CHARACTERIZATION AND SOURCE TRACKING OF DIARRHEAGENIC BACTERIA CONTAMINATING FISH IN LAKE VICTORIA, KENYA

BY

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DECLARATION

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

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DEDICATION

I dedicate this work to my late father John M. Wawire who was my inspiration and driver of my professional goals, and to my wife Rose, son Eugene, daughter Eda and my mother Alice whose unending love and support make everything I do possible.

ABSTRACT

Lake Victoria is an important source of fish. Nonetheless, fish has been reported to be of low microbiological quality as they have been reported to be contaminated with *Escherichia coli, Vibrio* spp, *Shigella* spp and *Salmonella.* Nevertheless these microbial contaminants of fish in Lake Victoria have not been linked to their possible reservoirs, therefore making it difficult for regulatory agencies and fishers minimize fish contamination. Furthermore, it is not known if *E. coli* contaminating fish from Lake Victoria is pathogenic or not. Similarly, is not known if antibiotic resistance encountered among microbes present in Lake Victoria fish linked to clinical or environmental sources. The study aimed at characterizing and source tracking diarrheagenic bacteria contaminating fish in Lake Victoria. Fish and environmental samples from 5 locations (Sirongo, Dunga, Homa bay, Mbita town, and Luanda Konyango beaches) and human stool specimen from Kisumu East Sub-County hospital were investigated for *E. coli*, *Salmonella* spp, *Shigella* spp and *V. cholerae*. Standard microbiological procedures, API 20 E, serotyping, multiplex PCR and antibiotic susceptibility testing were used to recover and characterize the microbes. Antibiotic inhbition zones were used to discriminate isolates. *E. coli* was detected highest in human (92.9%) and lowest in freshly caught Nile perch and *Rastriniobola argentea* at 7.3% and 3.5% respectively. *Shigella* spp was only recovered among human, *Salmonella* spp was detected in soil (16.7%), sundried *R. argentea* (8.6%), human (7.1%). No *V. cholerae* was detected. High levels of total coliforms counts were recorded for water at shores ($\log_{10}$ 3.75 ± 0.26 cfu/ml), with a significant decline from the shoreline (0m) towards offshore (150m) ($p = 0.0001$). Enterotoxigenic *E. coli* were detected among human stool. No *E. coli* virulence genes tested were detected among fish isolates. Overall antibiotic resistance rate of 49.7% was detected. *E. coli* isolates recovered from soil and *R. argentea* recorded the highest resistance to tetracycline (100%). Tetracycline, ampicillin, nalidixic acid and cifuroxime showed significant variation in sensitivity among different sources of *E. coli* ($p < 0.05$). Multiple Antibiotic Resistance (MAR) index of *E. coli*; grouped soil and fish isolates together, whereas DA with an average rate of correct classification (ARCC) of 41%, misclassified 38.5% of *E. coli* isolates recorvered from fish as soil isolates. The study therefore concludes that soil could be a major source of diarrheagenic bacteria contaminating Lake Victoria fish and recommeds MAR indexing of *E. coli* and DA as possible tools for determining sources of contamination among fish in the region.

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LIST OF ABRREVIATIONS

API	Analytical Profile Index
ANOVA	Analysis of Variance
ARCC	Average Rate of Correct Classification
AST	Antimicrobial Susceptibility Test
ATCC	American Type Cell Culture
BMU	Beach Management Unit
BOX –PCR	Extragenic Repeating Elements – Polymerase Chain Reaction
CDC	Center for Disease Control and Prevention
cfu	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
DA	Discriminant Analysis
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggrigative *Escherichia coli*
EDTA	Ethylene Diamine Tetracetic Acid
EFSA	European Food Safety Authority
EHEC	Enteroheamorragic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*

ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Sequence – Polymerase Chain Reaction
ETEC	Enterotoxicogenic *Escherichia coli*
EU	European Union
FAO	Food and Agriculture Organization
FBDs	Foodborne Diseases
FDA	Food and Drug Administration
GLP	Good Laboratory Practice
GOK	Government of Kenya
HIV	Human Immunodefficiency Virus
KEMRI	Kenya Medical Research Institute
KEMFRI	Kenya Marine and Fisheries Research Institute
LT	Heat Labile Toxin
LVB	Lake Victoria Basin
MAR	Multiple Antibiotic Resistance
MID	Minimum Infection Dose
MIL	Motility Indole Lysine
MST	Microbial Source Tracking or Microbial Subtyping
NTS	Non Typhoidal Salmonellae
PCR	Polymerase Chain Reaction

PFGE	Pulsed Field Gel Electrophoresis
REP-PCR	Repetitive Extragenic Palindromic Sequence - Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
ST	Heat Stable Toxin
TCBS	Thisulfate Citrate Bile salts sucrose
TSI	Triple Sugar Iron
USA	United States of America
USAID	United States Agency for International Development
UV	Ultra Violet
WHO	World Health Organization
XLD	Xylose Lysine Desoxycholate

OPERATIONILIZATION OF TERMS

Characterization: Implies to identify or discriminate or attributing of distinguishing traits. Making significance and meaning of what is observed about the microbes detected in the various experiments. Here bacteria were characterized based on their types, dues to response to biochemical tests, antimicrobial response or antisera or presence of certain genes.

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Diarrheagenic bacteria: A range of enteric bacteria causing diarrheal illness in humans. Here diarrheagenic bacteria are limited to members of *Salmonella*, *Escherichia coli*, *Shigella* and *Vibrio cholerae*.

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CHAPTER ONE: INTRODUCTION

1.1 Background

In Kenya, Lake Victoria is an important source of fresh water fish and contributes over 90% of the national total fish production. Lake Victoria supports valuable artisanal and commercial fishery (Abila and Jensen, 1997; Ogello *et al*., 2013), with the major commercial fish species including the Nile perch (*Lates niloticus*), "*omena/dagaa*" (*Rastrineobola argentea*), and Nile tilapia (*Oreochromis niloticus*) (Government of Kenya [GOK], 2006). Lake Victoria is an important source of employment, water, food and income to the local communities living within its surroundings (Ogello *et al*., 2013). To a majority of the local communities along the Lake, fish is the main source of protein both for humans and animal feed (Abila and Jensen, 1997; Kabahenda *et al*., 2011). In this region, fish is eaten either freshly cooked or after undergoing some form of preservation or processing like, sun drying, salting or smoking (Kabahenda *et al*., 2011). Fishing and fish processing or preservation operations in the region have been reported to be associated with production of fish of low microbiological quality (Mungai *et al*, 2002; Ogwang' *et al*, 2005; Sifuna *et al*, 2008). Lake Victoria fish have previously faced trade bans from accessing lucrative fish markets in the European Union (EU) (United States Agency for International Development [USAID], 2008). These bans have been associated with contamination of fish by pathogens (Abila, 2003; Ogwang' *et al*, 2005; USAID, 2008).

Although previous studies on Lake Victoria have established that *Escherichia coli, Vibrio* spp, *Shigella* spp and *Salmonella* are the major contaminant of fish offered for sale in local markets (Ogwan'g *et al.*, 2005; Sifuna *et al.*, 2008; Onyango *et al.*, 2009), linking the possible sources of diarrheagenic bacteria in Lake Victoria to those contaminating fish has not been done. Linking

reservoirs of *E. coli*, *Vibrio* spp, *Shigella* spp and *Salmonella* along Lake Victoria is important as the information will help fishers and regulatory agencies such as the State or Local County Departments of Fisheries Development prevent fish from coming in contact with possible sources of diarrheagenic bacteria.

