CHARACTERIZATION AND CONTROL OF PATHOGENIC PARASITES IN NILE TILAPIA (*OREOCHROMIS NILOTICUS* LINNAEUS 1758) IN FISH-FARMING SYSTEMS IN BUNGOMA COUNTY, KENYA

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DECLARATION

This thesis is my original work and has not been presented for degree or other awards in any other institution

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DEDICATION

I dedicate this work to my wife Deborah; children Keziah and Karlvin for being patient while I undertook this study and their unrelenting encouragement. Besides, this work is dedicated to my parents the late Dismas Waswa and Gladys Machuma for their care and financing of my studies during my earlier educational levels.

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LIST OF ABBREVIATIONS AND ACRONYMS

AU	African Union
APRM	African Peer Review Mechanism
ASM	American Society of Microbiology
BOD	Biochemical/Biological Oxygen Demand
CDC	Centres for Diseases Control
cfu	colony forming unit
CGoB	County Government of Bungoma
CLSI	Clinical Laboratory Standards Institute
CRS	Creative Research System
EAC	East African Community
FAO	Food and Agriculture Organization
FFEPP	Fish Farming Enterprise Productivity Programme
GISD	Global Investigative Species Database
ICMSF	International Commission of Microbiological Specifications and Foods
KNBS	Kenya National Bureau of Statistics
MALF	Ministry of Agriculture, Livestock and Fisheries
MIC	Minimum Inhibitory Concentration
MDP	Ministry of Devolution and Planning
MFD	Ministry of Fisheries Development
MR	Methyl red

NCCLS	National Committee for Clinical Laboratory Standards
NOFP	National Oceans and Fisheries Policy
PCR	Polymerase chain reaction
SDF	State Department of Fisheries
spp	Species
TCBS	Thiosulphate-Citrate-Bile salt-Sucrose agar
TSA	Tryptone Suaga agar
TSI	Triple Sugar Iron agar
TVACC	Total Viable Aerobic Colony Count
UNCLOS	United Nations Convention on the Law of the Sea
VP	Voges-Proskauer
WHO	World Health Organization

DEFINITION OF TERMINOLOGIES

Antimicrobial:	It is a a natural or synthetic chemical substance that kills, slow or inhibits the growth of microorganisms such as bacteria
Antimicrobial resistance:	Is the acquired ability of a microorganism to multiply or tolerate the effect of antimicrobial agent to which it is normally susceptible
Aquaculture:	It is farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants in either freshwater or salt water under controlled conditions
Bacteria:	They are groups of single-celled, microorganisms typically a few micrometers in length and have a wide range of shapes, ranging from spheres to rods and spirals.
Capture fisheries:	The commercial harvesting of wild fish
Exclusive Economic Zone:	Refers to a sea zone where a state has special rights regarding the exploration and use of marine resources including energy production from water and wind.
Intermediate:	Means that a higher dose of antibacterial agent is needed to prevent growth of bacteria.
Parasite:	An organism that lives on or in a host organism and gets its food from or at the expense of its host.
Plasmid:	It is an extra-chromosomal DNA coding virulent or non-virulent determinant
Resistance:	It is an adaptive phenomenon that bacteria have developed to survive in the presence of antimicrobials
Susceptible:	The microorganism cannot grow in the presence of the antibacterial, antiparasitics and antifungal agents

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ABSTRACT

In Kenya, fish farming has been expanding in recent years, with Government support through financial and input subsidies to small scale farmers. The sub sector generates a variety of benefits such as food and nutrition and develops trade. However, the sub sector faces the challenges of fish diseases, given that the fish production facilities support high-density living conditions that are favorable for spread of parasitic diseases. Besides, when fish that is infected is consumed by humans, the humans may be infected, especially when eaten fresh. Infected fish lowers their market value and sunctions may also be imposed on fish exports. Knowledge of fish parasites in aquaculture systems as well as treatment options available would enlighten fish farmers on the specific actions to take in order to harvest quality fish. The study was cross sectional carried out in Bungoma County from August 2015 to Decemebr 2017. The main objective of the study was to characterize and evaluate control options of pathogenic parasites present in fish farming systems in Bungoma County. Bungoma County was chosen for this study because it is one of the Counties that has high potential for fish farming. The County hosts one out of the public fish farms and hatcheries at Chwele besides authenticated private fish hatcheries. He hatcheries are sources of fish fingerlings as well as training and demonstration centres for fish farmers. At every fish pond, six water quality parameters were assessed using a portable auto sampler. Farmed Nile tilapia was randomly sampled to determine the pathogenic parasites and bacteria present in various organs. Commercial fish feeds were also purchased from two fish feed millers in Bungoma County and assessed for parasite presence. Polymerase Chain Reaction (PCR) and genetic sequencing analysis were conducted in order to assess the genes responsible for antimicrobial resistance. The data was entered into Micro Soft Excel (Windows 10) spreadsheet and analysed by SPSS Software. It was found that there was significant difference only in temperature of the pond water sampled across the sub counties ($F=15.5$; $df=5$; $p<0.001$). Helminthes recovered were *Pallisentis tetraodontis, Pallisentis* spp., *Acanthocephalus* spp., *Procamallanus* spp. and *Philometroides* spp. from Nile tilapia and *Cleidodiscus* spp. from pond water. Bacteria that were isolated from Nile tilapia were *Vibrio vulnificus, Vibrio parahaemolyticus, Aeromonas hydrophila,* and *Pseudomonas aeruginosa.* In fish pond water, *Aeromonas hydrophila* was isolated while *Streptococcus iniae* was isolated from fish feeds. Besides, there was a significant difference in distribution of total viable aerobic colony counts in fish ponds water across the different sub counties ($H=6$;df 5; $P< 0.041$). Furthermore, it was found that there was significant difference in the distribution of *Aeromonas hydrophila* in fish ponds across the different sub counties ($H=3$;df;$P< 0.016$). The external parasites were eliminated by formalin (36% formaldehyde) at concentration of 25ppm for 1h and Hydrogen peroxide at 75ppm for 30 min. The bacterial isolates were found to be significantly different in responses to antimicrobial agents (OLR;$df= 16$; $P<0.00$). When the bacterial isolates were subjected to PCR, all five bacterial pathogens isolated from fish, pond water and fish feeds were found to contain bla_{TEM} gene amplified at 424bp. The sequences bla_{TEM} gene returned 100% identity with GenBank Accession number; BankIt2236899 Seq1a MN114035 to Seq 9b MN114052). This study found that the bla_{TEM} gene was present in recovered *Vibrio parahaemolyticus, Aeromonas hydrophila, Pseudomonas aeruginosa* and *Streptococcus iniae*. It is recommended that Formalin and hydrogen peroxide as well as potassium permanganate should used to manage fish helminthes in the ponds.

CHAPTER ONE

INTRODUCTION

1.1 Background

Fish farming has been practiced for a period of time worldwide and world per capita fish consumption is 20 Kg per person per year. Kenya's per capita fish consumption increased from 3.2 Kg/person/ year in 2013 to 4.5 Kg/person/year in 2016 due to increased production from aquaculture; though still below the East African average of 7 Kgs/ person/ year and Africa's 10 Kgs/person/year (APRM, 2016). At global level, fish produced was 171 million metric tons (MT), with aquaculture contributing 80 million MT (FAO, 2016).

In Kenya, fish farming has been going on since 1920s in regions endowed with a lot of water resources. The fish species cultured in the Country are mainly Nile tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*), Common carp (*Cyprinus carpio*) and Rainbow trout (*Oncorhynchus mykiss*) (MFD, 2012a; MFD, 2008; Ngugi *et al.,* 2007). In order for the Country to maximize the aquaculture opportunities that exist including employment creation and income generation, the Government of Kenya launched and implemented a Fish Farming Enterprise Productivity Programme (FFEPP) 2009-2013, covering 219 constituencies (Charo-Karisa and Gichuri, 2010). To complement the FFEPP and reap more benefits from aquaculture, more people opted to construct their own fish ponds, thus increasing the area under aquaculture to about 20,000 hectares from 722 Ha in year 2008 (SDF, 2013; MFD, 2012b; MFD, 2008).

Eventually, the number of fish farmers rose to 59,095 with 55,750 stoked fish ponds country wide (MALF, 2016). The fingerlings used to stock the fish ponds and cages were sourced from both public (16) and private (40) authenticated fish farms and hatcheries (FAO, 2014). The aquaculture production was 14,952 metric tons (MT) worth Kshs. 4,254 million (MALF, 2016). This growth is expected to further expand so that the farmed fish bridges a huge gap between the supply and demand for fish occasioned by increased population and the shifting trends of Kenyans‟ preference towards fish consumption. coupled with reduced land acreage for animal and poultry rearing (MFD, 2012b).

The aquaculture development in Kenya is diverse and is carried out in various production systems. These include earthen ponds, earthen ponds with liners, net cages in ponds, dams or rivers, rectangular raceway tanks, circular or rectangular concreate tanks, lockable ponds and recirculating aquaculture systems (Munguti *et al.*, 2014; Nyonje *et al.*, 2011; MFD, 2010). In Bungoma County, apart from fish farming, communities residing close to dams and rivers engage in fishing activities on subsistence basis. The main types of fish produced through the County aquaculture programmes are tilapia and cat fish. (CIDP, 2018).

The occurrence of parasites in pond fish is anticipated due to crowded conditions in ponds. The parasitic composition on fish in turn depends on environmental (water quality) conditions (Lagrue *et al.*, 2015; Ali *et al.*, 2014; State and State, 2009); species of fish; species of microorganisms and and food habits (Huss, 1994; Cowx, 1992). Fish naturally harbour parasites on the skin and in the alimentary canal. At

certain thresholds, the parasites grow to large numbers and invade fish, leading to spread of diseases (Valerie *et al.,* 1993). *Vibrio vulnificus* have growth optimum temperature at 37^{o}C, with a temperature range for growth ranging between 8^{o}C and 43^{o}C while *Aeromonas* spp grow optimally at 28^{o}C and tolerates temperature range from 2°C to 45^{o}C and (Kovacek, 2003; ICMSF, 1996a; ICMSF, 1996b).

Fish diseases account for the largest source of economic loss in aquaculture production (Munguti *et al.,* 2014; Fiovarati *et al.,* 2007. Serious disease infections have been reported in areas with considerable aquaculture production (Adenike and Olabode, 2009). Some of the parasites that cause diseases in farmed tilapia are bacteria, nematodes, acanthocephalans and trematodes (Daisy, 2015; Orina *et al.,* 2014; Nganou *et al.,* 2011; Al-Harbi and Uddin, 2003; Al-Harbi, 1994).

Characterization of fish parasites is paramount for sustainable management of fish diseases. This makes it possible to effectively treat any diseases that affect the fish using specific drugs. Genetic analysis of antibacterial resistance genes in bacteria relies on DNA extraction, amplification and sequencing. This is an accurate method of determing detailed molecular characteristics of genes that cause resistance, with the ultimate goal of prescribing drugs that are able to eliminate the pathogens. The diseased farmed fish are normally treated using a variety of drugs comprising antibacterials and antiparasitics (Daniels *et al.,* 2000). The antibacterials include; β-lactams (penicillins, amoxillin, ampicillin and ampicillin-subacttum, cefepime, cefuroxime), tetracyclines (doxycycline, tetracycline and oxytetracycline), cephalosporins (cephalexin, cefotaxime and ceftazidime) and carbapenems

(imipenem). Others are quinolones and fluoroquinones (ciprofloxacin, nalidixic acid, ofloxacin and levofloxacin), aminoglycosides (amikacin, gentamicin and kanamycin), phenicols (chloramphenicols), macrolides (erythromycin, clarithromycin and azithromycin) as well as folote pathway inhibitors (trimethoprim-sulfamethoxazole), all being used to treat bacterial infections (Shaw *et al.,* 2014; Daniels *et al.,* 2000). On the other hand, the antiparasitics that eliminate trematodes, nematodes and acanthocephalans include; formalin, hydrogen peroxide, chloramines T, Potassium permanganate and Malachite green (Stoskopf, 2015).

The current study used morphological, microbiological/molecular methods to characterize the pathogenic helminthes and bacteria in Nile tilapia.

1.2 Problem Statement

Fish farming favours diseases outbreak because fish production facilities are closed systems that usually support high-density living conditions that confine fish. The high-density conditions are favourable for spread of parasitic diseases due to overcrowding and increased stress (Ponnerassery *et al.,* 2012). Besides, in Kenya, the water quality parameters of the fish farming systems are monitored and records kept. The National Aquaculture Policy provides for use of Good Aquaculture Practices including prudent application and use of veterinary drugs to ensure safety of fish and fishery products intended for human consumption (MFD, 2011). However, farmers use these drugs indiscriminately without reference to specific bacteria or parasites infecting the fish. This is coupled with inadequate knowledge on fish diseases and parasites by the fish farmers (MFD, 2011). Further, studies indicate that *Escherichia*

coli isolates have been shown to be resistant to tetracycline, co-trimoxazole, streptomycin, ampicillin, quinolones and third generation cephalosporins at varying frequencies. Some of the isolates were found to be resistant two or three antimicrobials (Kariuki *et al.*, 2013).

Most of the residents in Bungoma County are farmers either on large, medium or small-scale. However, fish farming in the Country is mostly practiced by medium and small-scale farmers (CGoB, 2013). The two species fish farmed in the County are Nile tilapia and cat fish with Nile tilapia contributing 99% whereas catfish contributes 1% (CIDP, 2018).

Diseases outbreak are common in fish production facilities because these are closed systems that usually support high-density living conditions that confine fish. The high-density conditions are favourable for spread of parasitic diseases due to overcrowding and increased stress (Ponnerassery *et al.*, 2012). Besides, when fish that is infected is consumed by humans, the humans may be infected especially when eaten fresh.

This study focused on characterizing parasites that are present in fish farming systems in Bungoma County and evaluating some drugs used in their control. The study also determined sensitivity of the bacterial pathogens against selected antimicrobial agents.

1.3 Justification

Infections of farmed fish by parasites and bacteria lead to loss of nutrition value inherent in fish and increase incidence of food poisoning. Again, fish is important as a source of trade development (African Union, 2014) but infections may result in fish export sanctions. This study focused on the parasites and bacteria due to the nature of water supply to fish ponds in Bungoma County. The ponds mostly receive water from rivers and springs. The riverine sources of water could introduce fish pathogens via contamination (Blanco *et al.,* 2000). Yet there is dearth of information on parasites that cause diseases in farmed fish and also whether the therapeutic drugs used to treat them are effective.

In Bungoma County, consumed fish is sourced from River Nzoia and other streams and farm supplies and also obtained from neighbouring Counties namely Kakamega, Busia and Trans Nzoia as well as Uganda. The major farmed fish species is Nile tilapia and yet, there are no documented studies on the kind of pathogenic parasites present in cultured Nile tilapia and possible sources of contamination. The current study therefore, focused on characterizing parasites present in fish farming systems in the County. The findings of this study could be useful in solving the problem of parasitic and bacterial infections and also guarantee quality of fish consumed. Further, the findings would be useful in designing disease prevention strategies and action plans by the County Government as well as the National Government. Besides, the findings would be usd by extension officers to advice fish farmers in the County on putting in place proper biosecurity systems to manage and control diseases.

1.4 Research questions

i. What is the level of selected water quality parameters in fish farming systems in Bungoma County?
ii. What are the pathogenic parasites present in fish farming systems in Bungoma County?
iii. What are the responses of the isolated parasites to antiparasitics and antimicrobials?
iv. What genomic antibacterial resistance gene is present in the pathogenic bacteria isolated from fish farming systems in Bungoma County?

1.5 Hypotheses

i. There is no significance difference in the levels of selected water quality parameters among subcounties of Bungoma County.
ii. There are no pathogenic parasites in fish farming systems in Bungoma County.
iii. There is no significance difference in pathogenic parasitic responses to antiparasitics and antimicrobials.
iv. There is no genomic antibacterial resistance gene in the pathogenic bacteria isolated.

1.6 Objectives

1.6.1 General objective

To characterize pathogenic parasites, present in farmed Nile tilapia, pond water and fish feeds in fish farming systems and evaluate the control methods of the parasites in Bungoma County.

1.6.2 Specific objectives

i. To determine the level of selected water quality parameters in fish farming systems in Bungoma County.

ii. To determine the pathogenic parasites, present in fish farming systems in Bungoma County.

iii. To assess the responses of pathogenic parasites to selected antimicrobials and antiparasitic drugs.

iv. To determine the genomic antibacterial resistance genes, present in the pathogenic bacteria isolated from fish farming systems in Bungoma County.

1.7 Significance of the study

The current study characterized pathogenic parasites present in pond water, fish feeds and Nile tilapia. The characterization is important because extension officers as well as fish farmers will be in a position to identify the common pathogenic parasites existing in a given geographic area and apply the specific drugs for the parasites. The findings are applicable in the management, control and prevention of disease outbreaks in aquaculture systems. Again, various drugs used to treat infected farmed fish are available in the markets. However, the drugs are not uniformly effective against paraites. Therefore, there is need to determine the drugs that are effective against each specific parasite isolated. Further, if the drugs are not well used, it may lead to development of resistance against them by the targeted pathogens.

1.8 Scope and Limitations of the study

The current study covered six sub counties in Bungoma County. The sub counties were Bumula, Bungoma South, Bungoma West, Bungoma North, Mt Elgon and Bungoma East (CGoB, 2014). This study covered six water quality parameters including temperature, dissolved oxygen, conductivity, pH, turbidity and BOD. There are other factors known to impact on fish disease causing parasites but were outside the scope of this study. The factors include salinity, Hydrogen sulphite, total dissolved solids, total suspended solids, ammonia, nitrate, nitrite and carbon dioxide (Zorriehzahra *et al.,* 2012). The current study has characterized specific parasites found within the geography of Bungoma County. Besides, the assumption of the study was that out of all existing active fish ponds, only those fish ponds already stocked were sampled during the period of the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 Water quality parameters

The water quality parameters of fish ponds affect physiology, growth rate and may cause mortality in farmed fish (Graig, 2008). High temperatures are reported to accelerate life cycles and thereby promoting spread of ectoparasites (Hassan, 1999). Further, multiplication of *Vibrio* spp. and *Streptococcus* spp. in fish ponds is linked to high temperatures (25^0 C-32^0 C) (Yanong and Francis-Floyd, 2016; Chatterjee and Haldar, 2012). A decrease in dissolved oxygen due to temperature rise above 25^0 C compromises immune system of fish as most warm water fish prefer dissolved oxygen above 2 mg / l (Suomalainen *et al.*, 2005).

