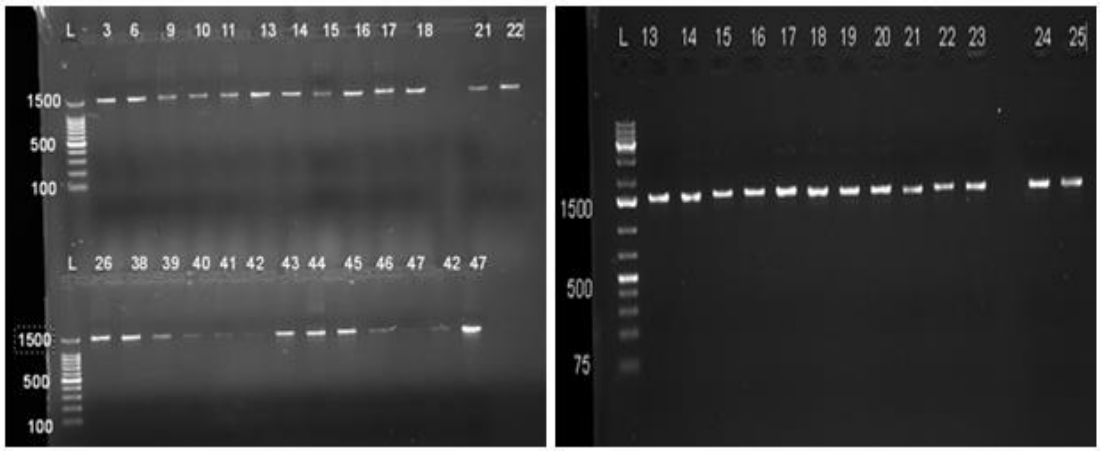
Appendix V: The heat-map shows the correlation coefficient (r) among various tested antibiotics in the current study. The intensity of colors indicates the numerical value of the correlation coefficient (r), Red and blue colors refer to the negative and positive correlations, respectively.

Appendix VI: Genomic DNA Gel Images



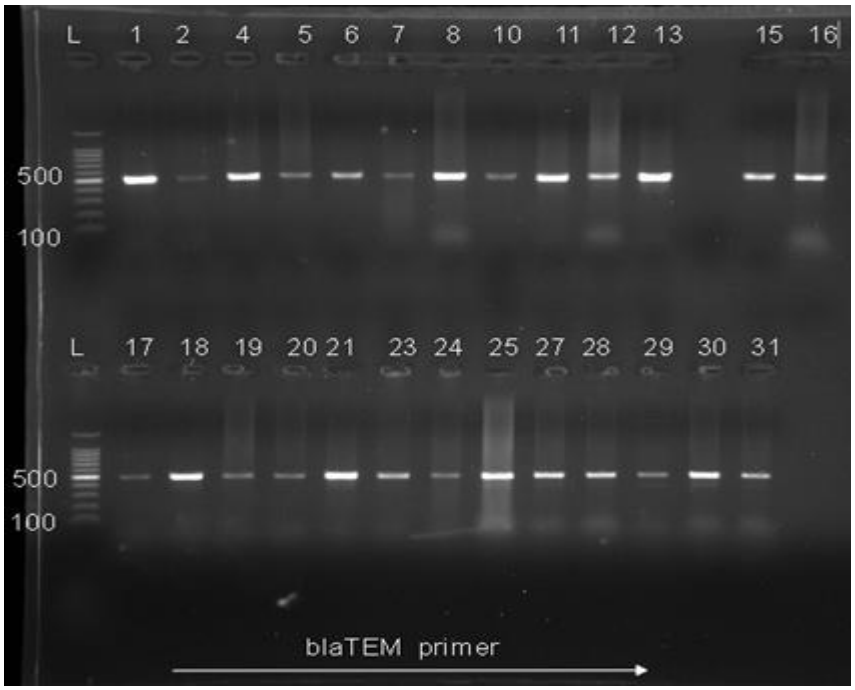
Agarose gel images showing extractedgenomicLane L: Molecular weight marker (100 bp), Lanes 1-47 represent genomic DNA.