Escherichia coli have been used as an important indicator of fecal contamination of fish in Lake Victoria (Sifuna *et al*, 2008). However, there are many strains of *E. coli* with, some being potential pathogens (Nataro and Kaper, 1998; Amisano *et al*., 2011). Based on the virulence properties, *E. coli* associated with enteric infections can be classified into at least five groups namely enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) serotypes (Donnenberg and Whittam, 2001; Amisano *et al*., 2011). Although studies have demonstrated the occurrence of *E. coli* in fish originating from Lake Victoria (Sifuna *et al*, 2008; Onyango *et al*, 2009), none has demonstrated if the *E. coli* recovered from fish are pathogenic or not. In Kenya some pathogenic strains of E. coli namely EPEC, ETEC and EAEC have frequently been recovered among patients especially children of below five years old with diarrhea (Sang *et al.,* 1997; Sang *et al.,* 2012). This study used Polymerase chain reaction (PCR) based approach to understand the distribution of these three *E. coli* pathotypes (EPEC, ETEC and EAEC) among the different possible sources investigated including fish. The PCR approach for determining *E. coli* pathotypes are based on the detection of specific virulence genes that are responsible for the different *E. coli* pathotypes and their characteristic pathogenicity displayed by each pathotypes (Kuhnert *et al*., 1997; deMoura, et al., 2012). Since virulence factors are directly involved in the mechanism of pathogenicity, their genes have been found ideal targets for the molecular analysis

of potential pathogenicity and typing of different pathotypes in medical diagnosis, food and water (Kuhnert *et al*., 2000; European Food Safety Authority Panel on Biological Hazards, 2015) and hence their use for discrimination in this study.

Previous studies by Sifuna *et al*, (2008) and Onyango *et al*, (2009) have also shown the emergence of antibiotic resistant diarrheagenic bacteria isolated from fish originating from Lake Victoria, Kenya. The emergance of antibiotic resistant microbes has been considered a worldwide threat to public health (Rice, 2009; Laxminarayan et al., 2015). Understanding the scientific basis of antimicrobial resistance has been considered essential to combating this public health threat. This study therefore attempted to link the emerging antibiotic resistance phenotypes among diarrheagenic bacteria originating from fish and those from clinical and environmental situations. The absence of an understanding of such linkage has continued to allow for contamination of fish originating from Lake Victoria with antibiotic resistant microbes. By comparing the levels of antibiotic resistance and antibiotic resistance patterns displayed by diarrheagenic bacteria recovered from different sources, the study established an association between contaminant and their possible sources. Such information can offer useful criteria and identification of practices that can be employed to minimize possible contacts with sources responsible for contamination of fish in Lake Victoria.

Studies investigating the microbial quality of fish originating from Lake Victoria have, attributed domesticated animals, human, soil and water as would be important sources of bacteria contaminating fish (Abila and Jensen, 1997; Mungai *et al*., 2002; Ogwang' *et al*., 2005 and Onyango *et al*., 2009). However, there hasn't been scientific evidence to link these sources to

contamination of fish. The identification of food safety risks associated with fish have generally relied on traditional methods such as total coliform, fecal coliform counts and presence or absence of pathogens such as *Salmonella* spps. Evidence shows that the coliforms ecology, prevalence, and resistance to stress differ from those of many of the pathogenic microorganisms they are proxy for (Desmarais *et al*., 2002). These differences (among coliforms and pathogenic enteric bacteria) are so great that they limit the utility of the coliforms as indicators of fecal contamination.

Generally foods contaminated with human or animal feces are regarded as a greater risk to human health, as they are more likely to also contain human-specific enteric pathogens, including *Salmonella enterica* serovar Typhi, *Shigella* spp., hepatitis A virus, and Norwalk-group viruses (Scott *et al*., 2002) among others. Coliforms especially *E. coli* as indicator of fecal contamination, has good characteristics of a fecal indicator, e.g. it being non pathogenic to humans, in several instances, and being present at concentrations much higher than the pathogens it predicts acts as a good indicator for the pathogen (Desmarais *et al*., 2002). However, as shown by Desmarais *et al*., (2002) *E. coli* may not be a reliable indicator in tropical and subtropical environments due to its ability to replicate in contaminated soils. Due to this ubiquitous nature of *E. coli*, the effectiveness of using it to predict the presence of human or animal waste impact and subsequent health risks has therefore been considered limited (Desmarais *et al*., 2002). Nevertheless, the usefulness of the microbial indicators as tools for risk assessment can be significantly enhanced by the development of testing methods and analysis techniques that can define specific sources of these organisms (Scott *et al*., 2002).

Microbial source tracking (MST), also referred to as bacterial or fecal source tracking, is a set of techniques used to determine the sources of fecal bacteria in the environment (Simpson *et al*., 2002). MST techniques attempt to determine sources of fecal bacteria introduced into water bodies by humans, wildlife, or domestic animal sources (Wiggins, 1996; Wiggins *et al*., 1999; Guan *et al*., 2002). Microbial sub typing is achieved by characterization of isolates of a specific pathogen by either phenotypic and or genotypic methods (Meays *et al*., 2004). Phenotypic methods of Microbial source tracking (MST) include and not limited to serotyping (Wachsmuth, 1986), phage typing (Zierdt *et al*., 1980), and antimicrobial susceptibility testing (Wiggins, 1996; Wiggins *et al*., 1999). In this approaches, differences within different lineages of bacteria usually focuses on traits that may have been acquired from exposure to different host species or environments. This study will employ some of these approaches to determine a relationship between diarrheagenic bacteria contaminating fish originating from Lake Victoria with those isolated from different sources within Lake Victoria; and therefore establishing potential sources responsible for contaminating fish.

Overall, this study therefore intended to characterize *Salmonella* spp, *E. coli*, *Shigella* spp and *Vibrio cholerae* from different reservoirs in Lake Victora. The study also aimed at tracking for potential sources responsible for contamination or transmission of these pathogens to fish in Lake Victoria. The study achived this objective by recovering diarrheagenic bacteria from different sources namely domesticated animals, fish, water and soil. The study also pathotyped *E. coli* with the view of distinguishing these fecal indicators based of their virulence gene carriage. Similarly antibiotic susceptibility tests (AST) against a panel of six commonly used antibiotics was determined for the diarrheagenic bacteria recovered from the different sources

with the view of understanding the antibiotic resistance levels and antibiotic resistance profiles. Finally, MST approaches was employed to help predict the possible origins of *E. coli* as an indicator of fecal contamination of fish in Lake Victoria, Kenya. A cross-sectional study design was adopted and information generated provides an overview of the prevalence and distribution of diarrheagenic bacteria with respect to the different sources studied. The findings are useful for health planning and food safety policy development as well as providing a method with a capability of determining specific sources of these diarrheagenic bacteria contaminating fish along Lake Victoria, Kenya.

1.2 Statement of the Problem

Fishing forms an integral part of the economy of communities living along riparian region. it forms a source of employment and secondly as source of food. However Lake Victoria fish has been shown to be associated with pathogenic bacteria creating a health risk pool (Onyango *et al.*, 2009). The region has also been reported to have a diarrheal infection prevalence of 16% among children below the age of 5 years (Kenya National Bureau of Statistics, 2013). Diarrheagenic bacteria that have been shown to occur in fish originating from Lake Victoria includes but not limited to *Shigella* spp, *Salmonella* spp, *E. coli* and *Vibrio* spp (Mungai *et al.*, 2002; Sifuna *et al.*, 2008; Onyango *et al.*, 2009). Nevertheless, the sources and reservoirs for these diarrheagenic bacteria have not been linked to those contaminating fish along Lake Victoria, although some studies have associated domesticated animals and human activity (Ogwang' *et al*., 2005), soil (Abila and Jensen, 1997) and water (Mungai *et al*., 2002; Onyango *et al*., 2009) as possible sources.