The values recommended for conductivity levels for fresh water fish culture which is 20 to 1500 µs / cm (Boyd, 1990). Turbidity level recommended for fish ponds is 30-40 ppm (Santhosh and Singh, 2007). Recommended dissolved oxygen levels above 2 mg/ l is necessary for the farmed Nile tilapia to survive favourably (Suomalainen *et al.*, 2005).

Biological oxygen demand in fish ponds is high when organic suspended solids are increased, thereby reducing dissolved oxygen (Chen *et al.*, 1994). Besides, suspended solids affect health of fish by clogging gills, rougthening the skin and impairing feeding (Wedemeyer, 1996). Some of the bacteria in fish ponds are known to convert suspended solids to carbon dioxide during respiration, resulting in water pH drop (Noble and Sommerfelt, 1996).

2.2 Fish parasites

Fish pathogenic parasites include flatworms, trematodes, nematodes, thorny/spiny worms and bacteria. Bacteria that cause diseases in Nile tilapia are either Gram positive or Gram negative species. Helminthes include *Polyacanthorhynchus, Amirthalingamia, Cyclustera, Proteocephalus, Contracaecum, Procamallanus, Contacaecum, Camallanus,* and *Acanthocephala* spp. (Kamundia, 2011; Gichohi, 2010; Aloo, 2002).

The Gram negative bacteria are; *Vibrio, Aeromonas, Pseudomonas, Flavobacterium, Yersinia, Edwardsiella,* whereas Gram-positive ones are namely *Streptococcus, Staphylococcus, Nocardia, Lactococcus,* and *Vagococcus* species (Emere and Dibal, 2014; Pridgeon and Klesius, 2012; Mastan *et al.,* 2009; Faruk *et al.,* 2004; A-Harbi and Uddin, 2003; Al-Harbi, 1994). Under stressful conditions, pathogenic parasites present in fish are able to grow to large numbers, thereby causing diseases to fish (Ponnerassery, 2012; Valerie *et al.,* 1993). Some of these parasites are discussed below.

2.2.1 Helminthes

Flatworms (platyhelmintes) are mainly found on the skin, fins and gills of fish and are rarely longer than about 2 cm. They lack respiratory, skeletal and circulatory systems attach to hosts using hooks, clamps and a variety of other specialized structures (Noga, 2000). The known species include *Gyrodactylus, Gyrodactyloides, Dactylogyrus, Cichlidogyrus* and *Gyrodactylus.* They cause epithelial hyperplasia or

hemorrhage in infected fish (Dezfuli *et al.*, 2007; Noga, 2000; FAO, 1996; Kabata, 1995).

The trematode (flukes) life cycle comprises of intermediate hosts (one or two types) and a definitive host. They have two suckers for attachment to their host; oral sucker surrounding the mouth and ventral sucker on the ventral surface (Purivirоskul, 2011). The trematode *Diplostomum* spp. and *Posthodiplostomum* spp. are known to cause an eye blackspot or fluke blindness in infected fish (Violante-Gonzalez, *et al.,* 2009). *Gymnarchus niloticus* are recognized to infect visceral organs and digestive tract in *Synodontis victoriae*, *Clarias* spp. and *Bagrus* spp (FAO, 1996).

Nematodes (roundworms) are bilaterally symmetrical, coelomate elongate worms with cylindrical bodies tapering at both ends. They possess a solid resistant cuticle that makes them last longer at postmortem conditions (Dick and Choundury, 1995). Nematodes are the most important helminthes in fish health; infections of cultured fish with helminthes is quite common (Williams and Jones, 1994). The pathogenic nematodes include *Anguillicola crassus*, *Dactylogyrus extensus*, *Dactylogyrus vastator, Dactylogyrus lamellatus, Pseudodactylogyrus* spp that infect farmed eels and *Diplectanum aequans*, that infect farmed sea bass (Buchmann and Bresciani, 1997).

Thorny-headed/ spiny worms (acanthocephalans) are are cylindrical and elongated worms with a body consisting of presoma and metasoma. They are mostly gut worms with at least one intermediate host in their life cycle (FAO, 1996). The anterior

retractile proboscis hook pearces host epithelium (Amin and Heckmann, 1992) and attachment point of acanthocephalans becomes necrotic leading to peritonitis (Ferguson, 1989).

2.2.2 Bacteria

The bacteria that infect tilapia include; *Aeromonas hydrophila*, *Edwardsiella tarda*, *Flavobacterium columnare*, *Streptococcus agalactiae*, *Streptococcus iniae* and *Yersinia ruckeri* (Iregui *et al.*, 2012; Abuselian *et al.*, 2011; Al- Harbi, 2011; Ye *et al.*, 2011; Mohamed and Saleh, 2010; Jimenez *et al.*, 2008; Eissa *et al.*, 2008). *Aeromonas* are among the most common bacteria in a variety of aquatic environments and are frequently associated with severe disease among cultured fishes (Martin-Carnaham and Joseph, 2005). *Aeromonas hydrophila* and *Aeromonas veronii* cause haemorrhagic septicemia primarily in freshwater fish such as catfish, tilapia and bass and are major economic problem in the fish-farming industry (Austin and Austin, 1999). *Pseudomonas* spp. is the etiological agents that causes septicemia disease. Common species include *Pseudomonas diminuta*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas aeruginosa*. The species cause diseases when the host is subjected to stress (Somsiri and Soontornvit, 2002; Roberts, 2001).

Streptococcus species are Gram positive cocci fish pathogens known to cause streptococcosis (Zadeh *et al.*, 2012; Caroll *et al*., 2007; Wardman, 2007). General pathological symptoms of streptococcosis in fish are haemorrhage, lethargy, congestion, dark pigmentation, erratic swimming, and exophthalmos with clouding of the cornea (Buller, 2004). The *Vibrio* species infect fish in all ecological systems

causing vibriosis (Bergh *et al.*, 2001). The known pathogenic species include *Vibrio vulnificus, Vibrio anguillarum*, *Vibrio harveyii*, *Vibrio ordalii*, *Aliivibrio salmonicida* and *Moritella viscosa* (Austin and Zhang, 2006; Benediktsdóttir *et al.*, 2000).

2.2.3 Control of fish parasites

When fish get infected with parasitic pathogens in aquaculture systems, they are often treated with a variety of drugs available on the market. The drug groups available on the market include antimicrobials (against bacteria and fungi), antiparasitics, anaesthetics, and anticoccidials, besides vaccines (Shaw *et al.*, 2014; Daniels *et al.*, 2000).

The antimicrobials treatment of infected fish is principally based on a minimum inhibitory concentration (mic). The mic is the lowest concentration of a drug which prevents visible growth of bacterium after overnight incubation (Andrews, 2001). The mic for each drug is further classified as S, I, R; where R=resistant, I=intermediate or S=susceptible based on established interpretation guidelines (CLSI, 2013; Truong *et al.*, 2008). Again, each category (S, I or R) has a mic break-point. The break-point is a cutoff for each interpretation category established and the break-points are specific to each bacterium and drug (CLSI, 2013).

It is documented that indiscriminate use of antimicrobials in aquaculture may lead to emergence of resistant strains of bacteria infecting farmed fish (Ginovyan *et al.*, 2017; Alvarez *et al.*, 2004). Besides, when the drugs are excreted from fish, they end up

interacting with soils, enter the food chain by plant uptake, leach into groundwater and find their way into surface water via runoffs and drain flows (Yang *et al.,* 2010; Tamtam *et al.,* 2008; Boxall *et al.,* 2002). This is because some of these antimicrobials especially flouroquinoles are known to persist in the environment after long term use (Thiele-Bruhn *et al.,* 2003). Therefore, use of antimicrobials in treatment of infected cultured fish should be monitored frequently in order to evaluate the emergence and spread of pathogenic bacteria resistance to antibiotics. Again globally, there is limited data on antimicrobial resistance of bacteria present in aquaculture systems and other cultured organisms (Kathleen *et al*., 2016; Lim and Kasing, 2013; Sharrif *et al*., 2000).

2.3 Approaches for isolating and identifying fish pathogens

Isolating and identifying fish pathogens is crucial since death of fish could lead to loss of nutrition value inherent in fish, increased incidence of food poisoning and fish export sanctions. A number of techniques used to isolate fish pathogens have been developed overtime and the techniques are phenotypic and molecular in nature.

2.3.1 Phenotypic techniques

The The phenotypic techniques include cultural, API 20E system, RapID^{TM} One system, Vitek system, immunological, chemotaxonomy and microscopy. Cultural techniques involve enrichment of growth media followed by plating onto selective agar. On other occasion, it involves plating directly onto selective agar without enrichment. Different media are composed of essential nutrients that promote the growth of specific micro-organisms. The media also contain reagents that inhibit the

growth of non-target microorganisms. The confirmation of presumptive bacteria colonies normally is through biochemical tests (Adzitey and Nurul, 2011; Corry *et al.*, 2003). In addition, the media may contain indicators which allow the target organism to be easily identified, often by a colour change in the medium.

The Analytical Profile Index (API) 20E system is a standardized method using 20 miniaturized biochemical tests for the identification of *Enterobacteriaceae* and associated species. It is widely used for the identification of bacterial pathogens isolated from agricultural products, water and in clinical tests (Sabae and Rabeh, 2007; Sundram *et al.*, 2000).

The RapID^{TM} One system is a qualitative method which uses both conventional and chromogenic substrates for the identification of *Enterobacteriaceae* without additional biochemical tests. The system is based on the ability of microorganisms to degrade specific substrates detected by various indicator systems (Kitch *et al.*, 1994).

The Vitek system is an automated microbiology system utilizing growth-based technology and it has three formats. The three formats differ in increasing levels of capacity and automation. The Vitek procedure involves inoculating colonies suspended in saline into Reagent Cards (Shetty *et al.*, 1998). The Reagent cards contain 64 wells (Appendix 2.1) each containing individual test substrate and one negative control cell to assess growth and viability of the suspension. The system has four varied card categories being GN-gram-negative fermenting and non-fermenting bacilli; GP-gram-positive cocci and non-spore forming bacilli; YST-Yeast and yeast

like organisms; and BCL-gram-positive spore forming bacilli (www.pda.org/bookstore). The four varieties of cards are able to identify different species of microorganisms. The incubation period varies from 2-15 hours depending on the growth rate of the organism. When the incubation period is completed, the reactions are analysed automatically. The Vitek programmed computer determines whether each well is positive or negative by measuring light attenuation with an optical scanner (Shetty *et al.*, 1998).

The immunological techniques have antibody-based procedures that include indirect fluorescent antibody test, whole-cell agglutination, precipitin reactions, complement fixation, immunodiffusion, antibody coated latex particles, co-agglutination using antibody coated staphylococcal cells, passive haemagglutination, immuno-India ink technique and Enzyme Linked Immunosorbent Assay (Adams and Thompson, 2008; Austin and Austin, 2007). The immunological techniques are sensitive, specific, rapid and reliable for the detection of pathogens. However, the antibody-based procedures may result in cross reactivities (Adams, 2009; Adams and Thompson, 2008).

Chemotaxonomy investigates chemical constituents of micro-organism. The molecules studied include fatty acids, polar lipids, lipopolysaccharide, menaquinones, naphthoquinones, ubiquinones, mycolic, teichoic and teichuronic acids, peptidoglycan, polyamines, and isoprenoid quinones (Scheifer, 2009). On the other hand, microscopy involves detection and identification of micro-organisms through microscopic examination of morphological features (Fried *et al.*, 2002; Sims *et al.*, 2002; Rogerson and Gwaltney, 2000).

Cultural techniques involve enrichment of growth media followed by plating onto selective agar. On other occasions, it involves plating directly onto selective agar without enrichment. Different media are composed of essential nutrients that promote the growth of specific micro-organisms. The media also contain reagents that inhibit the growth of non-target microorganisms. The confirmation of presumptive bacteria colonies normally is through biochemical tests (Adzitey and Nurul, 2011; Corry *et al.,* 2003). In addition, the media may contain indicators which allow the target organism to be easily seen, often by a colour change in the medium. Microscopy involves detection and identification of micro-organisms through microscopic examination of morphological features (Fried *et al.,* 2002; Sims *et al.,* 2002; Rogerson and Gwaltney, 2000).

2.3.2 Molecular techniques

The molecular techniques entail manipulation and analysis of RNA, DNA, proteins and lipid. The techniques have three levels; Polymerase Chain Reaction (PCR), typing and sequencing.

i. Polymerase Chain Reaction

Polymerase Chain Reaction procedures include; conventional, single, multiplex, real-time, reverse- transcription and nested polymerase chain reactions. Conventional polymerase chain reaction allows for the quick replication of a single specific gene by enabling accurate detection of genetic markers (Toze, 1999). This procedure does not

distinguish between a viable and non-viable organism, which implies that it is sensitive and will detect all organisms present in a sample (Thando, 2013).

Single Polymerase Chain Reactioninvolves use of a single primer set which targets a specific gene to detect an organism. The primer set can be designed for specific species and can detect the target organism in the presence of others. This kind of PCR can be applied to rapidly detect and identify bacteria directly from a sample with or without pre-enrichment (Abulreesh *et al.,* 2006; Josefsen *et al.,* 2004).

Multiplex Polymerase Chain Reaction involves targeting of multiple genes from the same or different organisms by the use of multiple primer sets in a single reaction tube to produce amplicons of different sizes. All primers need to have close annealing temperature and the amplicons must be markedly different in sizes. It is known to reducing cost, limits volume of samples and is rapid (Shi *et al.,* 2010). However, the multiple primers may interfere with each other during the amplification process (Elnifro *et al.,* 2000).

Real-time Polymerase Chain Reaction is a procedure which the target DNA is amplified and quantified simultaneously within a reaction. The amplified DNA is detected in real time as the reaction progresses instead of at the reaction end. It employs specific primer set, one or two probes and/or fluorescent dye to improve detection signals (Dhanasekaran *et al.,* 2010; Rensen *et al.,* 2006; Valasek and Repa, 2005; Gizinger, 2003). It has no post- PCR processing of products, can determine the

number of bacteria in various samples and reduces the risk of amplicon contamination by laboratory environments (Wong and Medrano, 2005; Heid *et al.,* 1996).

Reverse-transcription Polymerase Chain Reaction involves using RNA as the initial template instead of DNA. Reverse transcriptase is used to reverse transcribed target RNA into its DNA complement (cDNA) and amplified using PCR. It is useful in detecting only viable cells of pathogens (Shi *et al.,* 2010; Sharma, 2006).

Nested Polymerase Chain Reaction employs two sets of primers in two successive polymerase chain reactions. The first PCR runs and the final products are collected. The final products from the first PCR are then used as primers in the second PCR. The technique improves the sensitivity and specificity of detecting pathogens (Picken *et al.,* 1997; Olsvik *et al.,* 1991).

ii. Typing level

The Pulsed field gel electrophoresis (PFGE) is an agarose gel electrophoresis technique used for separating larger pieces of DNA by applying electrical current that periodically changes direction (three directions) in a gel matrix (Trindade *et al.,* 2003; Arbeit 1999). In PFGE, intact chromosomes are digested using restriction enzymes or restriction endonucleases to generate a series of DNA and patterns specific for a particular species or strain (Shi *et al.,* 2010). This procedure has good reproducibility, discriminatory power and typeability. But it is sensitive to genetic instability, has limited availability and takes 3–4 days to complete a test. Degrading of DNA during

PFGE process can make strains untypeable (Alonso *et al.,* 2005; CDC, 2002; Wassenaar and Newell 2000).

Multilocus sequence typing (MLST) is nucleotide-based technique for typing bacteria using the sequences of internal fragments of seven house-keeping genes (Spratt, 1999; Maiden *et al.,* 1998). Different sequences within a bacteria species are assigned as distinct alleles for each house-keeping gene and the alleles at each end of the seven loci define the allelic profile or sequence type for each isolate (Urwin and Maiden, 2003). It provides typing data that is unambiguous, portable, more accurate and more discriminatory for most bacteria (Dingle *et al.,* 2005; Urwin and Maiden, 2003; Enright and Spratt, 1999). Nonetheless, multilocus sequence typing is expensive compared (Chen *et al.,* 2007; 2005).

Random amplified polymorphism deoxyribonucleic acid (RAPD) is a technique in which arbitrary primers are used to randomly amplify segments of target DNA under low-stringency PCR condition (Wassenaar and Newell, 2000). This process leads to the amplification of one or more DNA sequences and generates a set of finger printing patterns of different sizes specific to each strain (Trindade *et al.,* 2003; Farber, 1996). The RAPD is relatively cheap, rapid, readily available, and easy to perform. In addition, it has low reproducibility, average discriminatory power and approximately 80 % typeability (Rezk *et al.*, 2012; Shi *et al.*, 2010; Wassenaar and Newell, 2000).

Enterobacterial repetitive intergenic consensus (ERIC) PCR uses primers specific for enterobacterial repetitive intergenic consensus sequences. These primers can be used

under high stringency conditions to match the target DNA to produce DNA finger prints that are different in sizes. It is quick, easy to perform and cost effective though reproducibility is low (Trindade *et al.,* 2003; Wassenaar and Newell, 2000).

In repetitive extragenic palindromic, repetitive DNA elements present within bacterial genome are amplified to produce finger prints of different sizes that are specific to each bacterial strain (Versalovic *et al.,* 1991). Trindade *et al.* (2003) reported that REP is cheaper, easy to perform and applicable to a number of isolates but have lower discriminatory power.

Amplified fragment length polymorphism (AFLP) involves the use of two restriction enzymes to digest total genome DNA, one with an average cutting frequency (4-bp recognition site) and the other with a higher cutting frequency (6-bp recognition site). It is followed by linking of adapters to the sticky ends of the restriction fragments and amplification of a subset of selected restriction fragments (Shi *et al.,* 2010). The AFLP has good discriminatory power, good reproducibility, typeability, needs no prior sequence information for amplification and insensitive to genetic instability. However, the procedure is complex and requires several days to complete a test (Meudt and Clarke, 2007).

Restriction fragment length polymorphism (RFLP) involves the use of restriction enzyme to digest DNA and to separate the resulting restriction fragments according to their length on agarose gel electrophoresis. Restriction fragments are then transferred into a membrane through Southern blot procedure and hybridized to a membrane

bound labelled DNA probe (Foley *et al.,* 2009). This method utilizes the variations in homologous DNA sequences to characterize bacteria. This technique is inexpensive, very sensitive for strain identification or differentiation (Babalola, 2003; Mohran *et al.,* 1996).