Appendix VII: Purified PCR Products (Amplicons) Gel Images

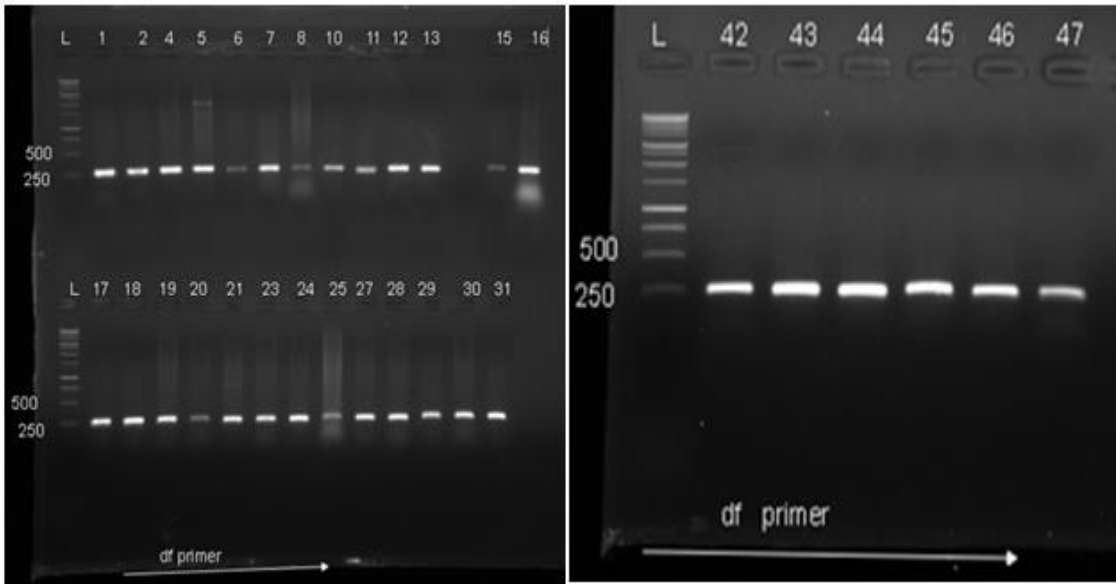


Agarose gel images showing amplification of 16SrRNAgenein purified PCR products (amplicons). Lane L: Molecular weight marker (100 bp and 1kb). Lanes 1-47 represent 16SrRNA gene.

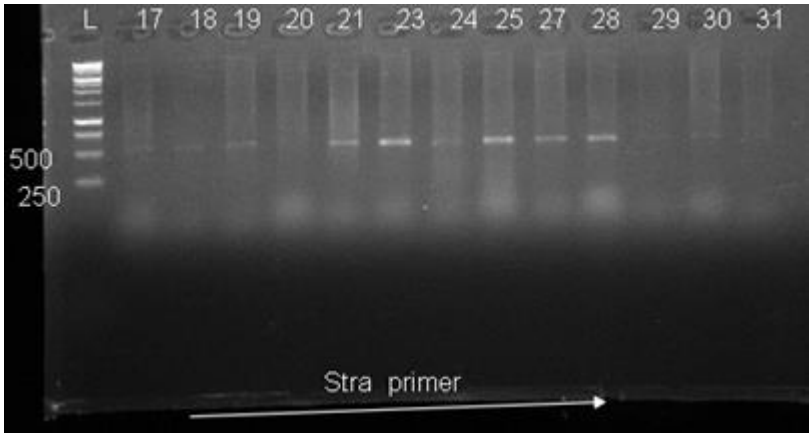
Appendix VIII: Amplification of other Antimicrobial Resistance Genes



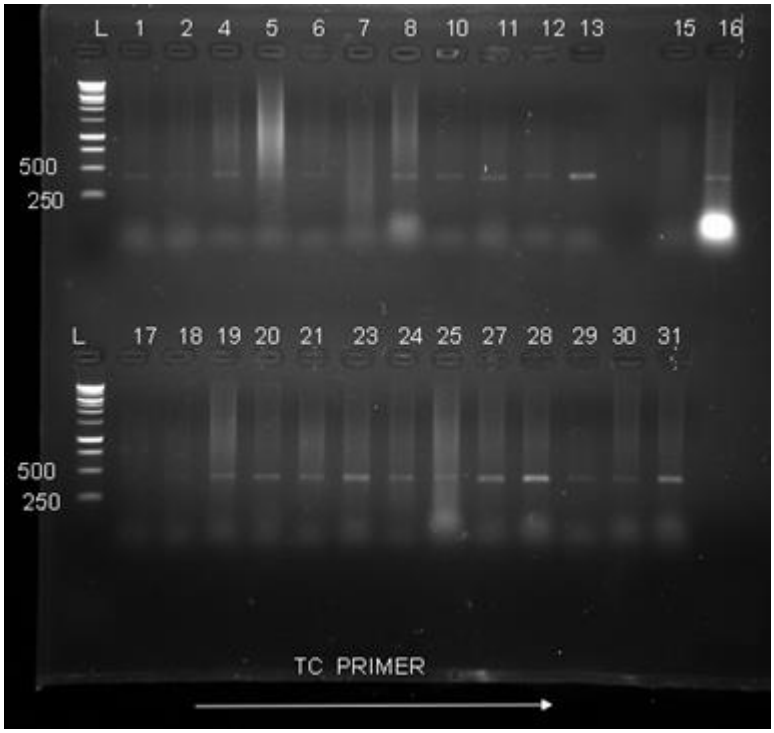
Agarose gel images showing amplification of blaTEM-1antimicrobial resistance gene. Lane L: Molecular weight marker.



Agarose gel images showing amplification of dfrA7 antimicrobial resistance gene. Lane L: Molecular weight marker.



Agarose gel images showing amplification of strA antimicrobial resistance gene. Lane L: Molecular weight marker.



Agarose gel images showing amplification of tetC antimicrobial resistance gene. Lane L: Molecular weight marker.

Appendix IX: Questionnaires for Fish Vendors in Retail Markets

Please tick where appropriate

1. Name of sub-County ……..
2. Name of the market ……
3. What is the source of your fish (Nile tilapia)?……..
4. How long does it take to transport fish from the source to the market?

   Less than 5 hours ……..

   Within 10 hours…….

   More than 10 hours ……..

5. How fresh are the fish by the time they reach the market?

   Very fresh …….

   Slightly fresh …….

   Stale ……..

6. Do you use ice or freezers when transporting fish from the source to the market?

   Yes ……

   No …….

Do you practice sanitation when handling fish from the source to the market?

Yes ……

No …….

7. Do you sell all your fish on every market day?

Yes ……

No …….