Although *E. coli* has been recovered from Lake Victoria fish, it is however not understood if they possess virulence genes. This is critical since EPEC, ETEC and EAEC have been frequently encountered among patients in Kenya (Sang *et al.*, 1997; Sang *et al.*, 2012). Further antibiotic resistant bacterial isolates have previously been recovered from Lake Victoria fish (Onyango *et al*., 2009), although no link and associations between the level and patterns of antibiotic resistance among diarrheagenic bacteria recovered from Lake Victoria fish and the environment the fish passes through have not been demonstrated. Therefore the origin of antibiotic resistant bacteria occurring in fish originating from Lake Victoria is not known.

Traditionally, methods relied upon to detect fecal contamination in fish include indicator microbes, which are ubiquitous therefore, making them difficult to be singly relied upon as a means of identifying fecal contamination in the environment and fish. Hence an alternative analysis method is needed, which can allow for the discrimination of *E. coli* as indicators of fecal contamination in fish. Knowing the source of fecal contamination of water or foods is necessary to determine the degree of risk associated with human health and to develop effective control and resource management strategies. In this study discriminant function analysis (DA) of antimicrobial resistance profiles techniques was employed to discriminate among sources of diarrheagenic bacteria contaminating Lake Victoria fish along Kenya shores. The techniques have been used to predict sources of surface water impairment in the Americas, but has not been tested in an environment such as one presented in this study.

1.3 General Objective

To characterize and source track diarrheagenic bacteria contaminating fish in Lake Victoria, Kenya.

1.4 Specific Objectives

1. To determine the distribution of *Shigella* spps, *Salmonella* spp *E. coli* and *Vibrio cholerae* among fish, water, domesticated animals and human stools and soil as sources of diarrheagenic bacteria in Lake Victoria, Kenya.
2. To determine the *E. coli* pathotypes (virulence genes) present among fish, water, domesticated animals, soil and humans.
3. To determine levels of antimicrobial resistance among diarrheagenic bacteria recovered from the fish, water, domesticated animals, soil and humans.
4. To compare *Escherichia coli* isolates from human, livestock, fish and environmental sources using discriminant function analysis (DA) method.

1.5 Null Hypothesis

1. Human, fish, water, domesticated animals, and soil are not reservoirs for diarrheagenic bacteria in Lake Victoria, Kenya.
2. *Escherichia coli* occurring in fish, human, domesticated animals, water and soil sourced in Lake Victoria, Kenya do not possess EPEC, ETEC and EAEC virulence genes.
3. Diarrheagenic bacteria in Lake Victoria, Kenya are not resistant to at least one of the common antimicrobial agents prescribed for diarrheal diseases.
4. Discriminant function analysis can not discriminate *E. coli* isolates sourced from human, livestock, fish and the environment

1.6 Justification and Significance of Study

Fish has been identified as an important source of long chain poly - unsaturated omega 3 fatty acids; it is also a source of white meat and rich source of proteins, vitamins and minerals (Huss, 1995). With the continued increase of fish consumption, it is important to understand the public health risks and especially those of microbiological importance that can be associated with the fish in the region.

It has also become necessary to understand the environment in which fish passes through from source to market and identify the possible sources of contamination that exist. By identifying points of contamination along Lake Victoria, Kenya fishers and regulatory agencies can be able to develop strategies to mitigate or minimize contamination and therefore minimize post harvest losses and ensure safe fish is available to the consumer.

One major challenge has been to develop and employ tools with the capability to source track contamination in fish sourced from Lake Victoria, Kenya. Concepts based on existence of selective pressure imposed on commensal gastrointestinal flora of animals and humans by antibiotic use or exposure such as Multiple Antibiotic Resistance (MAR) indexing and Discriminant Analysis (DA) can generate relationships that can be used to discriminate bacteria. These techniques have good power to differentiate between different bacterial linage and are also ease and less cost to deploy (Kaneene *et al*., 2007), making them suitable for a developing country like Kenya.

Lake Victoria, Kenya is an important source for fish production contributing to over 90% of total fish produced locally (GOK, 2006), and therefore any information identifying possible sources for fish contamination will go a long way in minimizing contamination of fish, reducing post harvest loses and reducing possible health risks that may be associated with eating contaminated fish at a national level.

1.7 Scope and Study Limitations

1.7.1 Scope

The study covered five landing sites and the Kisumu East Sub-County hospital (formally Kisumu District hospital). The respective fish landing sites in this study were purposively selected based on the frequencies of fishing and processing of fish especially sun-drying. Kisumu East Sub-County Hospital was also purposively selected since it is a tier 5 referral facility within Kisumu County, and receives patients from one of the landing site namely Dunga, where fish, animal, soil and water specimen were collected. It therefore presented a suitable site to present a picture of type's of diarrheagenic bacteria infecting people within the study site. The facility was also chosen because of ease of access from Maseno University where bacterial isolations were carried out since distant facilities may have had logistical and financial challenges in availing human stool specimen within acceptable time durations and conditions.

The study analyzed and recovered *E. coli*, *Salmonella* spp, *Shigella* spps and *Vibrio cholera* from fecal specimens of human, donkey, chicken, cattle and goat; and from water, fresh fish (Nile perch and *Rastrieobola argentea*) and sundried fish (*R. argentea*) and soil. Fish, animal stool and environmental samples were collected and processed for analysis the same day. Clinical samples were collected within the hospital laboratory facility. However, delivery of the clinical samples to the Microbiology laboratory at Maseno University was varied, but specimens were

processed for bacterial recovery within three days of collection. The human stool specimen were preserved on Cary-Blair transport medium (HIMedia Lab. Pvt. Mumbai, India) and refrigerated at 4^0C - 8^0C to inhibit further growth.

1.7.2 Limitation

The study utilized the dilution and streaking methods for the primary isolation of bacteria from samples. Typically this method results in about 100 well isolated colonies growing on the selective media used in this study. Consequently, this may have affected detection of target bacteria especially those with closely related characteristics on a selective medium.

Generally diarrheal infections are caused by viruses, bacteria, protozoa and helminths. The current study only focused on recovery of bacterial forms with specific interest in *E. coli*, *Salmonella* spp, *Shigella* spps and *Vibrio cholerae,* other microbes were not considered. Similarly, seasonal variations were not considered in this study since fishing and fish processing by sun-drying take places along the beaches throughout the year and assumed not to be affected by seasons.

CHAPTER TWO: LITERATURE REVIEW

2.1 Fish Production along Lake Victoria, Kenya

The fisheries sub-sector plays a significant role in the Kenyan economy (Abila *et al*., 2006; Lugo et al., 2014). The sector has grown rapidly and exports have generated considerable foreign exchange income. Lake Victoria, Kenya is an important source of fresh water fish contributing over 90% of the national total fish production (GOK, 2006; Njiru *et al*., 2006). Currently capture fisheries, mainly from Lake Victoria, Kenya earn local fishers over Ksh 7 billion, while exports earn Kenya Ksh 5 billion (US$ 50 million) in foreign exchange annually (United States Agency for International Development [USAID], 2008).