Plasmid Profile Analysis involves plasmid DNAs being extracted from bacteria and the DNA is separated on agarose gel electrophoresis. Plasmids are mobile extrachromosomal elements that can spontaneously be lost or readily acquired by bacteria and thus isolates that are related epidemiologically can easily display different plasmid profiles (Trindade *et al.,* 2003). Again, plasmids exist in a variety of spatial conformations (linear, nicked and supercoiled) which result in different migration velocities when submitted to agarose gel electrophoresis and this affects the reproducibility of the technique (Hartstein *et al.,* 1995). Ribotyping use selected restriction endonuclease to digest genomic DNA into small DNA fragments which are separated by gel electrophoresis and identified using Southern blot hybridization with a probe specific for rRNA genes (Shi *et al.,* 2010). Ribotyping has higher discriminatory power at the species and subspecies level compared to the strain level (Shi *et al.,* 2010; Denes *et al.,* 1997).

The RAPD technique was used in the current study to finger print the specific bacterial pathogens that were present in farmed Nile tilapia and were found to be resistant to any of the nine selected antibiotics tested. The RAPD procedure is simple, cost effective and gene specific with minimal contamination.

Multiplex PCR targets several genes from the same or different organisms using various primer sets in a single reaction tube to produce amplicons of different sizes (Shi *et al.,* 2010). The procedure was used as it is to target the resistant genes that were isolated from the pathogenic bacteria.

iii. Sequencing level

The It involves sequencing of 16S rRNA and 23S rRNA genes as well as DNA: DNA hybridization. The 16S rRNA is a common gene that is amplified for sequencing and subsequently for the identification, typing and taxonomic classification of the pathogen in question. The sequencing of the 16S rRNA gene (region) is an accepted procedure for developing phylogenetic relationships, identification and characterization of fish bacterial pathogens (Ransangan and Mustafa, 2009; Nomoto *et al.,* 2004; Chen *et al.,* 2002). The DNA hybridization has different protocols described (Abulreesh *et al.,* 2006; Newell *et al.,* 2000) and usually is preceded by Polymerase Chain Reaction (PCR) to amplify the targeted genes. The DNA sequencing procedure involves determining the order of the nucleotide bases (namely adenine, cytosine, guanine and thymine) in a DNA molecule (Adzitey *et al.,* 2013).

The study study undertook antimicrobial analysis of the bacteria recovered to find out the susceptibility responses. Only nine antibiotics out of the many available on the market were used in this study. The PCR, typing and sequencing procedures were applied to determine the resisntant genes present in the isolated bacteria. The current study further relied on cultural technique and microscopy to isolate and identify pathogenic bacteria and parasites. The techniques for bacteria identification involved

enrichment of growth media and biochemical tests such as gram stain, TSI, catalase, hydrogen sulphite, methyl red and voges-proskauer to identify potential pathogenic bacteria infecting farmed Nile tilapia, pond water and fish feeds.

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study area

The study was conducted in Bungoma County. Bungoma County lies between latitude 0^0 28' and latitude 1^0 30' North of the Equator, and longitude 34^0 20' East and 350 15' East of the Greenwich Meridian and an altitude of between 1,200m and 1,800m above sea level. The County covers an area of 3032.4 Km^2. It boarders the republic of Uganda to the North west, Trans-Nzoia County to the North-East, Kakamega County to the East and South East, and Busia County to the West and South West (Fig. 3.1).

The County experiences two rainy seasons, the long rains of March to July and short rains from August to October. The annual rainfall in the County ranges from 400mm (lowest) to 1,800mm (highest). The annual temperature in the County vary between 0°C and 32°C with mean annual temperature about 23^0C due to different levels of altitude, with the highest peak of Mt. Elgon recording slightly less than 0°C. The average wind speed is 6.1 Km/hr (CGoB, 2018).

The County has three agro-ecological zones namely lower midland, lower highland and upper highland. The major physical features include Mt. Elgon, several hills (Chetambe, Sang'alo and Kabuchai), rivers (Nzoia, Kuywa, Sosio, Kibisi and Sio-Malaba/Malakisi), waterfalls such as Nabuyole and Teremi. Mt. Elgon and Sang'alo hill have attractive caves. The altitude of the County ranges from over 4,321m (Mt. Elgon) to 1200m above sea level. The County has only one gazetted forest, the Mt.

Elgon forest reserve which measures 618.2Km2, and one National park, which measures 50.683 Km^2 (CGoB, 2014).

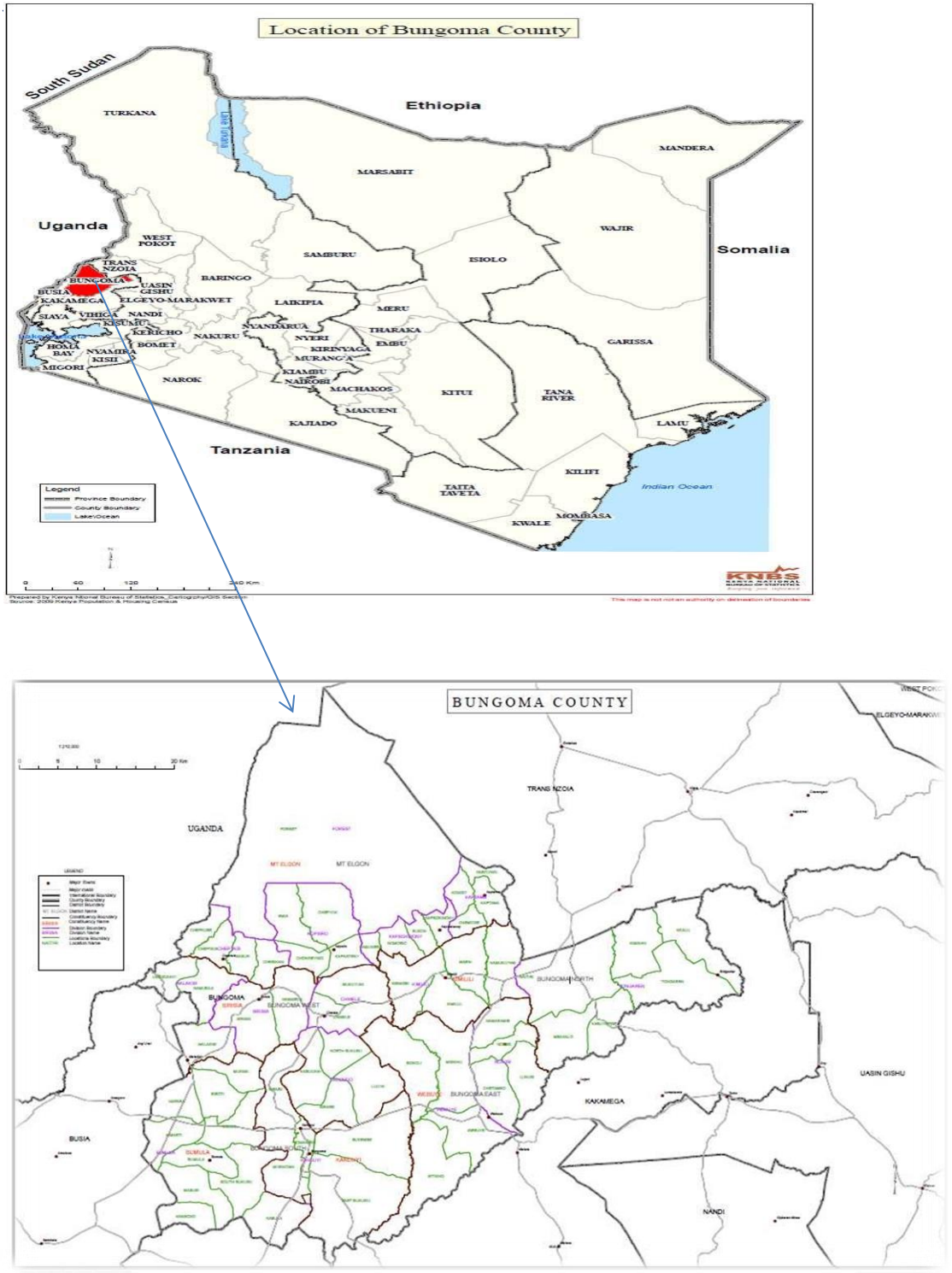

Figure 3. 1 : Map of Kenya showing 47 Counties (Bungoma County in red) and Map of Bungoma County: Sources: KNBS, Bungoma County) and County Government of Bungoma; mag. (18.52x16.09)

The County population is estimated at 1,375,063 (KNBS, 2010). During implementation of the Fish Farming Enterprise Productivity Programme (FFEPP), Bungoma was one of the succeful Counties (Charo- Karisa and Gichuri, 2010). To reap more benefits from aquaculture, more farmers in the County constructed their own fish ponds, besiedes, the County Government supporting the industry (CGoB, 2014; SDF, 2013; MFD, 2012b; MFD, 2008). The County also has a well-established Fish Farm and Hatchery at Chwele that is a source of fish fingerlings used to stock the fish ponds (CGoB, 2014). In the County, farmers grow fish either as a single production unit or practice integrated farming. Integrated fish farming involves combining production of fish growing with other farming activities such as poultry, bee keeping and crop farming.

3.2 Selection of study sites

The study was was purposively undertaken in six sub counties (Fig. 3.1) in Bungoma County. The six are based on the fact that prior to year 2013, when the Fish Farming Enterprise Productivity Programme (FFEPP) was implemented, the County had six constituencies currently sub counties where fish farming was supported. The sub counties are Bumula, Bungoma South, Bungoma West, Bungoma North, Mt Elgon and Bungoma East.

Bumula Sub County covers an area of 347.8 Km^2 with fertile soil for fish farming and crop production. The major rivers include Sio, Mayanja, Kimwanga and Nakhwana. It has hills such as Siboti, Malakisi and Nakuti. Bungoma South Sub County covers an area of 318.5 Km^2. It has the following resources; sunlight, rainfall, wind, fertile soils,

permanent rivers (Khalaba and Sio), Sanga'lo and Musikoma hills as well rocks and sand.

Bungoma West Sub County covers an area of 445.3 Km^2 with permanent rivers such as Chwele, Kuywa, Lwakhakha, Malakisi, Toloso, Ndakalu and Tisi. It has several hills namely; Kibichori, Kakichuma, Nabuloli, Sibanga, Chebukwa, Kabuchai hills, South Kulisiru, Yabeko, Cheptumi, Lukaala, Wekelekha, Malakisi and Bukokholoa.

Bungoma North Sub County covers an area of 559.6 Km^2 and is the bread basket of Bungoma County due its fertile land. Rivers; Nzoia, Kibisi, Sosio, Matili, Chelekeyi, Nandika, Kibingei, springs and wells. Bungoma East Sub County covers an area of 404.4 Km^2 and has fertile soils and is within the Nzoia River basin. It has fertie soils, streams, rivers and dams that support power generation, fishing and water treatment plants. Mt. Elgon Sub-County covers an area of 956.6Km^2 with fertile soils which are good for horticulture, tea and coffee and fish farming. Also, the Sub County has a forest reserve and National Park. The forest is one of the five water towers in the Country. The National park is famous for its caves.

3.3 Experimental design

A cross sectional study was undertaken from August 2015 to December 2017 comprising nine (9) sessions, each session taking place after every three months. On each sampling occasion, six water quality parameters taken, Nile tilapia fish, pond water, commercial fish feeds were purposively collected. During the study, out of 107

active fish ponds in the county, 78 farms randomly selected in six sub counties were sampled. The fish ponds were owned by individual farmers and institutions. The individual fish ponds sampled were 67 and those belonging to institutions were 11.

Fish was harvested using a seine net (Appendix II). Five whole farmed Nile tilapia of different sizes were harvested from each fish pond (Appendix III and IV). The fish ponds have an area of $300m^2$; 20 metres long by 15 metres width by 60-70 centimetres deep. Based on this area, $1m^2$ of the pond holds 3 fingerlings giving a total of 900 fingerlings for stocking in a single pond. However, the ponds are stocked with 1000 fingerlings of which 100 fingerlings are to compensate for any occurrence of mortality. The selection was based on the number of active ponds with a history that a pond had been stocked 4-6 months before sampling session. At 4-6 months, it is expected that Nile tilapia is above table size (250g) that can be harvested. Besides, any undersize fish that was harvested, it was returned to the fish ponds.

3.4 Sampling design

Bungoma County was chosen for this study because it is one of the Counties that has high potential for fish farming. The County hosts one out of the public fish farms and hatcheries at Chwele besides authenticated private fish hatcheries. The hatcheries are sources of fish fingerlings as well as training and demonstration centres for fish farmers.

The sampling procedures of farmed Nile tilapia were carried out according to procedures outlined by Emere and Egbe (2006). The sampling was comprised nine (9) sessions, each session taking place after every three months. On each sampling occasion, at every fish pond, 100 ml of water was collected in sterile glass bottles, stoppered, enriched in alkaline peptone water and then ice-packed. For commercial fish feeds, 1 Kg of fish feeds from each of the two feed millers who are the main suppliers operating in the county were purchased. The ice-packed water and purchased fish feeds were transferred to the Microbiology laboratory at Kenyatta University in Nairobi.

During the study, the total whole Nile tilapia sampled was determined using the to Creative Research System online survey formula (CRS, 1982). Formula given as:

$$n = \frac{Z^2 P (1-P)}{e^2}$$

Where,

Z is the *Z* value for the corresponding confidence level (1.96 for 95% confidence);

p is the estimated value/ percentage of picking a choice expressed as decimal (0.5 used for sample size needed);

e is the margin of error/ confidence interval as decimal (e.g. 0.05).

$$n = \frac{1.96^2 x\ 0.5\ (1-0.5)}{0.05^2}$$

n= 384

3.5 Data collection

3.5.1 Water quality parameters

On each sampling occasion, at every fish pond sampled, five water quality parameters namely temperature, dissolved oxygen, conductivity, pH and turbidity were measured by immersing a portable auto sampler (Hanna Instruments) (Appendix V) into pond water; quality parameter readings were observed and recorded in a notebook.

Biological Oxygen Demand (BOD) was measured and calculated as follows;

A) BOD=D2 –D1; Where D1=Initial amount of Oxygen and D2=present amount of Oxygen

B) $BOD = \frac{D_2 - D_1}{P}$

Where; $p = \frac{\text{Volume of water (e.g. pond)}}{\text{Capacity of reservoir (e.g. pond)}}$

The formula is calculated using raw data collected (i.e. from fish farm). The data was collected within a time range of 5 days in order to collect “D1: at the beginning and “D2” at the end of the time frame (Delzer and McKenzie, 2003).

3.5.2 Isolation and identification of parasites

3.5.2.1 Helminthes from Nile tilapia

The isolation procedures were carried out according to techniques described in previous studies (Emere and Egbe, 2006). The fish were harvested using a seine net on every sampling occasion. Fish skin and fins were sampled from each fish and

placed in sterile Bijou bottles. Fish were opened dorso- ventrally in aseptic conditions and gills, mouth, stomach and intestinal contents were washed into the sterile Bijou bottles containing 3ml of 0.9% physiological saline and stirred using a mounted pin. The Bijou bottles were then packed in a cooler box with ice and transferred to National Museums of Kenya Parasitology laboratory in Nairobi. In the laboratory, skin and fins were examined using hand lens for external parasites. To examine internal helminthes, 0.5 ml of the sampled gills, mouth, stomach and intestinal contents containing 3ml of 0.9% physiological saline solutions were collected using a dropper, placed on a slide, and then covered with a cover slip after which, observations were made using a compound light microscope at 100X - 400X magnification. Helminthes present were identified using the fact sheets on pictorial guide on fish parasites and counted (Pouder *et al.,* 2005).

3.5.2.2 Bacteria from Nile tilapia

Preparation of stock solutions

In the Microbiology laboratory, the sampled fish organs; mouth, scales, skin, gills, kidney, intestines and liver were individually and aseptically transferred into sterile 9 ml normal saline (0.85W/V) (Dentex,) and were homogenized thoroughly to make 1:10 M/V solutions. From the 1:10 stock solutions, 1 ml volumes were pipetted into sterile bottles containing normal saline solutions to make 10^{-1} solution. The same procedure was repeated for 10^{-2} solutions. The three categories of the solutions (homogenates) were all incubated for 24 h at 30^{0}C. The homogenates were used for all the bacteriological tests (Kagiko *et al.,* 2001). The plating technique used was pour plate and was done in triplicate for every sample.

Total Viable Aerobic Colony Counts

From each solution of the samples, starting at the 10^0 (stock solution), 1 ml volumes were taken and each volume transferred to a sterile plate (Petridish), 20 ml of Tryptone soya agar (TSA) (Himedia, India) added to the plate and mixed well to ensure an even distribution of colonies. The agars were allowed to settle and inverted plates transferred to the incubator (Biobase) at 30^0C for 24 h. After 24 h, the number of colonies present on the plates was counted using a colony counter machine (Gallenhamp, USA). The total viable aerobic colony counts were reported as cfu per ml of the stock solutions.

Biochemical tests

The pathogenic bacteria were isolated by taking 1 ml of the stock solution (10^0) and transferring it aseptically to flasks containing 9 ml of sterile salt-free alkaline peptone water (Himedia, India). The resulting homogenates were incubated at 30^{o}C for 24 h. Then a loopful was streaked on Thiosulphate-Citrate-Bile Salt (TCBS) agar (Himedia, India) and incubated for 24 h at 30^{o}C. After 24 h, suspected species of pathogenic bacteria colonies were picked and purified on TSA plates supplemented with 0.85 W/V Sodium chloride (NaCl) then incubated for 24 h. This was repeated for other concentrations or stock solutions. Thereafter, biochemical tests were carried out (A-Taee *et al.*, 2017; Bhaskar and Setty, 1994). The biochemical tests included Gram stain, TSI, methyl red, Voges- Proskauer and catalase (Dentex) (Table 3.1).

Table 3. 1 : Biochemical test confirmations

Biochemical test	**Observation**	**Confirmation**	
		Positive	**Negative**
Gram stain	Purple	+ve	
	Pink		-ve
TSI	Butts yellow with the production of gas as well as Hydrogen sulphite (H_2S) that blackens slants	+ve	
	Butts yellow with gas production and pink slants		-ve
Methyl red	Red colour	+ve	
	Yellow		-ve
Voges-Proskauer	Red ring	+ve	
	Yellow-brown		-ve
Catalase	Effervescence	+ve	
	No effervescence		-ve

3.5.2.3 Helminthes from pond water

For each fish pond that was sampled, 100 ml of water was collected in sterile Bijou bottles, after which 3ml of 0.9% physiological saline solution was added. The Bijou bottles containing sampled water were then packed in a cooler box with ice and transferred to National Museums of Kenya Parasitology laboratory in Nairobi. In the laboratory, helminthes were examined by placing 0.5 ml mixed solution using a

dropper on a slide. The drop was covered with a cover slip after which, observations were made using a compound light microscope at 100X - 400X magnification. Helminthes present were identified and counted.