In Lake Victoria, fishing activities are carried out at two levels namely artisanal and commercial fishing (Abila and Jensen, 1997). Lake Victoria has a multi-species fishery of tilapiines and haplochromines, cichlids and more than 20 genera of non-cichlid fish, including Mormyrus, catfish, cyprinids and lungfish (USAID, 2008). However, only the Nile perch (*Lates niloticus*), "*omena*/*dagaa*" (*Rastrineobola argentea*), and Nile tilapia (*Oreochromis niloticus*) are of commercial importance (GOK, 2006). Lake Victoria is also an important source of employment, water, food and income to the local communities (Abila *et al*., 2006; Lugo *et al*., 2014).

Domestic consumption of fish in Kenya has over the years increased, with high consumer awareness levels of the health benefits of eating fish (USAID, 2008). In Kenya, fish is eaten either freshly cooked or after undergoing some form of preservation or processing like, sun drying, salting or smoking (Abila and Jensen, 1997; USAID, 2008). Among Lake Victoria fish,

the Nile Perch has over the years dominated exports, with the European Union (EU) being a major market (Abila, 2003; USAID, 2008). However, between the years 1997 to 2000, Lake Victoria fish faced several trade bans from accessing the lucrative fish markets in the European Union (Abila, 2003). During this period when the EU had imposed an importation ban on Nile Perch from Kenya, the national fish exports declined by 68 percent nationally (Abila, 2003).

The fish export bans imposed on Kenya by the EU during this period were generally associated with contamination of fish with pathogens (Abila, 2003). Fish have a relatively short shelf-life, and rapidly lose quality and economic value if not handled properly (National Oceans and Fisheries Policy, 2008). It is therefore, necessary that fishers and regulatory authorities minimize post-harvest losses, assure fish quality and safety for human consumption, and comply with sanitary and phytosanitary (SPS) measures (National Oceans and Fisheries Policy, 2008).

According to National Oceans and Fisheries Policy, (2008) the Kenyan Government has plans to put in place measures for the maintenance of fish safety and quality assurance management systems to prevent, minimize or eliminate contamination of fish. If achived this should ensure the safety and quality of fish for human and animal consumption in the domestic and international markets. To achieve these ambitions, there is need to understand the environment in which fish passes through from the source to market and develop tools that will identify reservoirs and sources of contamination of fish with pathogens. This study attempted to address this challenge by characterizing some of the reservoirs of diarrheagenic bacteria along Lake Victoria, Kenya. It also utilized microbial source tracking techniques to identify possible sources of diarrheagenic bacteria contaminating fish. With the availability of such information

the Kenyan Government can be able to prioritize its resources of achieving the objective of minimizing or eliminating contamination of fish with pathogenic microbes along Lake Victoria, Kenya.

2.2 Food Safety Concerns Affecting Fish from Lake Victoria

Fishing and fish processing or preservation operations along Lake Victoria have been reported to be associated with production of fish of low microbiological quality (Mungai *et al*, 2002; Ogwang' *et al*, 2005; Sifuna *et al*, 2008). The National Oceans and Fisheries Policy, (2008) has identified poor design and construction of the fishing boats, the lack of cooling facilities on the fishing boats, possible cross contamination from the fishing crew and mixing of the catches as important factors responsible for the low microbiological quality of fish. On the other hand, Abila and Jensen, (1997) have associated contamination of fish with microbes to be due to poor processing practices as fish is dried on the ground (Figure 1), hence exposing them to both domesticated animals e.g. cattle, chicken and dogs and wildlife (birds and lizards) among others that may be carriers of pathogenic microbes.

Figure 1: The process of sundrying *R. argentea* practiced along Lake Victoria

Other fish processing techniques, along Lake Victoria include smoking and deep frying (Ogwang' *et al.*, 2005); and freezing (Mungai *et al.*, 2002). Fish targeted for this type of preservation are Nile perch of ≥ 1 kg in size. Studies have shown that products that go through these processing and preservation at the fish landing sites within Lake Victoria, are also prone to contamination due to poor handling practices (Mungai *et al.*, 2002; Ogwang' *et al.*, 2005; Onyuka *et al*., 2011).

2.3 Pathogenic Bacteria Associated with Fish

2.3.1 *Escherichia coli*

Escherichia coli are naturally present in the gastrointestinal tracts of humans and animals as part of the natural microflora. *Escherichia coli* has been used as indicator of fecal contamination, of food and water (Desmarais *et al*., 2002), hence being used as a representative of pathogenic

microbes of enteric origin. *Escherichia coli* can be found in fish intestines (Geldreich and Clarke, 1966). However Hansen *et al*. (2008) and Guzman *et al.* (2004) have observed that *E. coli* are not part of the permanent microflora in fish, but their occurrence is due to polluted water and feeding habits. In Kenya some studies have reported the occurrence of *E. coli* among fish originating from Lake Victoria (Ogwang' *et al.*, 2005; Onyuka *et al.*, 2011).

There are many strains of *E. coli* with, a few being potential pathogenes (Amisano *et al*., 2011). Pathogenic forms of *E. coli* associated with human and animal diseases are remarkably diverse. Pathogenic strains cause enteric diseases ranging in symptoms from cholera-like diarrhoea to severe dysentery; other *E. coli* may colonize the urinary tract, resulting in cystitis or pyelonephritis, or may cause other extraintestinal infections, such as septicemia and meningitis (Donnenberg and Whittam, 2001).

Based on the virulence properties, *E. coli* associated with enteric infections can be classified into five groups: enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) serotypes (Donnenberg and Whittam, 2001; Croxen and Finlay, 2010). In Kenya EPEC, ETEC and EAEC appear to be the most frequently encountered pathogens among children (Sang *et al.*, 1997; Sang *et al.*, 2012). Although studies have reported the occurrence of pathogenic *E. coli* among chicken and cattle in Kenya (Odwar *et al*., 2014, Macharia, 2015), no such study has attempted to determine the presence of these bacteria among fish.

Enteropathogenic (EPEC) serotypes: Enteropathogenic *E. coli* cause acute infantile diarrhoea in developing countries. The locus for enterocyte effacement (LEE) is a chromosomal pathogenicity island that confers a distinctive — attaching and effacing phenotype. Enteropathogenic *E. coli* do not carry genes for the phage-borne Shiga-toxins of enterohemorrhagic *E. coli*. Typical EPEC strains carry a virulence plasmid, which bears genes encoding bundle-forming pili, a plasmid that encode regulator and other putative virulence genes (Reid *et al*, 2000; Lacher *et al*, 2007). According to Okeke (2009), in the 70s' and 80s' classical EPEC serotypes were associated with disease in many parts of Africa, suggesting that EPEC was a predominant cause of diarrhea at that time. Among children EPEC has been associated with acute and persistent diarrhea leading to nutritional faltering and death (Levine and Edlman, 1984). In a recent study investigating the population-based burden of paediatric diarrheal disease in sub-Saharan Africa and south Asia, it was reported that there is association between typical EPEC and moderate-to-severe diarrhea, among children below 2 years. In addition, typical EPEC was significantly associated with death in infants aged ranging from 0 – 11 months (Kotloff *et al.*, 2013).

Enterotoxigenic (ETEC) serotypes: ETEC have been associated with traveler's diarrhea and infantile diarrhea in developing countries (WHO, 2006). These serotypes were observed to induce fluid accumulation in a ligated rabbit ileal loop model (Croxen and Finlay, 2010). Enterotoxigenic *E. coli* elaborate toxin very similar to cholera-toxin (Sack, 1975; WHO, 2006; Croxen and Finlay, 2010). Enterotoxigenic *E. coli* has elaborate heat labile toxin (LT) that is structurally and mechanistically similar to the cholera toxin as well as heat stable toxin (ST). There are two types of ST – the human (STh) and — porcine (STp) varieties, and the latter is

also implicated in human disease. There are also two types of LT but only LT-1 is believed to play a role in human disease (Croxen and Finlay, 2010; Hussain, 2015). In the 70's and early 80's, strains producing this heat labile enterotoxin and/or a heat stable enterotoxin were among the most common causes of diarrhea in travellers visiting Kenya (Sack *et al*., 1977 and 1978). Enterotoxigenic *E. coli* are believed to be primarily transmitted via contaminated food and water. In Kenya ETEC strains have been recovered from river water (Simiyu *et al.*, 1998) hence possibilities of them contaminating aquatic environment.