3.5.2.4 Bacteria from pond water

The enumeration of TVACC was by Pour plate method through use of Plate count agar and was incubated at 30^{o}C for 72 h (WHO, 1984). For pathogenic bacteria strains, 100 ml amounts of water were passed through membrane filters. The filters were then procedurally subjected to TSA, Gram stain, TSI, catalase, methyl red and Voges-Proskauer, and hydrogen peroxide tests to enumerate and detect pathogenic bacteria as outlined for fish samples above (Torimiro *et al.*, 2014).

3.5.2.5 Helminthes from fish feeds

Commercial fish feeds that farmers use to feed their cultured fish were purchased from two fish feed millers in Bungoma County. The purchased fish feeds were transported to National Museums of Kenya Parasitology laboratory in Nairobi for parasitological analysis. In the laboratory, 0.5 g of fish feeds were washed into the sterile Bijou bottles with 3ml of 0.9% physiological saline and stirred using a mounted pin. Then 1ml of the mixed solution was collected using a dropper, placed on a slide, covered with a cover slip, and observations made using a compound light microscope at 100X - 400X magnification. Helminthes present were identified and counted.

3.5.2.6 Bacteria from fish feeds

The purchased fish feeds were subjected to bacterial analysis by taking 1g of each fish feed and homogenizing into 9 ml of distilled sterile water using a sterile blender. Serial dilutions of the homogenates were made and then subjected to TSA, Gram stain, TSI, catalase, methyl red, Voges-Proskauer, and hydrogen peroxide tests to enumerate and detect pathogenic bacteria as outlined for fish sample tests (Torimiro *et al.*, 2014).

3.5.3 Parasitic responses to antiparasitics and antimicrobials

3.5.3.1 Response of helminthes to antiparasitics

In this study, the antiparasitics that were applied against isolated pathogenic parasites included Formalin (36% formaldehyde), Potassium permanganate and Hydrogen peroxide. The antiparasitics were prepared according to manufacturers' instructions. They were administered to infected fish via bath treatment for external parasites and oral method for internal parasites (Stoskopf, 2015).

3.5.3.2 Response of bacteria to antimicrobials

The isolated and identified pathogenic bacteria that had been stored in double strength nutrient broth (added glycerol) were subcultured on nutrient agar and incubated for 24h at 30^0C. Thereafter, using a sterile wireloop, each bacterial strain was spread on Mueller-Hinton agar (Himedia) plate. Impregnated discs were placed on each prepared Mueller-Hinton agar (Himedia) plate containing bacterial strains and incubated for 24h at 30^0C. After 24h, the diameter of developed zones of inhibition for each disc were measured in millimetres (mm) using a plate ruler and recorded.

The diameter of zones of inhibition for each disc was classified for antimicrobial agent (Table 3.2) as resistant, intermediate or susceptible as per the break-points (CLSI, 2013).

The antimicrobials beta lactams (β-lactams) (ampicillin, cefepime, cefuroxime, cefotaxime and ceftazidime), quinolones and fluoroquinones (ciprofloxacin and nalidixic acid) and aminoglycosides (amikacin and gentamicin) (Himedia) were used in this study. Phenicols (chloramphenicols) folote pathway inhibitors (trimethoprim-sulfamethoxazole) were not used because are banned for use in animal production (Shaw *et al.,* 2014; Kikuvi *et al.*, 2002; Daniels *et al.,* 2000). All the antimicrobial agents were placed on discs at a concentration of 30 µg per disc.

Table 3. 2 : Break-points for selected antimicrobials used in this study.

Antimicrobial agent	**Symbol of agent**	**Mean responses level (mm)**		
		S (≥)	**I (=)**	**R (≤)**
Ampicillin	AX	17	14-19	13
Ceftazidime	CAZ	21	18-20	17
Cefuroxime	CPM	18	15-17	14
Cefotaxime	CTX	26	23-25	22
Cefepime	CXM	25	19-24	18
Ciprofloxacin	CIP	21	16-20	15
Nalidixic acid	NA	19	14-18	13
Amikacin	AK	17	15-16	14
Gentamicin	GEN	15	13-14	12

Source: Chemoquip Limited, Nairobi Kenya

3.5.4 Genomic analysis using Multiplex Polymerase Chain Reaction

The isolated bacteria (*Aeromonas hydrophila, Vibrio vulnificus, Vibrio parahaemolyticus, Pseudomonas aeruginosa* and *Streptococcus iniae*) that had been refrigerated were taken to the National Museums of Kenya Biodiversity Genetic Laboratory. The isolates were subjected to genomic analysis using self-designed oligonucleotide primer bla_{TEM} gene based on nucleotide sequence of the bla_{TEM} gene listed in National Centre for Biotechnology Information (NCBI) GenBank Database. The ampicillin resistant *Escherichia coli* sourced from Kenyatta University Microbiology Laboratory was used as a control. The *E. coli* is known to resist

ampicillin 100% due to presence of bla_{TEM} gene that is amplified at 424bp (Fatma-Azzahra, 2017).

3.5.4.1 Bacterial plasmid DNA extraction

For each pathogen, 5 ml Lysogeny Broth (LB) medium containing antibiotics (Table 3.3) were inoculated with every single bacterial colony. The tubes were incubated at 37 ˚C overnight with vigorous shaking at 360 rpm. The bacteria from the culture were pelleted at 10,000 x g for 5 minutes at room temperature. The supernatant was then discarded. Bacterial pellets were resuspended in a total of 1 ml ice-cooled solution I (50 mM). It was pipetted up and down/vortexed as necessary to fully resuspended the bacteria. Two (2) ml room temperature 0.2 N NaOH/1.0% SDS was added to the suspension and mixed thoroughly by repeated gentle inversion without vortexing. A 1.5 ml ice-cold Solution III was added to the lysate and mixed thoroughly by repeated gentle inversion without vortexing. It was then centrifuged at 15,500 x g for 30 min at 4^oC to recover resulting supernatant. Then 2.5 volume isopropanol was added to precipitate the plasmid DNA. It was mixed thoroughly by repeated gentle inversion without vortexing then centrifuged at 15,500 x g for 30 min at 4^oC for the removal of the resulting supernatant. The pellet which is plasmid DNA was rinsed in ice-cold 70% EtOH and air-dried for about 10 min to allow the EtOH to evaporate. The pellets were eluted in ddH_2O. 2ul RNase A (10mg/ml) was added to the mixture and incubated for 20 min at room temperature to remove RNA. The plasmid DNA was quantified spectrophotometrically and 0.8% agarose gel electrophoresis with ethidium bromide staining and photographed using a digital camera.

Table 3. 3: Concentration of bacterial plasmid DNA by spectrophotometer

Bacterial strain			**A260/A280**	**A260/230**	**Conc. (µg/mL)**
Vibrio parahaemolyticus	GE	1	1.91	1.04	122.4
		2	1.95	1.24	150.7
Vibrio vulnificus	SJ	1	2.03	0.92	70.1
		2	1.89	0.92	89.5
Aeromonas hydrophila	KA	1	1.77	0.79	98.4
		2	2.05	1.11	90.3
Pseudomonas aeruginosa	IH	1	2.47	1.00	44.0
		2	2.14	1.11	58.0
Streptococcus iniae	JLA	1	2.03	1.16	52.6
		2	1.79	1.09	111.1
Aeromonas hydrophila	GL	1	1.93	1.16	100.9
		2	2.11	1.03	106.7
Aeromonas hydrophila	GJ	1	1.57	0.99	59.8
		2	1.81	0.93	81.2
Vibrio vulnificus	SKF	1	1.61	1.19	113.6
		2	1.73	1.19	86.0
Escherichia coli	Positive control	1	1.59	0.79	41.8
		2	1.21	0.71	30.4

3.5.4.2 Protocols for molecular identification of *bla$_{TEM}$* gene

Molecular detection of *bla$_{TEM}$* gene was carried out with Cyclone 96 thermo cycler (ThermoFisher.com) at National Museums of Kenya Molecular Genetics Laboratory. Polymerase Chain Reaction (PCR) was performed using gene specific forward primers *bla$_{TEM-1a}$* F 5'- ATCTCAACAGCGGTAAGATCCT-3' and reverse primer *bla$_{TEM-1a}$* R5'- AGTTAATAGTTTGCGCAACGT-3' targeting the flanking regions of *bla$_{TEM-1a}$* gene (Githui *et al*., 2016). The cycling conditions consisted of 5 min of initial denaturation at 95°C, followed by 35 cycles consisting of 1 min denaturation at 94°C, 45 secs annealing at 54ºC with 1 min extension period at 72°C and a final extension for 10 min at 72ºC. The amplification products were visualized on a U.V illuminator after electrophoresis on a 1% agarose (SIGMA®) gel and ethidium bromide (SIGMA®) staining then photographed using a digital camera (CamScanner).

3.5.4.3 Purification (Gene cleaning)

Target DNA bands were excised from agarose gel using straight-edge razor blade and placed in different well labeled microcentrifuge tubes. Three volumes (1.2 ml for 0.4 g slice) NaI stock solution was added. It was then incubated at 56°C in a water bath for 15-30 min, mixing after every 5 min until all agarose had melted. Glassmilk stock solution was vortexed to mix well then 20 µl glassmilk was added to the microcentrifuge tubes (Eppendorf®). The tubes containing the mixtures were incubated for 15 min on ice, mixing every 3 to 5 min to keep glassmilk in suspension. The tubes were then microcentrifuged for 5 secs to pellet the glassmilk. The supernatant was removed and placed into new tubes. A 500 µl (10-50 volumes) New Wash (stored at –20ºC, Bio 101) was added to silica gel pellet and the pellets

resuspended. The tubes were then microcentrifuged for 10 secs, and the supernatant discarded. The wash was repeated two more times (3 washes in total). After discarding the supernatant from the last wash, the pellets were spinned for 10 secs; and the residual new wash removed by pipette. The pellets were then resuspended in 15 µl nuclease free water; incubated for 5 min at 45°C to 55°C in a water bath and microcentrifuged for 30 secs to pellet silica gel. The DNA-containing supernatant was then transferred to new tubes. The tubes were then spinned for a few secs to remove any residual glassmilk carried over with supernatant transfer. The supernatant was then transferred to new tubes. The presence of DNA in elute was confirmed through an agarose (SIGMA®) gel electrophoresis.

3.5.4.4 Sequencing of the bla_{TEM} gene

The PCR amplified and purified products were submitted to Inqaba biotech™ Laboratory in South Africa for sequencing. The generated sequences (Appendix VI) obtained were compared with reference sequences present in the GenBank database. The activity was by means of BLAST online programme (NCBI, 2013).

3.5.4.5 Phylogenetic and diversity analysis

The bla_{TEM} gene sequence (Appendix VII) derived from various bacterial strains (were combined with related sequences obtained from NCBI‟s nucleotide database. Sequences were aligned utilizing the Clustal-W program in BioEdit (Version 7.05) and the phylogenetic relationships inferred from the aligned nucleotide sequences by the Maximum Likelihood method at Boostrap 1000 replicates using Phylip program (Felsentein, 1993) as implemented in the MEGA6 version suite (Tamura *et al.*, 2013).

3.6 Data analysis

The water quality parameters average (means) readings were determined and the One-way Analysis of Variance (ANOVA) (for continuous variables) was used to determine the significant differences at 99% confidence levels. The total counts of helminthes and bacteria represented as colony forming units per Gram (cfu/g) and colony forming units per millilitre (cfu/ml) were converted to their respective logarithmic$_{10}$ representations. The data was entered in Micro Soft Excel (Windows 10) spreadsheet and analyzed using SPSS Software. Confidence levels of 99.99% were considered significant. Kruskal-Wallis test was used to determine occurrence of significant differences between and within the sub counties for bacteria counts (complete). The zones of inhibition of antimicrobials tests obtained were analyzed using Ordinal (for class/discontinuous data) Regression test for significant differences (Mariita *et al.*, 2010). Where significance was indicated, Post hoc Tukey test was used to separate the means. The genomic resistance gene in bacteria isolates was determined by use of bands and its molecular weights based on known standards (Sifuna, 2009).

CHAPTER FOUR

RESULTS

4.1 Water quality parameters

The fish ponds sampled in Bumula Sub County had the highest temperature ($\bar{x}$ =30.35^{0}C±1.77) followed by Bungoma East with mean of 29.85^{0}C±1.18. Bungoma South had mean pond temperature of 29.11^{0}C±3.28 and Bungoma West had a mean of 26.74^{0}C±3.79. Mt. Elgon Sub County ponds had mean temperature of 23.64^{0}C±2.24 while Bungoma North had mean temperature of 21.70^{0}C±1.32 (Table 4.1). It was found that there was significant difference in temperature of sampled fish ponds water (F=15.5; df= 5; P<0.001) (Appendix VIII).

Table 4. 1 : Mean values of water quality parameters of fish ponds

Sub County	**Farms**	**Temperature (^{0}C)** Mean±SE	**Dissolved Oxygen (mg/l)** Mean±SE	**Conductivity (µs/ cm)** Mean±SE	**pH** Mean±SE	**Turbidity (ppm)** Mean±SE	**Biological Oxygen Demand** Mean±SE
Bumula	10	30.35±1.77	2.80±0.40	88.20±21.41	6.72±0.57	15.00±4.42	8.27±4.19
Bungoma South	18	29.11±3.28	2.52±0.41	103.33±70.33	6.73±0.55	18.61±4.79	8.88±3.11
Bungoma West	25	26.74±3.79	3.04±0.82	83.64±18.60	6.63±0.34	15.40±4.25	8.74±2.30
Bungoma North	12	21.70±1.32	2.71±0.11	97.17±20.70	6.83±0.43	14.92±4.27	9.22±2.87
Mt. Elgon	5	23.64±2.24	2.85±0.21	91.00±23.97	6.71±0.52	17.40±3.97	8.81±0.81
Bungoma East	8	29.85±1.18	2.94±0.25	87.38±22.75	6.76±0.77	14.75±4.36	8.74±0.63

Mean temperature significantly different, $P<0.001$. Means separated using Tukey HSD test.

Dissolved oxygen levels in Bungoma West Sub County was the highest ($\bar{x}$ =3.04±0.82mg/l) Bungoma East had a mean of $\bar{x}$ =2.94±0.25mg/l with Mt. Elgon having a mean of 2.85±0.21mg/l. Bumula had a mean of 2.80±0.40mg/l, Bungoma North had dissolved oxygen of 2.71±0.11mg/l while Bungoma South had a mean of 2.52±0.41mg/l. Among the sub counties, there was no significant difference in dissolved oxygen (F=2.10; df= 5; P=0.081) (Appendix VIII).

Conductivity was highest ($\bar{x}$ =103.33±70.33µs/ cm) in Bungoma South followed by Bungoma North with a mean of 97.17±20.70µs/ cm, whereas Mt Elgon Sub County had a mean of 91.00±23.97µs/ cm. Further, Bumula had a mean of 88.20±21.41µs/ cm, with Bungoma East having a mean of 87.38±22.75µs/ cm and Bungoma West Sub County had a mean of, 83.64±18.60µs/ cm (Table 4.1). Further, the conductivity levels across the sub counties had no significant difference (F=0.631; df =5; *P*=0.677) (Appendix VIII).

The lowest pH ($\bar{x}$ =6.63±0.34) was in Bungoma West Sub County followed by Mt Elgon Sub County with a mean of 6.71±0.52), and Bumula had a mean of 6.72±0.57. Furthermore, Bungoma South had a mean of 6.73±0.55, Bungoma East a mean of 6.76±0.77 while highest mean of 6.83±0.43 was in Bungoma North Sub County. There was no significant difference in pH levels among the fish ponds water (F=0.27; df 5; *P*=0.928) (Appendix VIII).

Among the six sub counties, Bungoma South had the highest ($\bar{x}$ = 18.61±4.79ppm) turbidity levels, Mt. Elgon Sub County was found to have the second highest ($\bar{x}$ = 17.40±3.97ppm) turbidity then followed by Bungoma West with mean of 15.40±4.25ppm. Bumula Sub County had a mean of 15.00±4.42ppm, while Bungoma North had a mean of 14.92±4.27ppm and finally Bungoma East had a mean of 14.75±4.36ppm (Table 4.1). Besides, there was no significant difference in turbidity (F=1.83; df =5; *P*=0.0110) (Appendix VIII).

The Biological Oxygen Demand (BOD) mean for Bumula Sub County was =8.27±4.19mg/l, Bungoma South Sub County had the BOD mean of 8.8±3.11mg/l, while Bungoma West Sub County had the mean of 8.74±2.30mg/l. Further, Bungoma North Sub County recorded the highest mean of 9.22±2.87mg/l. Finally, Mt Elgon Sub County had the BOD mean of 8.81±0.81mg/l while Bungoma East Sub County had a mean of 8.74±0.63mg/l (Table 4.1). There was no significant difference in BOD levels (F=1.018; df 5; *P*=0.414) (Appendix VIII).

4.2 Parasites

4.2.1 Helminthes in Nile tilapia

For external helminthes, they were recovered from tail fin of fish samples from Bungoma West Sub County and Bungoma South Sub County. The recovered helminthes in Bungoma West had a mean of 2.0±0.89 and Bungoma South had a mean of 1.0±0.44 (Table 4.2). The recovered helminthes were *Acanthocephalus* spp. and *Pallisentis tetraodontis* in Bungoma West Sub County and one recovered from Bungoma South Sub County was *Pallisensis* spp. There was significance difference in the distribution of the helminthes in tail fins (H= 4; df 5; *P*<0.041) (Appendix X).

Table 4. 2: Mean number of helminthes isolated from intestines, tail fins, gills and stomach of Nile tilapia

Sub County	**Number of farms**	**Organ**	**Parasitic Species**	**Mean±SE**
Bungoma South	5	Intestine	*Acanthocephalus* spp.	2.50±1.09
	1	Tail fin	*Pallisentis* spp.	1.0±0.44
Bungoma West	2	Tail fin	*Acanthocephalus* spp.	2.0±0.89
			Pallisensis tetraodontis	2.0±0.89
	5	Gills	*Philometroides* spp.	2.50±0.47
	3	Stomach	*Philometroides* spp.	1.33±1.02
Bungoma North	3	Stomach	*Procamallanus* spp.	3.33±1.64

Helminthes were isolated from fish intestines in Bungoma South Sub County with a mean of 2.5±1.09. The isolated species waas *Acanthocephalus* spp. and there was significant difference in the distribution of the total helminthes in the intestines (H=4; df 5; $P<0.035$) (Appendix X).