Enteroaggregative (EAEC) serotypes: Enteroaggregative *E. coli* exhibit characteristic aggregative pattern of adherence and produce persistent gastroenteritis and diarrhea in infants and children in developing countries (Donnenberg and Whittam, 2001; Croxen and Finlay, 2010). However, EAEC is now known to be a predominant cause of diarrheal disease in developed as well as developing countries (Rappelli *et al*, 2005; Nataro *et al*, 2006; Hussain, 2015). Enteroaggregative *E. coli* were originally associated with persistent diarrhea but are now known to be associated with a wide range of diarrheal syndromes, ranging from watery to invasive diarrhea, which may be acute or persistent (Okeke and Nataro, 2001; Huang *et al*, 2004; Huang *et al*, 2006). Enteroaggregative *E. coli* related diarrhea has also been associated with HIV-infected patients in Africa (Mwachari *et al*, 1998; Gassama *et al*, 2001; Okeke, 2009). They are commonly recovered from asymptomatic individuals, reflecting partly strain heterogeneity, infection load and inter-individual variations in susceptibility (Jiang *et al*, 2002; Samie *et al*, 2007). This may imply that some individuals carrying EAEC strains may be reservoirs for bacteria that could cause disease in more susceptible individuals (Okeke, 2009).

Enteroinvasive (EIEC) serotypes: Enteroinvasive *E. coli* produce disease resembling shigellosis in adults and children (WHO, 2006). Over the last decade, several studies have tried to investigate the role of EIEC in diarrheal infections in Africa, but this was not reported even in a case study in Gabonese population (Okeke, 2009). Studies conducted in Kenya, Mozambique, Ghana and Nigeria only identified a small number of EIEC isolates but saw no significant association with clinical manifestation (Okeke *et al*, 2003; Rappelli *et al*, 2005; Bii *et al*, 2005; Opintan *et al*, 2009).

Enterohemorrhagic (EHEC) causes sporadic cases and outbreaks of hemorrhagic colitis characterized by bloody diarrhea, and hemolytic uremic syndrome (HUS) associated with renal failure; (Nataro and Kaper, 1998; Croxen and Finlay, 2010). Among the numerous groups of pathogenic *E. coli* the EHEC strains have certainly the widest notoriety in human infections and severity (Donnenberg and Whittam, 2001; Croxen and Finlay, 2010). EHEC produce a toxin active on Vero cells *in vitro*. The most famous EHEC strain belongs to the O157:H7 serotype (Donnenberg and Whittam, 2001; Hussain, 2015). Infections of humans with the O157:H7 serotype occurs most frequently as food–borne outbreaks in communities, such as families, schools, elderly homes and day-care centers in the Developed World (Mainil and Daube, 2005). Food vehicles involved in the outbreaks of *E. coli* strain O157:H7 EHEC is mainly of ruminant origin such as raw or inadequately cooked meat products; or unpasteurized milk. Strain O157:H7 EHEC are being carried by healthy cattle and to a lesser degree, other ruminants in the gastro – intestinal tract (Mainil and Daube, 2005).

In Africa the first documentation of EHEC outbreak was a sporadic case of hemorrhagic uremic syndrome caused by EHEC O157:H7 reported in South Africa in 1990 (Browning *et al*., 1990). Three years later, another outbreak was reported in Swaziland, and resulted in approximately 2,000 deaths (Isaacson *et al.*, 1993; Effler *et al.*, 2001). In the latter case, the spread of the organism was likely to have been augmented by contamination of surface water by dead cattle, or consumption of meat of dead cattle, a condition that was later exacerbated by drought (Effler *et al.*, 2001). Previous studies have focused solely on recovery of EHEC belonging to O157 serotype from stool, water and food sources (Rajii *et al.*, 2008; Abong'o and Momba, 2008). Majalija *et al*, (2008) carried out thorough searches for EHEC and STEC in bovines and humans. They recovered strains using polymerase chain reaction (PCR) belonging to a range of serotypes but none of these were O157 strains. That study demonstrated that molecular tests of EHEC virulence genes, or possibly toxin-detection tests, offer the most promising means of detecting these pathogens in Africa (Okeke, 2009).

Recently an *E. coli* O104:H4 strain linked to raw sprouts with a combination of two different pathotypes (entero-aggregative *E. coli* (EAEC) and EHEC) was reported in Germany (Brzuszkiewicz *et al.*, 2011). A proposed name for the new pathotype has been given as entero-aggregative-hemorrhagic *Escherichia coli* (EAHEC). The natural reservoir of this new EAHEC type is unknown but human or animal origins have been suggested (Beutin and Martin, 2012), although strong evidence points to human (Baquero and Tobes, 2013). Such situations call for a more precise method of identification.

To control *E. coli* infections in communities, employing the principles of food hygiene at the processing and retail levels of a food chain remain the most effective approach (Caprioli *et al.*, 2005). In particular, cross contamination between raw and ready to eat products must be avoided (Cowden *et al.*, 2001). According to Brown *et al.* (2002) and McDowell and Sheridan (2001) environmental contamination with *E. coli* plays an important role in the transmission of the infection to humans, and therefore the handling of animal waste represents an important aspect of transmission. Some Pathogenic *E. coli* e.g. EHEC have been reported to be able to survive in bovine faeces for a considerable time. Therefore practices such as sterilization of animal faeces in form of manure and slurries by properly composting can ensure the reduction of the microbial load and therefore minimize environmental contamination by *E. coli* (Jiang *et al.*, 2003a; Jiang *et al.*, 2003b).

2.3.1.1 Techniques of identifying *E. coli* pathotypes

The identification of different diarrheagenic *E. coli* types isolated from stool, environmental or food samples can be assayed for by biochemical reactions, serotyping, and genotypic assays based on virulence characteristics, using molecular methods such as polymerase chain reaction (PCR) (Nataro and Kaper, 1998; Carlos *et al.*, 2010).

One of the first techniques used to identify different *E. coli* types was the High-Performance Liquid Chromatography (HPLC) method. In the HPLC method, antigenic proteins of *E. coli* types such as formyl-methionyl-leucyl-phenylalanine can be detected and their function deduced \(Marasco *et al.*, 1984). This approach has also been used to detect specific receptors, like the iron receptor for *E. coli* types, which are important virulence factors for binding iron in body fluids (Hantke *et al.*, 2003). However, this approach is not suited for the routine detection of

diarrheagenic *E. coli* types, as these receptors are produced by many pathogenic members of the *Enterobacteriaceae* family such as *E. coli* and *Salmonella enterica* (Earhart, 1996) and therefore lack specificity (Haag *et al*., 1993).