In Bungoma West, there were helminth recovered from gills and stomach that had mean values of 2.5±0.47 and 1.33±1.02 respectively (Table 4.2). The helminth in was *Philometroides* spp. In Bungoma North Sub County, the helminth recovered from the stomach was *Procamallanus* spp. with a mean of 3.33±1.64 (Table 4.2). The *Philometroides* spp. in gills had no significance difference in the distribution

(Kruskal-Wallis (H=4 df=5; *P*= 0.484) (Appendix X). Besides, *Philometroides* spp. and *Procamallanus* spp. isolated from the stomach had no significance difference in distribution (H=4; df 5; *P*=0.469) (Appendix X).

4.2.2 Bacteria in Nile tilapia

4.2.2.1 Total viable aerobic colony counts

The total viable aerobic colony counts (TVACC) on scales among the six sub counties had Bungoma West Sub County with the highest mean of 121.76±224.66cfu/g, Bumula had mean of 119.50±114.10cfu/g, and Bungoma East had a mean of 98.87±41.84cfu/g. Bungoma North had a mean of 84.91±73.52cfu/g, Bungoma South with a mean of 49.55±39.10cfu/g, and finally Mt Elgon Sub County had the lowest mean of 42.40±7.16cfu/g (Table 4.3). There was no significant difference in the number of TVACC (H= 6; df 5; *P*=0.363) (Appendix XI).

Table 4. 3 : Mean numbers of total viable aerobic colony counts in Nile tilapia sampled from Bungoma County, Kenya

Sub County	**Farms**	**Mean (cfu/g) ±SE**						
		Scales	***Skin***	***Mouth***	***Gills***	***Intestine***	***Kidney***	***Liver***
Bumula	10	119.50±114.10	77.70±82.65	138.30±110.45	79.90±83.38	103.80±108.62	3.20±3.82	11.60±6.88
Bungoma South	18	49.55±39.10	70.05±76.07	93.83±99.88	73.16±86.55	88.55±100.03	7.44±9.31	10.77±15.22
Bungoma West	25	121.76±224.63	85.36±74.87	134.96±118.15	112.44±98.69	90.48±85.48	9.40±11.36	9.92±5.89
Bungoma North	12	84.91±73.52	86.08±75.08	72.41±75.51	166.50±108.73	69.00±95.83	10.41±10.56	8.50±7.74
Mt. Elgon	5	42.40±7.16	88.40±119.26	104.80±115.89	76.00±49.93	3.40±2.40	10.00±5.78	7.00±7.77
Bungoma East	8	98.87±41.84	57.75±35.04	103.00±87.58	81.00±59.50	85.00±90.83	13.00±17.29	9.00±5.89

On the skin, Bungoma East had the lowest mean of 57.75±35.04 cfu/g, followed by Bungoma South with a mean of 70.05±76.06cfu/g, and Bumula a mean of 77.70±82.65cfu/g. Bungoma West Sub County had the mean of 85.36±74.87cfu/g, Bungoma North with a mean of 86.08±75.08cfu/g while Mt Elgon Sub County had the highest mean of 88.40±119.26cfu/g (Table 4.3). Further, it was found that there was no significant difference on the distribution of TVACC in the skin (H= 7; df 5; *P*=0.067) (Appendix XI).

From the mouths, Bumula Sub County had the highest mean counts of 138.30±110.45cfu/g followed by Bungoma West Sub County with a mean of 138.30±118.15cfu/g, and Mt Elgon Sub County had a mean of 104.80±115.89cfu/g. Further, Bungoma East Sub County had a mean of 103.00±87.58cfu/g, Bungoma South Sub County had a mean of 93.83±99.88cfu/g and finally Bungoma North Sub County had the lowest mean count of 72.41±75.51cfu/g (Table 4.3). It was found that there was no significant difference in distribution of TVACC in the mouths (H=7; df 5; *P*=0.252) (Appendix XI).

In the gills, Bungoma North Sub County had the highest TVACC ($\bar{x}$ =166.50±108.73 cfu/g), followed by Bungoma West with a mean of 112.44±98.69cfu/g, and Bungoma East had a mean of 81.00±59.50cfu/g. Bumula had a mean of 79.90±83.38cfu/g, Mt Elgon Sub County recorded a mean of 76.00±49.93cfu/g and the lowest was in Bungoma South with a mean of 73.16±86.55cfu/g (Table 4.3). It was found that distribution of TVACC in gills was not significantly different (H=7; df =5; *P*=0.381) (Appendix XI).

For the intestines, Mt. Elgon Sub County had extremely low TVACC ($\bar{x}$ =3.40±2.40cfu/g), while Bungoma North Sub County had a mean of 69.00±95.83 cfu/g. Bungoma West had a mean of 90.48±85.48cfu/g, Bungoma East had a mean of 85.00±90.83cfu/g, Bungoma South with a mean of $\bar{x}$ = 88.55±100.03cfu/g, and Bumula had the highest mean of 103.80±108.62cfu/g (Table 4.3). It was found that there was no significant difference on the distribution of TVACC in the intestines (H= 7; df 5; *P*=0.307) (Appendix XI)

In kidneys, Bungoma East had the highest TVACC mean counts of 13.00±17.29cfu/g. It was followed by Bungoma North with a mean of 10.41±10.56cfu/g, and Mt Elgon Sub County had a mean of 10.00±5.78cfu/g. Bungoma West Sub County had a mean of 9.40±11.36cfu/g, with Bungoma South having a mean of 7.44±9.31cfu/g) and the lowest mean counts of 3.20±3.82cfu/g were recorded in Bumula Sub County (Table 4.3). Besides, it was found that the kidneys had significance difference in the distribution of TVACC (H=7; df 5; $P<0.015$) (Appendix XI).

The liver had the highest TVACC mean of 11.60±6.88cfu/g in Bumula Sub County, followed by Bungoma South with a mean of 10.77±15.22cfu/g, and Bungoma West with a mean of 9.92±5.89cfu/g. Bungoma East had a mean of 9.00±5.80cfu/g, Bungoma North had a mean of 8.50±7.74cfu/g and Mt Elgon Sub County had the lowest with a mean of 7.00±7.77cfu/g (Table 4.3). There was no significant difference in distribution of TVACC in the liver H=7; df 5; $P=0.903$) (Appendix XI).

4.2.2.2 Pathogenic Bacteria in Nile tilapia

The bacteria colonies on TCBS agar were; *Vibrio vulnificus* were yellow green, *Vibrio parahaemolyticus* were bluish with green centres, *Aeromonas hydrophila* were cream yellow/ yellow shin and *Pseudomonas aeruginosa* were deep green. Other biochemical tests results were as shown in Table 4.4 below.

Table 4. 4 : Biochemical tests confirmation for pathogenic bacteria isolated from Nile tilapia sampled from Bungoma County, Kenya.

Biochemical test	**Bacteria species**			
	Vibrio vulnificus	***Vibrio parahaemolyticus***	***Aeromonas hydrophila***	***Pseudomonas aeruginosa***
Gram stain	-ve	-ve	-ve	-ve
TSI	-ve	-ve	+ve	-ve
Catalase	+ve	+ve	+ve	+ve
Methyl red	-ve	+ve	+ve	-ve
Voges-Proskauer	-ve	-ve	+ve	-ve

There were no bacteria recovered from any of the organs of Nile tilapia sampled from farms in Bumula Sub County and Mt. Elgon Sub County. However, the other four sub counties (Bungoma South, Bungoma West, Bungoma North and Bungoma East) recorded presence of bacterial pathogens (Table 4.5).

Table 4. 5 : Mean number of pathogenic bacteria isolated from scales, skin, intestines and gills from Nile tilapia sampled from Bungoma County, Kenya.

Sub County	**Farms**	**Organ**	**Bacterial Species**	**Total colony counts**	**Mean (cfu/g) ±SE**
Bumula	0	0	0		0.00
Bungoma South	5	Scale	*Vibrio vulnificus*	10	2.00±1.19
Bungoma West	9	Skin	*Vibrio vulnificus*	38	4.22±2.51
	6	Intestine	*Pseudomonas aeruginosa*	6	1.00±0.50
Bungoma North	9	Gill	*Vibrio vulnificus*	27	3.00±6.99
	6	Gill	*Vibrio parahaemolyticus*	12	2.00±3.10
Mt Elgon	0	0	0		0.00
Bungoma East	5	Gill	*Aeromonas hydrophila*	3	0.60±0.51

Vibrio vulnificus was recovered from scales in Bungoma South with a mean of 2.00±1.19cfu/g (Table 4.5). There was no significant difference in the distribution of the *Vibrio vulnificus* (H=5; df 5; *P*=0.975). In Bungoma West, *Vibrio vulnificus* with a mean of 4.22±2.51cfu/g was isolated from skin while *Pseudomonas aeruginosa* with a mean of 1.00±0.50cfu/g was isolated from the intestines (Table 4.5). There was

no significant difference in their distribution (H=9/6; df 5; *P*=0.992). In Bungoma North Sub County, *Vibrio vulnificus* and *Vibrio parahaemolyticus* were isolated from gills with a mean of 3±6.99cfu/g and 2.00±3.10cfu/g respectively (Table 4.5). There was a significant difference in distributions of bacteria in gills (H=9/6; df 5; *P*< 0.001) (Appendix XII). Besides in Bungoma East, *Aeromonas hydrophila* were recovered from gills with a mean of 0.60±0.51cfu/g. There was a significant difference in distributions of bacteria in gills (H=5; df 5; *P*< 0.001) (Appendix XII).

4.2.3 Helminthes in pond water

The helminthic parasite, *Cleidodiscus* spp. (Appendix X) was recovered from pond water sampled from Bungoma North Sub County with a mean of 8.74±3.44.

4.2.4 Bacteria in pond water

4.2.4.1 Total viable aerobic coony counts

The total viable aerobic colony counts (TVACC) were present in all water samples from Bungoma County (Table 4.6). Water sampled from Mt Elgon had the highest TVACC ($\bar{x}$ = 189.00±152.02cfu/ml) followed by Bungoma North ($\bar{x}$ =141.75±114.02cfu/ml) while Bungoma West had mean of 128.44±115.64cfu/ml. Furthermore, Bumula, Bungoma South and Bungoma East Sub Counties had TVACC mean values of 100±cfu/ml, 60.30±87.70cfu/ml, 91.67±101.70cfu/ml and 92.50±88.62cfu/ml respectively. However, it was found that there was a significant difference in distribution of TVACC in fish ponds water (H=6; df 5; *P*< 0.041) (Appendix XIII).

Table 4. 6 : Mean numbers of total viable aerobic colony counts in pond water

Sub County	Farms	Mean (cfu/ml) ±SE
Bumula	10	60.30±87.70
Bungoma South	18	91.67±101.70
Bungoma West	25	128.44±115.64
Bungoma North	12	141.75±114.02
Mt. Elgon	5	189.00±152.02
Bungoma East	8	92.50±88.62

4.2.4.2 Pathogenic Bacteria in pond water

The bacteria recorded was *Aeromonas hydrophila*, whose colonies on TCBS agar were cream yellow/ yellow shin. Other biochemical tests results were as shown in table 4.7 below.

Table 4. 7 : Biochemical tests confirmation for bacteria (*Aeromonas hydrophila*) isolated from pond water

Biochemical test	Bacteria species *Aeromonas hydrophila*
Gram stain	-ve
TSI	+ve
Catalase	+ve
Methyl red	+ve
Voges-Proskauer	-ve

Aeromonas hydrophila was recovered in three sub counties namely, Bumula, Bungoma South and Bungoma West (Table 4.8). The other three sub counties; Bungoma North, Mt. Elgon and Bungoma East had no pathogenic bacteria recovered.

Table 4. 8 : Mean numbers of bacteria (Aeromonas hydrophila) isolated from pond water

Sub County	**Farms**	**Bacterial species**	**Mean(cfu/ml) ±SE**
Bumula	2	*Aeromonas hydrophila*	8.50±4.42
Bungoma South	9	*Aeromonas hydrophila*	2.80±2.50
Bungoma West	17	*Aeromonas hydrophila*	1.18±2.06

Findings showed that water samples from Bumula, Bungoma South and Bungoma West respectively had *Aeromonas hydrophila* with a mean of 8.50±4.42cfu/ml in Bumula Sub-County, followed by Bungoma South Sub-County with mean of 2.80±2.50cfu/ml counts, while Bungoma West Sub County had a mean of 1.18±2.06cfu/ml counts. Furthermore, it was found that there was significant difference in the distribution of *Aeromonas hydrophila* in fish ponds (H=3; df 5; $P<$ 0.016) (Appendix XIV).

4.2.5 Bacteria in fish feeds

4.2.5.1 Total viable aerobic colony counts in fish feeds

At the time of this study, there were only two fish feed millers operating in Bungoma County. The two millers were each located in different sub counties; Bungoma South and Bungoma West Sub Counties. The fish feed miller in Bungoma South Sub County had a TVACC mean of 122±86.26cfu/g whereas the one in Bungoma West Sub County had a mean of 97±68.58cfu/g TVACC.

4.2.5.2 Pathogenic bacteria present in fish feeds

Streptococcus iniae on TCBS agar were grayish. Other biochemical tests results were as shown in table 4.9 below.

Table 4. 9 : Biochemical tests confirmation for pathogenic bacteria (*Streptococcus iniae*) isolated from fish feeds

Biochemical test	**Bacteria species** *Streptococcus iniae*
Gram stain	+ve
TSI	+ve
Catalase	+ve
Methyl red	+ve
Voges-Proskauer	-ve

4.2.6 Control of helminthes in Nile tilapia

The antiparasitics were administered to infected fish via bath treatment for external parasites and oral method for internal parasites. Formalin and hydrogen peroxide were used to treat external parasites isolated in the current study whereas, Potassium permanganate were used against internal parasites. The external parasites were eliminated by formalin (36% formaldehyde) at concentration of 25ppm for 1h and Hydrogen peroxide at 75ppm for 30 min (Table 4.10).

Table 4. 10 : Antiparasitics recommended dosage and clearance time

Antiparasitics	**Optimal dose**	**Time frame**
Formalin	25 ppm	1 h
Hydrogen peroxide	75 ppm	30 min
Potassium permanganate	1 mg/l	4 h

4.2.7 Sensitivity tests of bacteria

4.2.7.1 Sensitivity test of bacteria isolated from Nile tilapia

The four bacterial strains recovered from Nile tilapia had different zones of inhibition value ranges (Fig. 4.1). *Vibrio vulnificus* isolated from scales samples from ponds in Bungoma South had diameter ranges of 22mm to 35mm while *Vibrio vulnificus* recovered from skin samples from Bungoma West had diameter ranges of 19mm to 30mm. *Vibrio parahaemolyticus* isolated from gills from Bungoma North was found resistant to ampicillin and cefuroxime with a diameter range of 0 mm. *Aeromonas hydrophila* isolated from gills samples from Bungoma East Sub County was susceptible to amikacin, ceftazidime, cefepime, cefuroxime and nalidixic acid with diameter range from 22mm to 26mm. However, *Aeromonas hydrophila* isolated from gills from Bungoma South Sub County was susceptible to amikacin, ceftazidime, ciprofloxacin, cefepime and gentamicin with higher diameter ranges of 24mm to 32mm. *Pseudomonas aeruginosa* isolated from intestine samples from Bungoma West Sub County was susceptible to amikacin, ceftazidime, ciprofloxacin, cefepime, cefotaxime, gentamicin and nalidixic acid with diameter ranges of 11mm to 35mm.

Overall, it was found that there was no significant difference in sensitivity tests among the bacteria isolated from Nile tilapia (OLR=11.061; df= 24; *P*=0.087) (Appendix XV). Further, there was no significant difference in sensitivity tests among the bacteria isolated from Nile tilapia (OLR=6.136; df= 16; *P*=0.189) (Appendix XVI).

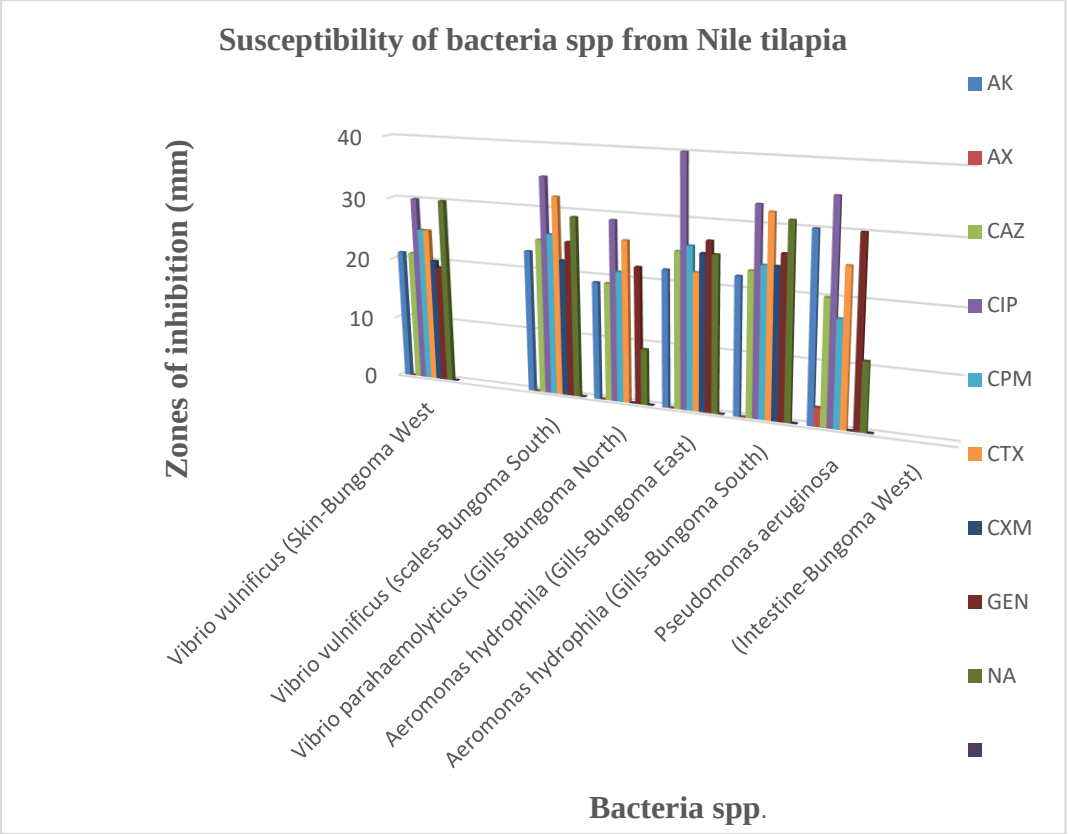

Figure 4. 1: Sensitivity tests for pathogenic bacteria isolated from Nile tilapia against selected antimicrobial agents.
AK-Amikacin, AX-Ampicillin, CAZ-Ceftazidime, CIP-Ciprofloxacin, CXM-Cefuroxime, CTX- Cefotaxime, CPM-Cefepime, GEN-Gentamicin, and NA-Nalidixic acid

4.2.7.2 Sensitivity test of bacteria isolated from pond water

Aeromonas hydrophila was isolated from pond water sampled from Bumula, Bungoma South and Bungoma West Sub Counties (Fig. 4.2). *Aeromonas hydrophila* from ponds water in Bumula Sub County had diameter of zones of inhibition from 51mm to 38mm. *Aeromonas hydrophila* from pond water in Bungoma South was found to have zones of inhibition diameter ranging from 0 mm to 28mm. On the other hand, in Bungoma West, *Aeromonas hydrophila* had zones of inhibition diameter from 0mm to 34mm.

The bacterial isolates were found to be significantly different in sensitivity tests (OLR=44.455; df 16; *P*<0.00) (Appendix XVII). However, among the sub counties, the bacterial isolates were not significantly different in sensitivity tests (OLR=6.629; df 16; *P*=0.357) (Appendix XVIII).

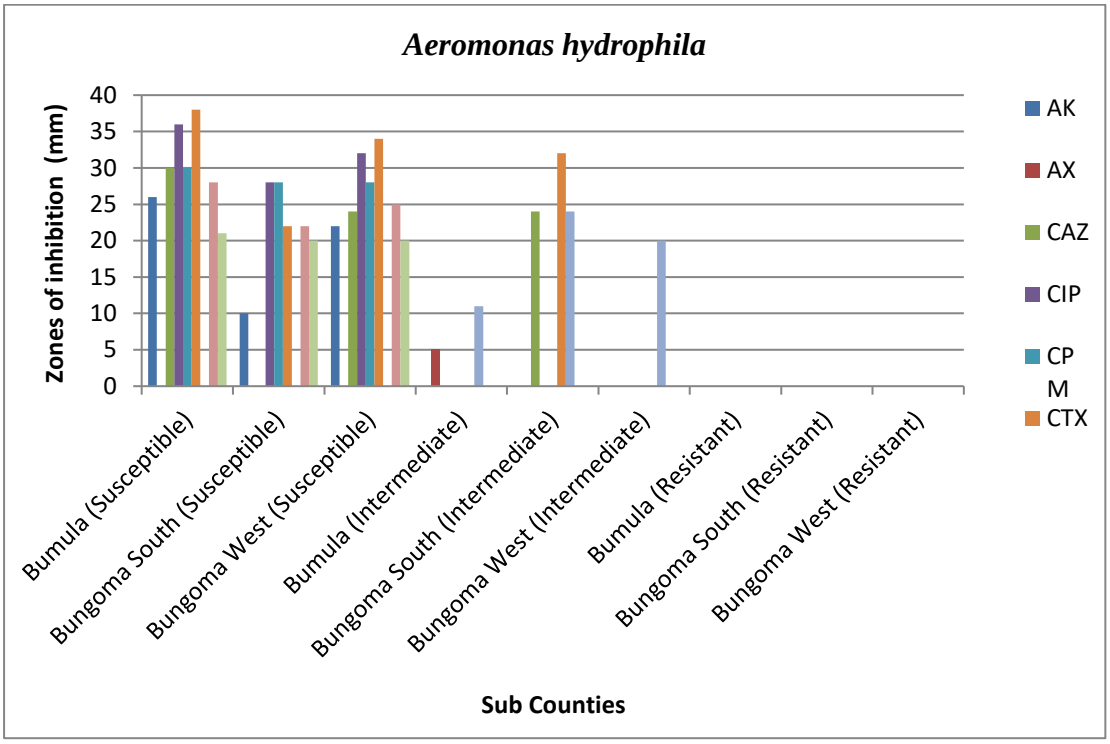

Figure 4. 2: Sensitivity tests for pathogenic bacteria isolated from pond water against selected antimicrobial agents.
AK-Amikacin, AX-Ampicillin, CAZ-Ceftazidime, CIP-Ciprofloxacin, CXM-Cefuroxime, CTX- Cefotaxime, CPM-Cefepime, GEN-Gentamicin, and NA-Nalidixic acid

4.2.7.3 Responses of pathogenic bacteria present in fish feeds

Streptococcus iniae isolated from fish feeds sourced from Bungoma West Sub County had varying sensitivity levels towards the tested antibacterial agents (Fig. 4.3). It was found to have zones of inhibition diameter ranges from 0mm to 30mm.

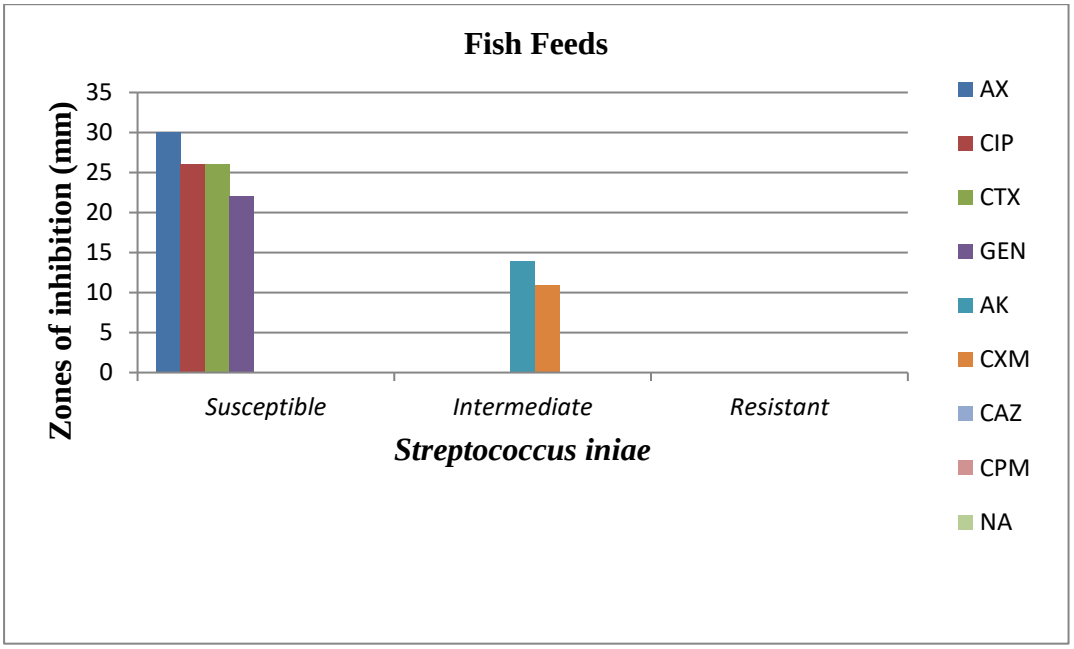

Figure 4. 3: Sensitivity tests of bacteria (*Streptococcus iniae*) isolated from fish feeds in Bungoma West Sub County against selected antibacterials

AK-Amikacin, AX-Ampicillin, CAZ-Ceftazidime, CIP-Ciprofloxacin, CXM-Cefuroxime, CTX- Cefotaxime, CPM-Cefepime, GEN-Gentamicin, and NA-Nalidixic acid

4.2.8 Genopic resistance gene *bla$_{TEM}$* gene

The *bla$_{TEM}$* gene was present in all the five pathogenic bacteria isolated from Nile tilapia, pond water and fish feeds. The *bla$_{TEM}$* gene was amplified about 424bp (Fig. 4.4). Further, nucleotide sequences showed 100% sequences identity with the *bla$_{TEM}$* gene (GenBank Accession Numbers: BankIt2236899 Seq1a MN114035, BankIt2236899 Seq1b MN114036, BankIt2236899 Seq2a MN114037, BankIt2236899 Seq2b MN114038, BankIt2236899 Seq3a MN114039, BankIt2236899 Seq3b MN114040, BankIt2236899 Seq4a MN114041, BankIt2236899 Seq4b MN114042, BankIt2236899 Seq5a MN114043, BankIt2236899 Seq5b MN114044, BankIt2236899 Seq6a MN114045, BankIt2236899 Seq6b MN114046, BankIt2236899 Seq7a MN114047,

BankIt2236899 Seq7b MN114048, BankIt2236899 Seq8a MN114049, BankIt2236899 Seq8b MN114050, BankIt2236899 Seq9a MN114051, and BankIt2236899 Seq9b MN114052).

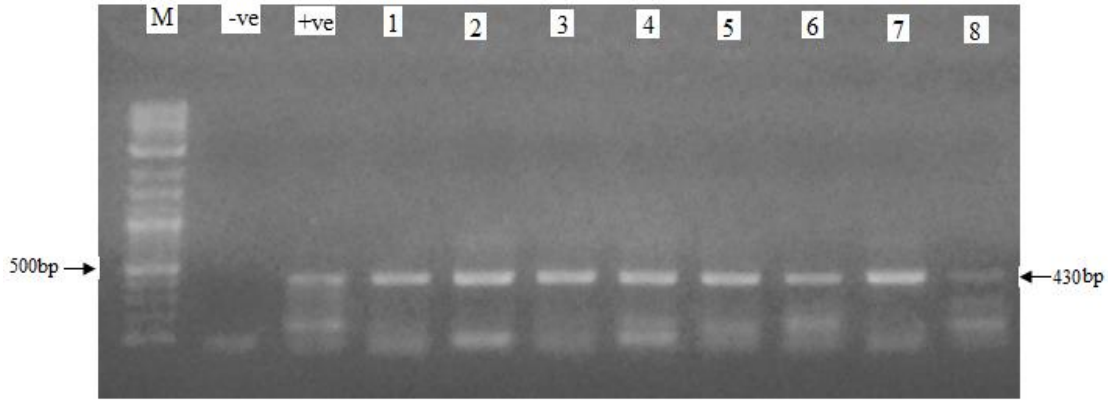

Figure 4. 4: Molecular detection of presence of bla_{TEM} gene in bacteria isolated from Nile tilapia, pond water and fish feeds

Molecular detection of presence of *bla*$_{TEM}$; M; 100bp ladder, Negative control, Positive control-*Escherichia coli*, lane[1]*Vibrio parahaemolyticus* isolated from gills in Bungoma North; lane[2]*Vibrio vulnificus* isolated from fish scales in Bungoma South; lane[3]*Aeromonas hydrophila* isolated from pond water in Bungoma South; lane[4]*Pseudomonas aeruginosa* isolated from fish intestines in Bungoma West;lane[5]*Streptococcus iniae* isolated from fish feeds in Bungoma West; lane[6]*Aeromonas hydrophila* isolated from gills in Bungoma East; lane[7]*Aeromonas hydrophila* isolated from gills in Bungoma South; and lane[8]*Vibrio vulnificus* isolated from fish skin in Bungoma West; mag. (0.01x 0.02)

The phylogenic tree with the highest log likelihood (-560.5035) (Fig. 4.5) and the percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 373 positions in the final dataset.

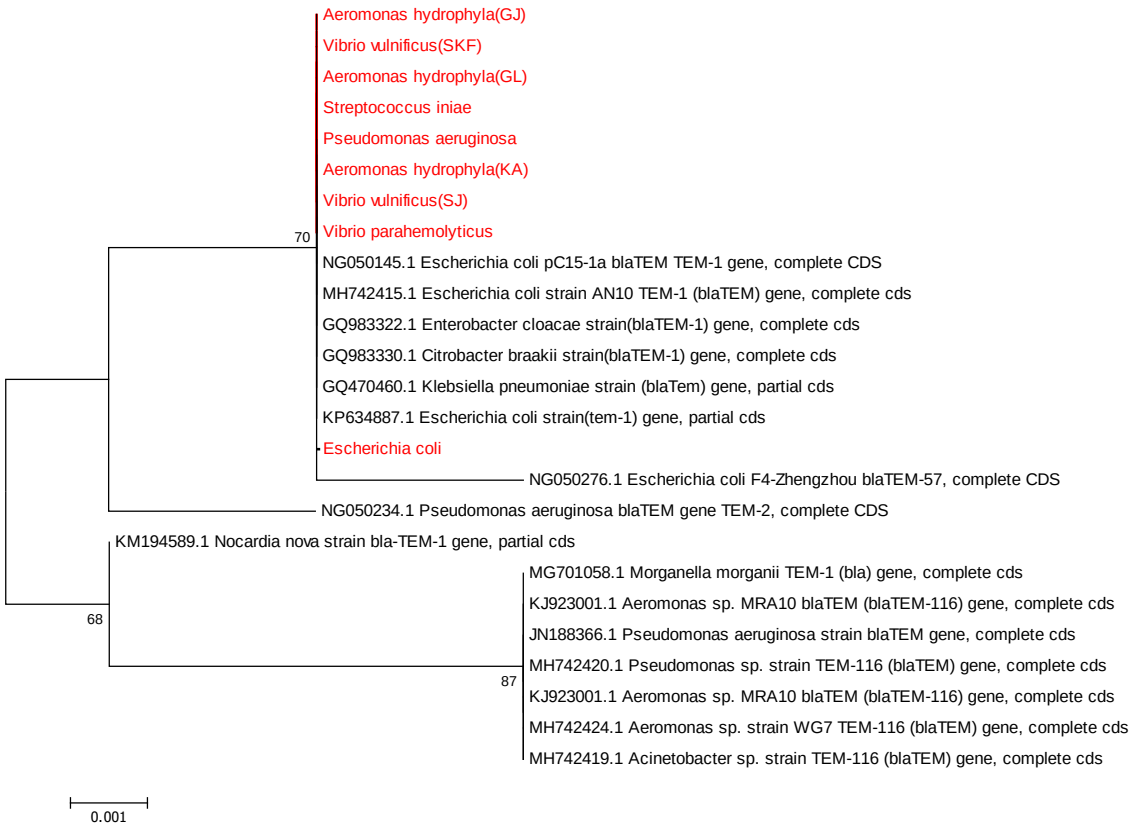

Figure 4.5: Phylogenetic tree of the extracted bacterial strains together with other strains with *bla*$_{TEM}$; gene indicating similarities to other strains

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Water quality parameters

The varying mean temperature ranges reported in the current study could be due to different levels of altitude and agro-ecological zones. The required temperature in Kenya for farming fresh warm water fish is 22-32^{0}C and 14-17^{0}C for cold water fish (MALF, 2015). Other studies have recommended temperature ranges from 15^{0}C-35^{0}C (Anita and Pooja, 2013). This implies that as much as each sub county had a different temperature ranges, the ranges were within recommended levels for fish ponds water without predisposing farmed Nile tilapia to parasitic invasions.

The highest mean value of dissolved oxygen in Bungoma West could imply minimal contamination of fish ponds water in the Sub County. However, dissolved oxygen levels (less than 3 mg/l) in other sub counties could be attributed to pollution through runoffs during rainy season, floating artificial fish feeds that farmers use to feed their fish and manure applied by farmers to fertilize the fish ponds before restocking.

Conductivity of fish ponds is depended on soil composition (Rusell *et al.*, 2011) and therefore, the high levels of conductivity ($\bar{x}$ =103.33±70.33µs/ cm) recorded in Bungoma South Sub County could be attributed to key economic activity of sugarcane production. The sugarcane production is highly depended on inorganic

fertilizer whose ions would be washed into the fish ponds water. The conductivity ranges are consistent with previous studies that showed values more than 100 µs / cm (Crane, 2006) and recommended ranges of between 100 and 2000 µs/ cm (Stone *et al*., 2013). Tough other five sub counties recorded conductivity values of less than 100 µs / cm, still the values were within ranges documented by other studies with values of 9-400 µs / cm in delta region, Nigeria (Njoku *et al*., 2015) and recommended ranges for fish ponds water from 20 to 1500 µs / cm (Boyd, 1990).

The lower pH levels in Bungoma West and Bumula could be due to acidic soils characteristic of the two sub counties adjacent to each other and whose farmers rely on inorganic fertilizers for sugarcane production. During runoffs, the fertilizer remnants could be washed into fish ponds increasing pH values. Again, the farmers in the two sub counties could be using the inorganic fertilizers instead of manure in fertilizing their fish pond. In Kenya, the recommended pH values are 6.5 to 8.5 for both cold and warm water fish ponds water (MALF, 2015). Therefore, the pH ranges of 6.63±0.34 - 6.83±0.43 in Bungoma County are consistent with the national recommended levels. Nevertheless, in Nigeria's Niger delta region, the pH values ranged from 6.5 to 9.5 for fish ponds sampled (Njoku *et al*., 2015) while other recommendations for pH for fish ponds water are in a range of 4 to 11, (Anita and Pooja, 2013; Bryan *et al*, 2011; Ntengwe and Edema, 2008; Mohammed, 2005). This further implies that the pH in Bungoma County fish ponds water is consistent with other studies.

Turbidity levels in Bungoma County during the study ranged from 14.75±4.36ppm to 18.61±4.79ppm being less than the recommended levels in the Country at 30-45ppm for freshwater fish ponds (MALF, 2015). The variation is also consistent with other documented recommendations of 30-40 ppm (Santhosh and Singh, 2007) and 30-80 ppm and (Bhatnagar *et al*., 2004). This could arise from the fact that farmers were put more manure and/ or inorganic fertilizer in fish ponds than recommended because all fish ponds are normally manured before stocking and restockings are done. The recommended amount of manure is 20g/m^2 while inorganic fertilizer is 2-3g/m^2 (MALF, 2015). However, the lowest turbidity level of 14.75±4.36ppm recorded in Bungoma East Sub County is still above the reported level of less than 12 ppm that may cause stress in farmed fish (Bhatnagar *et al*., 2004).