DNA hybridization has also been used to detect different diarrheagenic *E. coli* types, for example, DNA probe for specific genes that encode for virulence proteins of EIEC, in combination with invasion assays (Boileau *et al.,* 1984). This technique cannot however determine the serotype because it is based on lipopolysacharide (LPS) composition of the bacteria (Boileau *et al.,* 1984). The synthesis of LPS structures, which consist of lipid A, the core and antigen O, begins in the cytoplasm, where these structures are assembled. In the synthesis of LPS, a large number of genes participate, many of which are part of clusters located in different regions of the bacterial chromosome and, in some organisms, in plasmids (Schnaitman and Klena, 1993; Marolda and Volvano, 1998). This method therefore only identifies bacteria through their particular pathogenic potential i.e., pathovar (Boileau *et al.,* 1984). However, the sensitivity and specificity of DNA hybridization tests is relatively high when compared to the HPLC based and cell culture invasion methods. Furthermore testing of the bacterial colonies using specific antisera is needed to confirm their serotype (Boileau *et al.*, 1984). Another limitation of DNA hybridization is the large amount of time required to obtain the result from colonies on plate.

Serotyping is another method for detecting pathogenic *E. coli*. The technique is based on a specific combination of O and H antigens that define the "serotype" of an isolate (Nataro and Kaper, 1998). Although, *E. coli* of specific serogroups can be associated reproducibly with

certain clinical syndromes, however it is not in general that these serologic antigens themselves confer virulence displayed by the bacteria. Rather, the serotypes and serogroups serve as readily identifiable chromosomal markers that correlate with specific virulent clones (Whitttam *et al*., 1993). In application of this technique, monoclonal antibodies against the antigen for *E. coli* are used, in combination with latex agglutination, to determine specific types (Nataro and Kaper, 1998).

Polymerase chain reaction (PCR) has become the most commonly used method that gives rapid, reliable results with a high sensitivity and specificity (Nataro and Kaper, 1998). The most promising technique is the (multiplex) PCR, which uses specific target genes for the different diarrheagenic *E. coli* types. Detection of diarrheagenic *E. coli* types is based on detection of several genes: *stx1, stx2, eae, bfpA, invE, aggR, esth, estp, elt,* and *astA*. These genes are specific for EHEC, EPEC, EAEC pathotypes (Fujioka *et al.*, 2009; Fujioka *et al.*, 2013). The multiplex PCR can simultaneously detect different types of *E. coli* in one sample. However, there is a problem with detection based upon these target genes and a large overlap between different strains. The same *eae* probes used to identify EPEC can also be used to identify EHEC if they are derived from the highly conserved 5' end of the gene (Nataro and Kaper, 1998). Specific PCR pimers have been developed to detect sequences in the 3' end of *eae* genes from EPEC O127:H6, EHEC O157:H7 and EHEC O111 strains (Gannon *et al*., 1993; Louie *et al*., 1994; Heuvelink *et al*., 1995). Primers for *eae* have been combined with primers for *stx1* and *stx2* in multiplex PCRs to distinguish EHEC from EPEC (Fratamico *et al*., 1995; Heuvelink *et al*., 1995). However, *stx*-negative O157:H45, O157:H8 and O157:H39 strains have been found to possess 3' *eae* sequences nearly identical to those found in EPEC O127:H6 but quite different

from the O157:H7 *eae* sequences (Schmidt *et al*., 1993; Willshaw *et al*., 1994). These observations, therefore suggest that design and choice of primers should be carefully made for accurate distinction of some EHEC from EPEC strains by multiplex PCRs.

2.3.2 *Vibrio cholerae*

Vibrio cholerae is a Gram negative, highly motile, curved or comma-shaped rod bacterium that produces cholera enterotoxins and responsible for the life threatening secretory diarrhoea (Faruque and Nair, 2008). *Vibrio cholerae* is considered the only causative agent of epidemic cholera which represents major public health problem and causes explosive epidemics in developing countries (Rivas *et al.*, 1993; Siddique *et al.*, 1996; Ali *et al.*, 2012). Three groups of *V. cholerae* strains are recognized: serogroups O1, O139 and non-O1 (Farmer *et al.*, 1985; Ali *et al.*, 2012). In Kenya studies by Mungai *et al*, (2002) and Onyuka *et al*, (2011) reported the occurrence of *V. cholerae* among fish sourced from Lake Victoria.

Vibrio cholerae O1 is the serogroup traditionally associated with cholera cases, involving severe, watery diarrhea through the action of cholera toxin (Ali *et al.*, 2012). The serogroup is divided into the classical and EL Tor biotypes (Peterson, 2002). Serotype *V. cholerae* O139 emerged in India in 1992 and has spread to neighbouring countries (Chun *et al.*, 2009). It also causes cholera, but many secondary infections are asymptomatic (Albert, 1996; Chun *et al.*, 2009). Non-O1 *V. cholerae* has also been associated with diarrhoeal disease, although not as severe as the diseases caused by *V. cholerae* O1 (Carvajal *et al.*, 1998; Mekalanos *et al*., 2012).

In the absence of disease, the *Vibrio* life cycle consists of a free–swimming phase in marine and estuarine environments in association with zooplankton, crustaceans, insects and water plants. *Vibrio* interacts with various surfaces found in the environment to generate biofilms which may promote survival (Watnick *et al.*, 1999). Within the host the motile vibrios penetrate the mucus layer covering the intestinal villi, adhere to and colonize the epithelial surface of the small intestine, assume a non-motile phase, and replicate and cause disease by secreting numerous exoproteins at the site of infection (Oliver and Kaper, 1997).

The origin of epidemics has not yet been fully understood, although Venkateswaran *et al,* (1989) has suggested autochthonous marine *V. cholerae* to be the cause after the contribution of urban nutrients to the marine environment. Aquatic species living in brackish and estuarine waters have also been considered to be an important source of *V. cholerae* (Kayser *et al.,* 1987). Venkateswaran *et al,* (1989) suggested that *V. cholerae* associated with plankton, copepods and crustaceans could be a source of gastrointestinal infections. Other hypotheses have suggested migration, transport, contaminated water, vegetables, animals and marine species as other factors that affect transmission of *V. cholerae* (Carvajal *et al.,* 1998). Although, contamination of Lake Victoria fish with *V. cholerae* has been well documented by Mungai *et al,* (2002) and Onyuka *et al,* (2011), the origin of the contaminantion is not well understood.

2.3.3 *Salmonella* spp

These are Gram negative, facultative anaerobic, non-spore forming bacilli that can be split into more than 2,500 serotypes according to a system based on somatic (O) capsular (Vi) and flagella (H) antigens, known as the Kauffmann-White scheme (Grimont and Weill, 2007; Malorny *et al*., 2011). These mesophilic organisms are widely distributed in nature and are commonly found in

the intestinal tracts of animals and human beings and in environments polluted with animal or human excreta (Jay, 1992). However, *Salmonella* spp can multiply and survive in the estuarine and tropical fresh water environments for weeks (Rhodes and Kater, 1988). Recent studies on Lake Victoria fish have shown the occurrence of *Salmonella* (Onyango *et al.*, 2009; Onyuka *et al.*, 2011). Contamination fish has been associated to poor fish handling practices such as use of contaminated water, transportation of fish in dirty fishing boats and dirty packaging baskets by the fisher folks (Onyango *et al.*, 2009; Onyuka *et al.*, 2011).

Salmonella harbor several genes that have been suggested to be involved in their survival in nonhost environments (Andino and Hanning, 2015). These genes encode products involved in nutrient acquisition and utilization, motility, and transcriptional regulation, as well as genes having unknown functions (Hilbert *et al*., 1999), which increases their cabapility to withstand harsh conditions (Andino and Hanning, 2015).