The highest Biological Oxygen Demand ($\bar{x}$ =9.22±2.87mg/l) value in Bungoma North could linked to organic materials that reach the Sub County via streams emanating from Mt Elgon and Cherangani Hills through River Nzoia all tranversing the Sub County. During the rainy season, the organic materials could be washed into fish ponds via runoffs. Normally, high BOD is an indicator of organic pollutants from food wastes and sewage flow that have high nutrient loading (Divya, 2018). The high levels of BOD minimize dissolved oxygen levels in fish ponds thereby creating stressful conditions. However, the recorded BOD values in the current study were within recommended levels for fish ponds water. The recommendation being values not more than 10.0 mg/l (Ekubo and Abowei, 2011).

5.1.2 Pathogenic parasites

In the current study, the three acanthocephalins were recovered from the external orgn, the tail fin. However, the three helminthic species *Pallisentis* t*etraodontis, Pallisentis* spp. and *Acanthocephalus* spp. are mostly isolated from gastrointestinal organs (Gautam *et al*., 2018; Gupta and Ramakant, 2015; Purivirojkul and Nontawith, 2008). That implies that may be tail fins picked up the parasites while swiming from the excreta. Alternatively, the contrast could be that in the current study, fish samples were drawn from fish ponds while earlier studies relied on wild fish samples.

The recorded *Acanthocephalus* spp. consistently mirrors what has been documented in other studies. *Acanthocephalus* spp. was found in the intestines of farmed Nile tilapia in Tetu Sub County in Nyeri, Kenya (Mavuti *et al.,* 2017) and Kikuyu Sub County in Kiambu County (Maina *et al.,* 2017). Furthermore, *Oreochromis niloticus* sampled from Lake Victoria in Homa Bay County (Kamundia, 2011) and River Tana (Gichohi, 2010) was found infested with *Acanthocephalus* spp. Regionally, *Acanthosentis tilapiae* was reported in Bahr Youssef and Fayoum fish farms in Egypt (Dayhoum, 2003). This implies that acanthocephalans are widely distributed and not limited to one region.

The presence of nematodes namely *Philometroides* spp. and *Procamallanus* spp. in farmed tilapia implies that intermediate hosts (copepods) for the nematodes exist in the farms in which fish were harvested. This is because nematodes require at least one intermediate host before it infects fish (Paperna, 1980). Alternatively, it could be that the fingerlings that were stocked already had larval stages of the nematodes and

therefore, the source of contamination was the fish hatcheries and farms where fingerlings had been sourced. The recoveries of nematodes in this study is consistent with the occurrence of nematodes in other counties in the Country. Nematodes have been documented in Sangoro (Kisii County) and Sagana fish farms (Kirinyaga County) that are located in Lake Victoria basin and Mt Kenya highland regions respectively (BOMOSA, 2009).

The helminthic parasite, *Cleidodiscus* spp. recovered from pond water sampled from Bungoma North Sub County could have originated from contaminated run offs where the parasites could have found their way into rivers passing through the sub county. The rivers in the county are the main source of water supply for fish ponds. The presence of *Cleidodiscus* spp. in pond water is in concurrence with other reports showing that it is a fresh water parasite in tropical regions (Noga, 2000). In the current study, the parasite was not recovered from sampled fish. This could imply that the parasite had not multiplied heavily in order to be swallowed by fish. It is known to infest gills and nasal cavities of fish though *Cleidodiscus* spp. is endoparasitic (Hoffman, 1998). Therefore, in conditions that favour the parasite to multiply; fish in fish ponds where it was recovered could be infected.

Determination of TVACC is very important to assess the extent of contamination in fish and aquatic environment. This is because high TVACC is normally due to either surface contamination or because of phenomenal growth of bacteria already present in farmed fish. According to International Commission on Microbiological Specifications for Foods (ICMSF), the TVACC in fish should be less than $5x10^5$

(ICMSF, 2007; ICMSF, 1986). Generally, the observed TVACC ($3.4x10^0$-$1.67x10^2$cfu/g) in Nile tilapia fall within the recommended levels by ICMSF implying less contamination levels in the fish farming systems. Further, TVACC ranges of 10^2 - 10^7 cfu/g for tropical fish species in the wild had previously been documented (Davaraju and Setty, 1985; Liston, 1980). Besides, catfish sampled from fish farms in Kano State, Nigeria had mean TVACC of $1.26x10^4$cfu/g. However, *Tilapia mossambicus* and *Tilapia rendali* sampled from Fletcher Dam in Gweru, Zimbabwe had TVACC ranges between $8.60x10^6$ and $25.60x10^6$cfu/g (Sichewo *et al*., 2013). The higher TVACC counts in dams could be due to huge runoffs from sources of water supply to the dam unlike the low levels of water supply to fish in the current study farms. The current study found mean TVACC of $6.03x10^1$-$1.89x10^2$cfu/ml in pond water. The higher counts could be due to huge runoffs from sources of water supply to the fish ponds during rain season. Also, most fish ponds are fed by rivers and streams and could be a source of contamination.

Pseudomonas aeruginosa was isolated from gills; it had also been recovered from gills, skin and intestines of fish in previous studies (Lerma *et al*., 2014; Tripathy *et al*., 2007). The occurrence could be attributed to uptake from pond water sediment during feeding and breeding. The species was documented as present in Nile tilapia culture pond sediment in Hai Duong Province in Vietnam (Huong *et al.,* 2014). *Pseudomonas aeruginosa* is an opportunistic pathogen in fish farms and causes septicemia under strenuous conditions of the fish ponds (Lerma *et al*., 2014; Austin and Austin, 2007). Furthermore, in fish farming, the method of harvesting (which usually involves the dragging of a seine net on the bottom of the pond) may contaminate the fish with mud and silt which may contain microorganisms (Cowx, 1992; Sikorki *et al*., 1990). Some

othe species namely *Pseudomonas aeruginosa* when present in food, they get into the body and then release endotoxins which irritate the stomach and the bowels (Huss, 1994; Bryan, 1980). Therefore, its presence indicates that contamination of harvested fish and if not well handled may cause food poisoning. Besides, when fish has been harvested and exposed to high temperature, the it may cause spoilage threby reducing quality (Gram, 1990).

Aeromonas hydrophila from pond water in adjacent sub counties of Bumula, Bungoma South and Bungoma West Sub Counties in the present study could be attributed to cross contamination from source of pond water supply. This is because during rainy season, runoffs could spread the bacteria. This finding is consistent with reported occurrence of *Aeromonas* spp. present in water drawn from concrete ponds in delta region in Nigeria (Njoku *et al.,* 2015).

The *Streptococcus iniae* was isolated from fish feeds with a mean of 12±8.48cfu/g from Bungoma West Sub County whereas the feeds from Bungoma South Sub County returned negative results. The fish feeds could be a source of *Streptococcus iniae* contamination for fish ponds during feeding of the farmed fish. Feeds are known to contaminate fish ponds (Okpokwasili and Ogbulie, 1999). The bacterium is known to cause streptococcosis in tilapia (Zadeh *et al*., 2012; Caroll *et al*., 2007; Wardman *et al*., 2007).

5.1.3 Parasitic responses to antiparasitics and antimicrobials

Formalin is normally applied for treatment of trematodes like *Cleidodiscus* spp. where fish is immersed in 30,000mg/l to 70,000mgl solution for 5-10 min (Stoskopf, 2015). It has been documented that antiparasitics are mixed with pond water; the treatment is effective against external infestations (Yanong, 2017). In this study, internal parasites were cleared by Potassium permanganate at a concentration of 1mg/l for 4h. In other studies, Potassium permanganate was effective against endoparasites (Flores-Crespo *et al.,* 1995).

Vibrio vulnificus isolated from scales samples from ponds in Bungoma South while *Vibrio vulnificus* recovered from skin samples from Bungoma West were found to be susceptible to all tested antimicrobials except ampicillin. However, *Vibrio vulnificus* isolated from oysters of Louisiana Gulf in USA were found susceptible to ampicillin (Han *et al.,* 2007) probably because Louisisna Gulf is a wild environment while the current study focused on controlled aquatic system. Further, *Vibrio vulnificus* was isolated from German coastal (wild environment) waters were found susceptible to ampicillin (Nadja *et al.,* 2015).

Vibrio parahaemolyticus isolated from gills from Bungoma North was found resistant to ampicillin and cefuroxime both agents but susceptible to amikacin, ceftazidime, ciprofloxacin, cefotaxime, cefepime, gentamicin and nalidixic acid with zones of inhibition diameter ranges between 9mm and 29mm. Other studies have also shown *Vibrio parahaemolyticus* recovered from shellfish in Selangor in Malaysia being resistant to ampicillin (Letchumanan *et al.,* 2015). This implies that *V.*

parahaemolyticus irrespective of the nature of aquatic environment, it had developed resistance against ampicillin. On the contrary, it was observed that *V. parahaemolyticus* isolated from Korean seafood to be resistant to cefotaxime and ceftazidime (Jun *et al.*, 2012).

Aeromonas hydrophila isolated from gills samples from Bungoma East Sub County was susceptible to amikacin, ceftazidime, cefepime, cefuroxime and nalidixic. However, *Aeromonas hydrophila* isolated from gills from Bungoma South Sub County was susceptible to amikacin, ceftazidime, ciprofloxacin, cefepime and gentamicin. *Aeromonas hydrophila* recovered from fish and crabs in Western Australia were susceptible to amikacin, cefepime, ciprofloxacin, gentamicin, ceftazidime and nalidixic acid (Aravena-Roman *et al.*, 2012). This implies that the isolated *Aeromonas hydrophila* strains in the current study and that from Western Australia have not developed resistance against the aforementioned antibacterial agents. At the same time, *A. hydrophila* isolated in Bungoma East and Bungoma South sub counties were moderately susceptible to ciprofloxacin, cefotaxime as well as gentamicin and cefotaxime, cefuroxime and nalidixic acid respectively. This implies that the strain could be possessing geographically different genes that confer mild resistance against antibacterial agents selected in this study.

Pseudomonas aeruginosa isolated from intestine samples from Bungoma West Sub County was susceptible to amikacin, ceftazidime, ciprofloxacin, cefepime, cefotaxime, gentamicin and nalidixic acid. But *Pseudomonas aeruginosa* was found resistant to ampicillin and cefuroxime. Uniquely, the isolates had resistance radia at 3

mm against ampicillin unlike other isolated bacterial pathogens in this study that had radia at 0mm against ampicillin. This implies that *Pseudomonas aeruginosa* is sparingly resistant to ampicillin compared to other recovered bacterial isolates. *Pseudomonas aeruginosa* isolated from Armenian fish farms was also found resistant to ampicillin. This could indicate probability of *Pseudomonas aeruginosa* having developed ampicillin resistant genes (Ginovyan *et al*., 2017).

Aeromonas hydrophila isolated from water in the current study is consistent with other studies on its resistance to ampicillin. Locally the bacterium has been recovered from River Njoro in Nakuru County (wild aquatic environment) and found to be resistant to ampicillin, gentamicin and ceftazoxime (Njeru *et al*., 2012). Besides, *Aeromonas hydrophila* isolated from wastewater in Eastern Cape Province, South Africa were resistant to ampicillin (Okoh and Igbinosa, 2012). Also, *Aeromonas hydrophila* isolated from sea sediments in Melaka in Malaysia were resistant to ampicillin (Olumide and Ahmad, 2017). This could mean that *Aeromonas hydrophila* has developed tolerance against ampicillin over time being an autochthonous bacterium.

Streptococcus iniae recovered from fish feeds in this study were resistant to ceftazidime, cefepime and nalidixic acid but were intermediate to amikacin and cefuroxime. The strain was susceptible to ampicillin, ciprofloxacin, cefotaxime and gentamicin. *Streptococcus iniae* obtained from fish farms in Jeju Island, Korea were documented as being susceptible to cefotaxime (Park *et al*., 2009). This implies that

though the *Streptococcus iniae* were isolated from different geographical areas, they probably possess similar sensitivity mechanisms against cefotaxime.

5.1.4 Genomic resistance of pathogenic bacteria

The plasmid-mediated bla_{TEM} gene was isolated from bacteria recovered from Nile tilapia, pond water and fish feeds. The recovery of bla_{TEM} gene in the five bacterial pathogens demonstrates that antimicrobial resistance was due to its presence. Further, the bla_{TEM} gene was isolated from plasmids meaning antibacterial resistance was plasmid-mediated. This observation concurs with other studies that had shown presence of bla_{TEM} gene in *Aeromonas hydrophila*. The bla_{TEM} gene was present in *Aeromonas hydrophila* recovered from River Njoro, Nakuru in Kenya (Njeru *et al*., 2012) and in *Aeromonas hydrophila* isolated from wastewater samples in South Africa (Okoh and Igbinosa, 2012). Besides, the bla_{TEM} were detected in fish and water sampled from brackish-water fish farms in Damietta and Kafr El-Sheikh along the Mediterranean Sea in Egypt (Yojiro *et al*., 2010). Most of the plasmids in many species of bacteria have been associated with resistance to multiple antibiotics (Aoki, 1992). Cefuroxime resistance by bacteria is due to existence of the resistant gene bla_{TEM} (Devarajan *et al*., 2017). In *Pseudomonas* spp., other than bla_{TEM-1}, *blaPSE* gene also confers resistance against antibacterial agents (Jiang *et al*., 2014). The bla_{TEM} gene was also present in *Aeromonas hydrophila* isolated from wild water in Brazil (Balsalobre *et al*., 2010). However, another study disagrees with this study as it had observed that bacterial pathogens including *Aeromonas hydrophila* resistance against antimicrobials is chromosomally mediated (Fosse, 2004).

In the current study, it was found that *Streptococcus iniae* was phenotypically resistant to ciprofloxacin, ceftazidime and nalidixic acid. The recovery of bla_{TEM} gene from *Pseudomonas aeruginosa* in the current study is in concurrence with earlier reports (Jog *et al.,* 2013). Previous studies have shown that *Pseudomonas aeruginosa* have TEM (Temoneira) (Jog *et al.,* 2013).

5.2 Conclusions

i. This study found that temperature varied among the six sub counties.

ii. The fish farming systems in Bungoma County were found to harbour helminthic parasites; *Pallisentis* spp, *Pallisentis tetraodontis*, *Philometroides* spp, *Procamallanus* spp, *Acanthocephalus* spp and *Cleidodiscus* spp. Further, pathogenic bacteria namely; *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Streptococcus iniae* were found present in the farming systems.

iii. The helmunthic parasites were susceptible to to tested antiparasitic drugs. On the other hand, isolated pathogenic were resistant to one or more antimicrobials.

iv. The bla_{TEM} gene was present in *Vibrio parahaemolyticus*, *Aeromonas hydrophila, Pseudomonas aeruginosa* and *Streptococcus iniae*.

5.3 Recommendations

i. The fish farming systems management to monitor water quality parameters so that the current levels are maintained.

ii. Formalin and Hydrogen peroxide as well as Potassium permanganate are viable at recommended doses for clearing parasites that infest Nile tilapia.

iii. That ampicillin should not be used to treat farmed Nile tilapia as it was found to be resisted by *Vibrio vulnificus, Vibrio parahaemolyticus, Aeromonas hydrophila, Pseudomonas aeruginosa* and *Streptococcus iniae* during the study.

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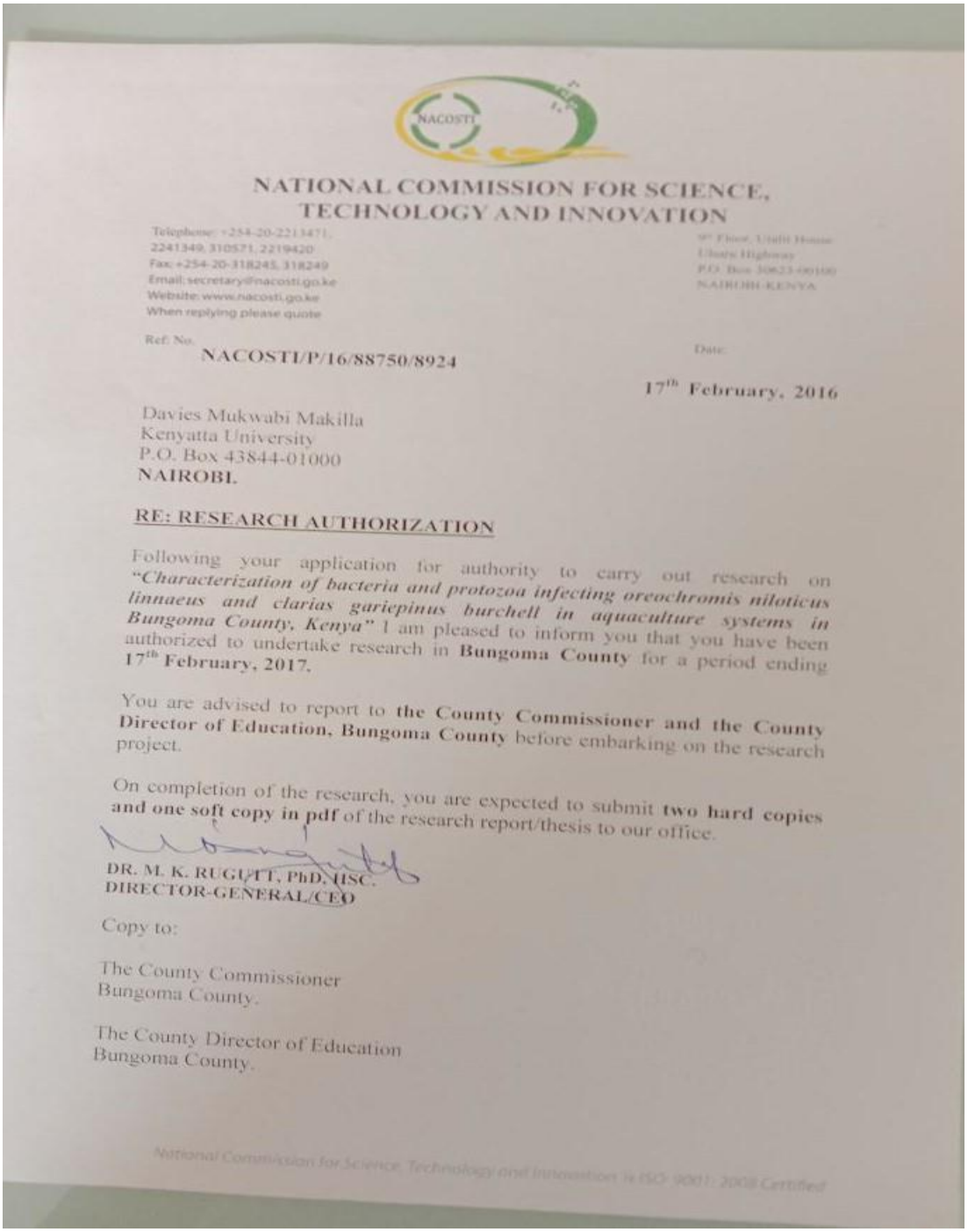
APPENDICES

Appendix I : Research authorization certificates



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NAIROBI-KENYA

Ref: No. NACOSTI/P/16/88750/8924

Date: 17th February, 2016

Davies Mukwabi Makilla
Kenyatta University
P.O. Box 43844-01000
NAIROBI.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Characterization of bacteria and protozoa infecting oreochromis niloticus linnaeus and clarias gariepinus burchell in aquaculture systems in Bungoma County, Kenya"*** I am pleased to inform you that you have been authorized to undertake research in **Bungoma County** for a period ending **17th February, 2017.**

You are advised to report to **the County Commissioner and the County Director of Education, Bungoma County** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

DR. M. K. RUGUTT, PhD, HSC.
DIRECTOR-GENERAL/CEO

Copy to:

The County Commissioner
Bungoma County.