Two clinical manifestations caused by *Salmonella* are recognized (enteric fever and the more common foodborne illness syndrome) and in both cases, the responsible micro- organisms enter the body via the oral route (Institute of Food Technologists, 2004). The principal symptoms of salmonellosis are non-bloody diarrhoea, abdominal pain, fever, nausea, and vomiting which generally appear about 24hrs after ingestion (Harrigan and Park, 1991). However, symptoms may vary considerably from grave typhoid-like-illness to more serious complications. The infective doses in healthy individuals vary according to serovars, foods involved, and susceptibility of the individuals. There is evidence for a minimum infective dose (MID) of as little as 20 cells (Varnam & Evans, 1991) while other studies have consistently indicated > 10^6

cells (Jay, 1992).

Salmonella are highly host adapted, where they infect only a limited number of species, or can be much more ubiquitous (Hoelzer *et al.*, 2011). The most significant human host-adapted organism is *S. enterica serovar* typhi, the causative organism for typhoid fever. Human is the only known reservoir for these isolates (Andrews-Polymenis *et al.*, 2010). Similarly, *S. enterica serovar* pullorum and *S. enterica serovar* gallinarum are poultry associated organisms that are so host adapted that even upon transmission to human they usually remain non-pathogenic (Ziprin and Hume, 2001). More frequently, animal host-adapted organisms can be transmitted to human causing symptomatic disease (Ziprin and Hume, 2001). *Salmonella enterica serovar* choleraesuis is normally a porcine organism though it can cause gastroenteritis and enteric fever, when transmitted to human (especially in children) (Chiu *et al.*, 2004). Other organisms, such as *S. enterica serovar typhimurium*, have a broad host range and these serotypes are responsible for the majority of human infections (Molbak *et al.*, 2006; Bell and Kyriakides, 2002).

In parts of sub-Saharan Africa, nontyphoidal *Salmonella* spp (NTS) are important causes of life-threatening bacteremia (Kariuki *et al*, 2006a; Karuiki *et al*, 2006b). Studies in Kenya have found that community-acquired NTS are among the top 3 causes of death among children <5 years of age (Berkley *et al*, 2005; Karuiki *et al*, 2006b). By using microbial subtyping tools, Kariuki *et al.* (2006a) demonstrated that most life-threatening salmonellosis in Kenya is caused by isolates that are clonal in origin. In their study, Karuiki *et al.* (2006b) reported that children from slums and poor backgrounds of Kenya were significantly more likely to be infected with multidrug-

resistant NTS than were children from middle-income families, indicating that environment may be important in transmission of antibiotic resistance microbes.

Salmonella infections in humans often result from the ingestion of contaminated foods, such as poultry, beef, pork, eggs, milk, seafood, and fresh produce (Gomez *et al.*, 1997). Sifuna *et al*. (2008) and Onyango *et al*. (2009) have reported the occurrence of *Salmonella* spps in Lake Victoria fish. Factors that influence the contamination of Lake Victoria fish with *Salmonella* are not clearly understood, although Onyuka *et al*. (2011) and Ogwang' *et al*. (2005) have associated these to poor fish handling and processing practices.

Direct contact with animals has been reported to result in transmission of *Salmonella* to humans (Fey *et al.*, 2000). Different control measures that can reduce incidence of *Salmonella* contamination exist depending on the mode of food contamination. Employing general food safety practices such as avoiding cross contamination, thoroughly cooking foods, and storing foods at the right temperatures prevent *Salmonella* from occurring in the home or food service environments (Institute of Food Technologists, 2004). Other approaches targeting animals include reduction of incidences of *Salmonella* carriage, by vaccination, and use of probiotics (Institute of Food Technologists, 2004).

2.3.4 *Shigella* spp

The genus *Shigella* is composed of Gram-negative, facultatively anaerobic, non-spore-forming organisms that do not ferment lactose and are non-motile (Cheesbrough, 2000). This genus consists of four distinct species i.e. *S. dysenteriae, S. flexneri, S. Boydii* and *S. Sonnei*

(Cheesbrough, 2000). *Shigella* is a major cause of dysentery throughout the world and is responsible for 5 – 10% of diarrhoeal illnesses in many areas (Ahmed *et al.,* 2006). Each year, 1.1 million people are estimated to die from *Shigella* infections (Kotloff *et al.*, 1999). *Shigella* food poisoning (shigellosis), which is an infection of the gut, may vary in severity from asymptomatic infection to fulminating dysentery (Guerrant, 1985).

The organism is host-adopted to humans and higher primates, and its presence in the environment is associated with faecal contamination (Food and Drug Administration [FDA], 2001). Even though, Onyango *et al*. (2009) reported the occurrence of *Shigella* in Lake Victoria fish, it is however not clear how Lake Victoria fish is contaminated with these pathogens, although *Shigella* strains have been reported to be able to survive in water for up to six months (Wachsmuth and Morris, 1989).

Generally, the majority of outbreaks of shigellosis worldwide are associated with drinking contaminated water and person-to-person transmission may also spread the disease through faecal-oral route (Eley, 1994; Kotloff *et al.*, 1999). In the USA, an estimated 20% of the total number of cases of shigellosis involves food as the vehicle of transmission (Mead *et al.*, 1999). Shigellosis is characterized by seasonality, with the largest percentage of reported cases occurring between July and October, and the smallest proportion occurring in January, February, and March (Gupta *et al.,* 2004) in the west. However, sporadic infections account for the majority of cases reported (Haley *et al.*, 2010). *Shigella* species are transmitted by the fecal-oral route, and most infections are transmitted from person to person, reflecting the low infectious dose (Gupta *et al.,* 2004). A few as 10 *Shigella* bacteria can result in clinical infection (DuPont

et al., 1989). Poor hand hygiene, ingestion of contaminated food or water, inadequate sanitation and toileting, overcrowding, and sexual contact are identified as important risk of *Shigella* transmission and infection (DuPont *et al.*, 1989; Cramer *et al.*, 2008). *Shigella* bacteria are present in the stools of infected persons while they are sick and for up to a week or two afterwards (DuPont *et al.*, 1989; DuPont, 2000). In developing countries, harvesting of seafood from faecal contaminated waters and use of unclean water in food preparation may be source of infection (Institute of Food Technologists, 2004).

In Sub-Saharan Africa, epidemic dysentery due to *Shigella dysenteriae* type I was first reported in Northeast Democratic Republic of Congo in the late 1979, but quickly spread within the country, reaching neighboring Rwanda and Burundi in 1981, and Tanzania in 1982 (Mhalu *et al.*, 1984; Ebright *et al.*, 1984; Huppertz, 1986). Unlike endemic shigellosis, attack rates were higher in adults than children (Ebright *et al.*, 1984; Huppertz, 1986). Case-fatality rates of 2–6% were reported but tended to be lower when appropriate therapy was used (Huppertz, 1986).

Prevention and control of shigellosis requires either that infected humans not be permitted to handle foods or that they practice good personal hygiene (Institute of Food Technologists, 2004). In developing countries improved hygiene and waste-handling practices may reduce the incidence of shigellosis (Institute of Food Technologists, 2004).

2.4 Tracking Sources of Food Contamination

According to EFSA (2008) the successful control of any food borne pathogen requires knowledge about the most important source or reservoirs as well as the principal routes of

transmission. According to Swaminathan *et al*. (2001) food borne outbreaks surveillance to be effective, isolates must be sub-typed routinely and the data analyzed promptly. This process allows for clusters to be detected locally using both advanced techniques and identified by traditional epidemiologic methods.