The County Director of Education
Bungoma County.

National Commission for Science, Technology and Innovation is ISO: 9001: 2008 Certified

REPUBLIC OF KENYA

THE PRESIDENCY

MINISTRY OF INTERIOR AND COORDINATION OF NATIONAL GOVERNMENT

Telephone: 055- 30326
FAX: 055-30326
E-mail: ccbungoma@yahoo.com
When replying please Quote

Office of the County Commissioner
P.O. Box 550 - 50200
BUNGOMA

ADM/15/13/218

Date; 7th March, 2016

TO WHOM IT MAY CONCERN

RE: RESEARCH AUTHORIZATION

The bearer of this letter Davies Mukwabi Makilla, a student of Kenyatta University has sought authority to carry out a research on **"Characterization of bacteria and protozoa infecting oreochromis niloticus Linnaeus and clarias gariepinus burchell in aquaculture systems *in Bungoma County*** for a period ending 17th ***February, 2017.***

The authority granted to him by the National Commission for Science, Technology and Innovation is hereby acknowledged and appreciated.

Any assistance accorded to him in this pursuit would be highly appreciated.

COUNTY COMMISSIONER
BUNGOMA

Phides N. Njeru
For: County Commissioner,
BUNGOMA COUNTY

REPUBLIC OF KENYA

MINISTRY OF EDUCATION, SCIENCE AND TECHNOLOGY
State Department of Education – Bungoma County

When Replying please quote
e-mail: bungomacde@gmail.com

Ref No: BCE/DE/19 VOL I/199

County Director of Education
P.O. Box 1620-50200
BUNGOMA
Dates: 7th March, 2016

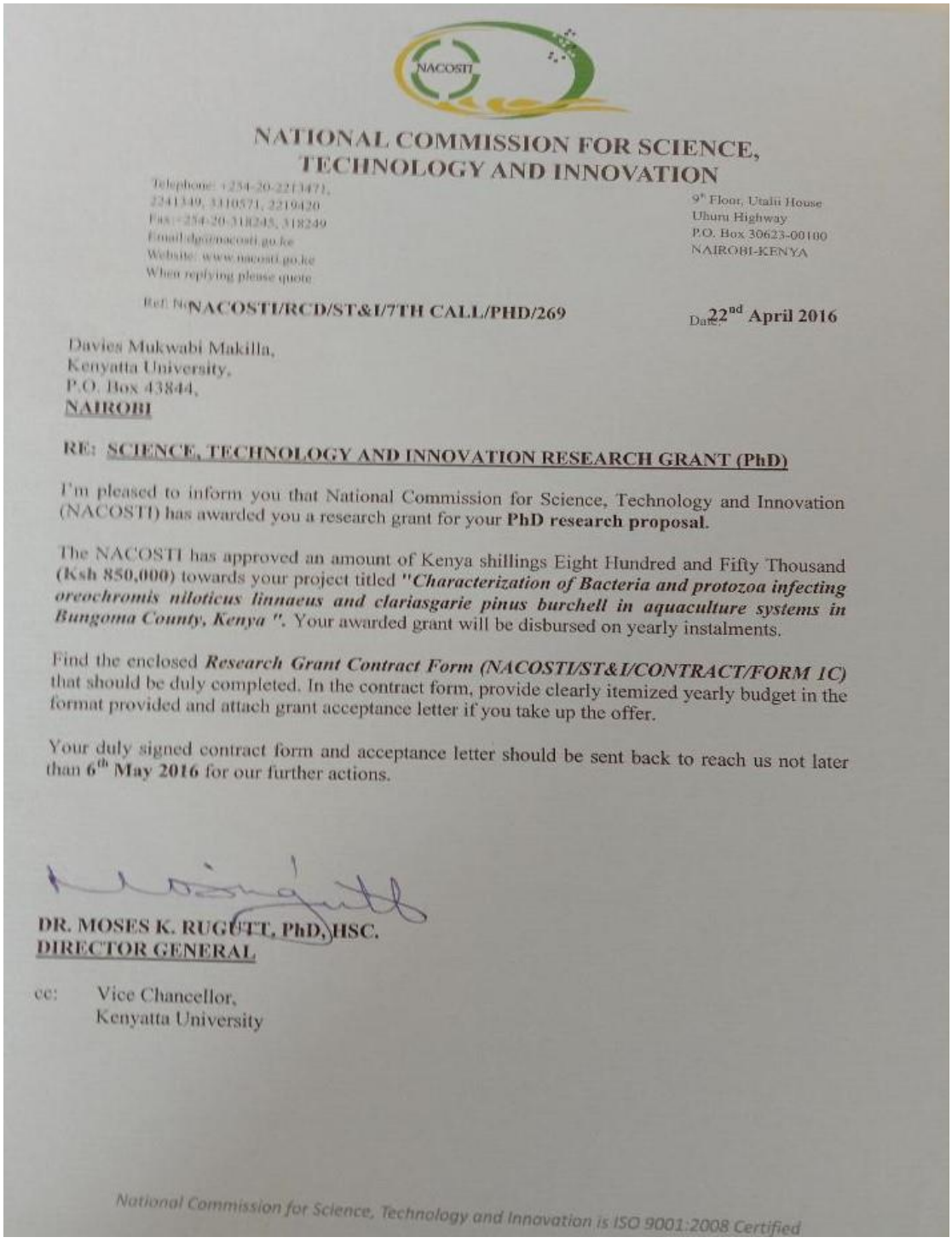
The Sub - County Directors of Education
BUNGOMA COUNTY

RE: AUTHORITY TO CARRY OUT RESEARCH – DAVIES MUKWABI MAKILLA ADMISSION NO- P/16/88750/8924

The bearer of this letter Davies Mukwabi Makilla is a student of Kenyatta University – He has been authorized to carry out research on ***"Characterization of bacteria and Protozoa infecting oreochromis niloticus Linnaeus and clarias gariepinus burchell in aquaculture systems in Bungoma County, Kenya"*** the research period runs up to **17th February, 2017**

Kindly accord him the necessary assistance.

CHARLES .A ANYIKA
COUNTY DIRECTOR OF BUNGOMA
BUNGOMA COUNTY.



NACOSTI

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone: +254-20-2213471,
2241349, 3310571, 2219420
Fax: +254-20-318245, 318249
Email:dg@nacosti.go.ke
Website: www.nacosti.go.ke
When replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/RCD/ST&I/7TH CALL/PHD/269**

Date: **22nd April 2016**

Davies Mukwabi Makilla,
Kenyatta University,
P.O. Box 43844,
NAIROBI

RE: **SCIENCE, TECHNOLOGY AND INNOVATION RESEARCH GRANT (PhD)**

I'm pleased to inform you that National Commission for Science, Technology and Innovation (NACOSTI) has awarded you a research grant for your **PhD research proposal.**

The NACOSTI has approved an amount of Kenya shillings Eight Hundred and Fifty Thousand **(Ksh 850,000)** towards your project titled ***"Characterization of Bacteria and protozoa infecting oreochromis niloticus linnaeus and clariasgarie pinus burchell in aquaculture systems in Bungoma County, Kenya "***. Your awarded grant will be disbursed on yearly instalments.

Find the enclosed ***Research Grant Contract Form (NACOSTI/ST&I/CONTRACT/FORM 1C)*** that should be duly completed. In the contract form, provide clearly itemized yearly budget in the format provided and attach grant acceptance letter if you take up the offer.

Your duly signed contract form and acceptance letter should be sent back to reach us not later than **6th May 2016** for our further actions.

DR. MOSES K. RUGUTT, PhD, HSC.
DIRECTOR GENERAL

cc: Vice Chancellor,
Kenyatta University

HIGHER EDUCATION LOANS BOARD

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Website: www.helb.co.ke

Anniversary Towers
18th Floor, University Way
P.O. Box 69489-00400
Nairobi, Kenya

HELB/45/003/VL.1/187

14th February, 2014

Makilla Davies Mukwabi
Kenyatta University
NAIROBI

Dear Sir/Madam,

HELB POSTGRADUATE SCHOLARSHIP AWARD 2013/2014

This is to inform you that you have been nominated for the award of the Higher Education Loans Board 2013/2014 Postgraduate Scholarship.

We take this opportunity to congratulate you on this well-deserved achievement. Your scholarship is valued at **Kshs.450,000.00** and will cover your tuition fees only. The award will be disbursed in annual installments to your university during your studies upon receipt of **a satisfactory Academic Progress Report from your respective faculty.** Please ensure that this report is availed to the Board at the beginning of each academic year.

By a copy of this letter, the university is also informed of the award and is requested to get in touch with Higher Education Loans Board in the event that the student is on any other institutional scholarship.

Yours faithfully,

ESTHER N. MICHIEKA (MRS.)
FOR: BOARD SECRETARY/CEO

Cc: Finance Officer - Kenyatta

Appendix II : Seine Net

Plate 3.1: A typical seine net spread in readiness to harvest farmed fish at Chwele; mag. (3.65) x0.24

Appendix III : Nile tilapia

Plate 3.2: Harvested farmed *Oreochromis niloticus*; mag. (2.93x8.13)

Appendix IV : Fish pond

Plate 3.3: Earthen fish pond (R); mag. (2.79x3.93) and polylined pond (L); mag. (2.80x4.72)

Appendix V: Portable autosampler

Plate 3.4: A portable auto sampler; mag. (5.55) x2.65

Appendix VI : Generated sequences of isolated bacteria

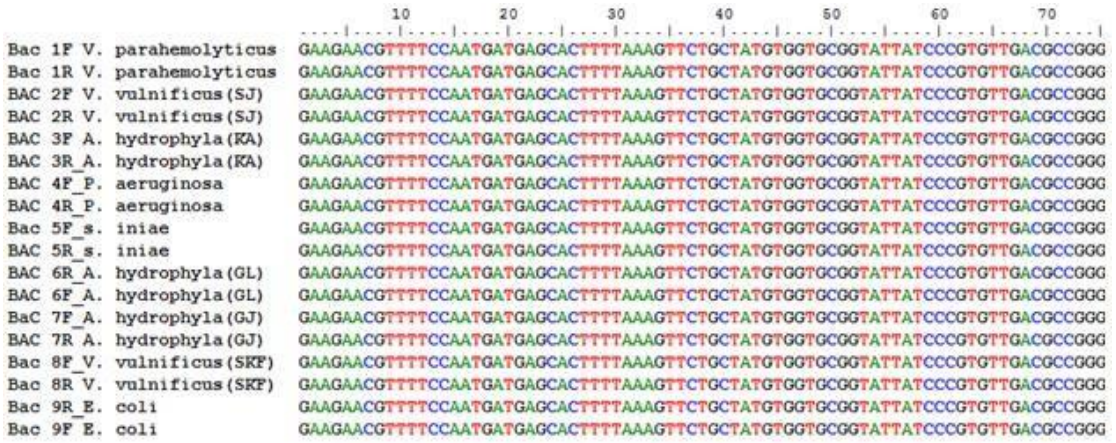

Plate 3.5: Sequences as aligned in the Clustal-W program in BioEdit (Version 7.05)

Appendix VII : A chromatogram files showing *bla*$_{TEM}$ gene sequence

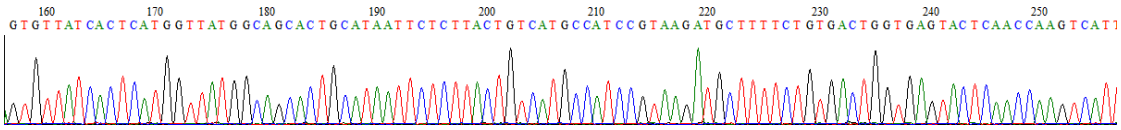

Plate 3.6: A chromatogram files showing the gene sequence

Appendix VIII: One-way Analysis of variance (ANOVA) for water quality parameters

S/No	Dependent variable	Independent variable	Sums of Squares	Degrees of freedom	Mean Sums of Square	F	P
1	Temperature	Sub-counties	652	5	130	15.5	<0.001
2	Oxygen	Sub-counties	3.10	5	0.61	2.10	0.081
3	Conductivity	Sub-counties	4693	5	938	0.631	0.677
4	pH	Sub-counties	0.34	5	0.069	0.27	0.928
5	Turbidity	Sub-counties	177	5	35	1.83	0.110
6	Biological Oxygen Demand	Sub-counties	736.035	5	147.207	1.018	0. 414

Appendix IX: Flatworm isolated from pond water sampled from Bungoma North Sub County

Plate 4.3: *Cleidodiscus* spp. ; mag. 0.01x (1.93)

Appendix X: Analysis of helminthes isolated from Nile tilapia

Independent variable	**Dependent variable**	**Null Hypothesis**	**Test**	**Kruskal Wallis Test statistic**	**Pvalue**	**Decision**
Sub-counties	Total Parasite Number (tail fin)	The distribution of total aerobic colonies counts in ponds is the same across the sub-counties	Independent samples Kruskal wallis test	4	<0.041	Reject null hypothesis
Sub-counties	Total Parasite Number (stomach)	The distribution of total parasite number in the stomach of Nile tilapia is the same across the sub-counties	Independent samples Kruskal wallis test	4	0.469	Accept null hypothesis
Sub-counties	Total Parasite Number	The distribution of total	Independent samples Kruska	4	0.484	Accept null hypothesi

	(gills)	parasite number in the gills of Nile tilapia is the same across the sub-counties	wallis test			s
Sub-counties	Total Parasite Number (intestine)	The distribution of total parasite number in the stomach of Nile tilapia is the same across the sub-counties	Independent samples Kruska wallis test	4	<0.035	Reject null hypothesis

Appendix XI : Analysis of total viable aerobic colony counts isolated from Nile tilapia

S/No.	Independent variable	Dependent variable	Null Hypothesis	Test	Kruskal Wallis Test statistic	Pvalue	Decision
1.	Sub-counties	TVACC Number (intestine)	The distribution of total parasite number in the intestine of Nile tilapia is the same across the sub-counties	Independent samples Kruska wallis test	7	0.035	Reject null hypothesis
2.	Sub-counties	Total Viable Aerobic Colony Counts (kidneys)	The distribution of TVACC in the kidneys of Nile tilapia is the same across the sub-counties	Independent samples Kruska wallis test	7	0.015	Reject null hypothesis
3.	Sub-counties	Total TVACC (gills)	The distribution of TVACC in the gills of Nile tilapia is the same across the sub-counties	Independent samples Kruska wallis test	7	<0.001	Reject null hypothesis

Appendix XII : Analysis of bacteria isolated from Nile tilapia

Independent variable	**Dependent variable**	**Null Hypothesis**	**Test**	**Kruskal Wallis Test statistic**	**Pvalue**	**Decision**
Sub-counties	Total Pathogenic Bacteria (Scales)	The distribution of TPB in the stomach of nile perch is the same across the sub-counties	Independent samples Kruskal wallis test	5	0.975	Accept null hypothesis
Sub-counties	Total Pathogenic Bacteria (Skin)	The distribution of TPB in the skin of nile perch is the same across the sub-counties	Independent samples Kruskal wallis test	9	0.992	Accept null hypothesis
Sub-counties	Total Pathogenic Bacteria (gills)	The distribution of TPB in the gills of nile perch is the same across the sub-counties	Independent samples Kruskal wallis test	9/6	<0.001	Reject null hypothesis
Sub-counties	Total Pathogenic Bacteria (Intestine)	The distribution of TPB in the intestine of nile perch is the same across the sub-counties	Independent samples Kruskal wallis test	9/6	0.737	Accept null hypothesis

Appendix XIII: Analysis of total viable aerobic colony counts isolated from pond water

Independent variable	**Dependent variable**	**Null Hypothesis**	**Test**	**Kruskal Wallis Test statistic**	**Pvalue**	**Decision**
Sub-counties	TVACC pond	The distribution of total aerobic colonies counts in ponds is the same across the sub-counties	Independent samples Kruskal wallis test	6	<0.041	Reject null hypothesis

Appendix XIV : Analysis of bacteria (*Aeromonas hydrophila*) isolated from pond water

S/No.	**Independent variable**	**Dependent variable**	**Null Hypothesis**	**Test**	**Kruskal Wallis Test statistic**	**Pvalue**	**Decision**
4.	Sub-counties	Pathogenic Bacteria-*Aeromonas hydrophila* (Ponds)	The distribution of PB in the ponds is the same across the sub-counties	Independent samples Kruska wallis test	3	0.016	Reject null hypothesis

Appendix XV: Ordinal Regression analysis of sensitivity tests among bacteria isolated from Nile tilapia

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.061[a]	6	.087
Likelihood Ratio	13.007	6	.043
Linear-by-Linear Association	1.585	1	.208
N of Valid Cases	45		

Appendix XVI: Ordinal Regression analysis of sensitivity tests among bacteria isolated from Nile tilapia across the sub counties

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.136[a]	4	.189
Likelihood Ratio	7.743	4	.101
Linear-by-Linear Association	1.973	1	.160
N of Valid Cases	45		

Appendix XVII: Ordinal Regression analysis of sensitivity tests among bacteria (*Aeromonas hydrophila*) isolated from pond water

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	44.455[a]	16	.000
Likelihood Ratio	37.583	16	.002
Linear-by-Linear Association	.315	1	.575
N of Valid Cases	45		

Appendix XVIII: Ordinal Regression analysis of sensitivity tests among bacteria (*Aeromonas hydrophila*) isolated from pond water across the sub counties

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.629[a]	6	.357
Likelihood Ratio	8.150	6	.227
Linear-by-Linear Association	1.821	1	.177
N of Valid Cases	45		