Microbial sub-typing allows for the possibility to make inferences about sources of human microbial infections by considering microbiological analysis and comparing data on the occurrence of pathogen in potential sources and/or comparing pathogen subtypes isolated from humans with subtypes isolated from animals and foods (EFSA, 2008). Using different subtyping techniques to Enterohaemoragic E. *coli* O157:H7 and *Yersinia enterocolitica* biotype 4, serotype O: 3 have revealed that the dominating reservoirs of these pathogens are cattle and pigs, respectively (EFSA, 2008).

Microbial source tracking (MST) refers to a specific application of microbial sub typing, in which markers from an isolate can be used to trace that isolate back to a specific animal source (Hald *et al.*, 2004). The basic idea is that even though the same pathogen might be found in different animal species, these sources might be host to unique populations of subtypes of these pathogens (Wiggins *et al*., 1999). In Denmark, microbial sub typing is considered an overall approach to identify specific bacterial and viral isolates and differentiate them from one another. Isolates recovered from human, animal, and food sources are sub typed and compared while illnesses are attributed to sub-type by matching animal sources (Pires *et al.*, 2014).

Microbial source tracking (MST) methods were originally developed specifically for identifying and tracking sources of microbial pollution in natural waters—such as lakes, rivers, and streams—failing to meet regulatory standards (Scott *et al.*, 2002). The field of MST involves a suite of discriminatory methods which have the potential to distinguish host sources (Simpson *et al.*, 2002; Field and Samadpour, 2007).

The methods used in MST studies can be divided into two categories viz: genotypic and phenotypic methods (Meays *et al*., 2004). In addition, MST techniques may also be categorized as library dependent (Butaye *et al.*, 2001) and library independent (Carson *et al.*, 2005). A "library" is a collection of microorganisms from different potential sources as well as from the target (Wiggins *et al*., 1999). Most MST techniques used have depended on the development of comprehensive libraries. Although the latter libraries are often made of several hundreds of isolates per potential source impacting the particular target (i.e., human, wildlife, and livestock) (Meays *et al*., 2004), the minimum number of isolates needed to perform statistically sound studies have not been determined.

Library-dependent methods are culture based and rely on isolate-by-isolate typing of microbes cultured from various fecal sources and from water samples (Scott *et al*., 2002). These isolates are matched to their corresponding source categories by direct subtype matching (Butaye *et al.*, 2001; Meays *et al.*, 2006) or by statistical means such as Discriminate analysis (DA) (Hagedorn *et al.*, 1999; Dombek *et al.*, 2000; Meays *et al.*, 2006).

Library-independent methods frequently are based on sample-level detection of a specific, host associated genetic marker in a DNA extract by PCR (Bernhard and Field, 2000; Carson *et al.*, 2005). Analyses of certain chemicals associated with sewage, including fecal sterols (Gilpin *et al*., 2002; Gilpin *et al.*, 2003), optical brighteners (Gilpin *et al.*, 2003; Martellin *et al.*, 2005**)**, and host mitochondrial DNA (Martellin *et al.*, 2005), have also been utilized for what can be more broadly termed fecal source tracking.

2.4.1 Phenotypic Methods used in MST

Many phenotypic methods have been suggested for use in discriminating among various groups of bacteria. Some of the methods include biochemical tests (Olsen *et al.*, 1992), phage susceptibility (Zierdt *et al.*, 1980), outer membrane protein profiles (Barenkamp *et al.*, 1981), antibody reactivity (Wachsmuth, 1986), fimbriation (Latham and Stamm, 1984) among others. These methods have disadvantages, including unstable phenotypes, low sensitivity at the intra-species level, and limited specificity (Scott *et al.*, 2002) that lead to poor identification and classification of the isolates. Some phenotypic methods have however, been used successfully as source tracking methodologies namely the multiple antibiotic resistance (MAR) analysis (Krumperman, 1983; Graves *et al.*, 2002) and the immunological methods (Orskov and Orskov, 1981; Parveen *et al.*, 2001)

2.4.1.1 Multiple Antibiotic Resistances (MAR) Analysis.

The multiple-antibiotic-resistance (MAR) test is based on detection of bacterial resistance to a panel of antibacterial agents (Guan *et al.*, 2002). The MAR patterns reflect the selective pressures imposed on the gastrointestinal floras of humans and animals during antibiotic use. The MAR test has been reported to be capable of identifying the sources of fecal streptococcal contamination in water (Harwood *et al.*, 2000) and distinguishing between *E. coli* strains from

specific point sources, such as industrial and municipal effluents, and strains from nonpoint sources, such as land runoff, that are dispersed over wide areas (Parveen *et al.*, 1997). The use of this method is based on the underlying principle that the bacterial flora present in the gut of various types of animals are subjected to different types, concentrations, and frequencies of antibiotics. Over time, selective pressure within a specific group of animal selects for flora that possess specific "fingerprints" of antibiotic resistance (Scott *et al.*, 2002).

Multiple-antibiotic-resistance testing involves the isolation and culturing of a target organism, then subjecting the isolates to antibiotic susceptibility testing. The organisms are then scored according to their susceptibilities to various antibiotics to generate an antibiotic resistance profile. These fingerprints are then characterized and analyzed by discriminate (or cluster) analysis (Scott *et al.*, 2002). The MAR approaches have also been used successful in discriminating *E. coli* or fecal streptococci isolated from specific animal sources such as wildlife, livestock (cattle, pigs, horses, and chickens), and humans (Harwood *et al.*, 2000; Graves *et al.*, 2002).

2.4.1.2 Multiple Antibiotic Resistance (MAR) indexing of *E. coli*

Multiple Antibiotic Resistance (MAR) indexing of *E. coli* has been used to identify the source of fecal pollution based on antibiotic resistance (Krumperman, 1983; Kaspar *et al*., 1990). According to Krumperman (1983), MAR indexing of *E. coli* can provide a useful tool for better risk assessment by identifying contamination from high-risk environments and also supplement traditional food microbiological evaluation approaches by providing additional information about the origin of contamination. The presence or absence of MAR *E. coli* can give more significance to current arbitrary numerical standards (Krumperman, 1983). In any case, an

unexpected increase in the MAR index of *E. coli* isolates from food should prompt an immediate investigation even though the number of *E. coli* organisms present may be below the established guideline or standard.

While developing MAR indexing of *E. coli* as a methodology to identify sources of fecal contamination among foods, Krumperman (1983), choose MAR index of 0.200 to differentiate between low- and high-risk contamination. Primary reservoirs for high-MAR *E. coli* were observed by Krumperman (1983), to be the major reservoirs for enteric diseases which are transmitted to humans through food and water, although indices of between 0.200 and 0.250 can be looked at as range of ambiguity, and samples in this range may require careful scrutiny.

Although, these methods have received significant attention as viable tools for tracking the sources of fecal pollution, antibiotic resistance determinants are often carried on mobile genetic elements e.g. transposons, integrons and plasmids, which can be lost from cells via cultivation and storage or by changes in environmental conditions (Prescott *et al*., 2000). In addition, strains from different locations may show variations in specific sensitivities due to variable antibiotic use among humans and livestock species (Witte, 1998).

2.4.1.2 Multiple Antibiotic Resistance (MAR) indexing of *E. coli*

Another method is Multiple Antibiotic Resistance (MAR) indexing of *E. coli* which has also been used to attempt to identify the source of fecal pollution based on antibiotic resistance (Krumperman, 1983; Kaspar *et al*., 1990). According to Krumperman (1983), MAR indexing of *E. coli* can provide a useful tool for better risk assessment by identifying contamination from

high-risk environments and also supplement traditional food microbiological evaluation approaches by providing additional information about the origin of contamination. The presence or absence of MAR *E. coli* can give more significance to current arbitrary numerical standards (Krumperman, 1983). In any case, an unexpected increase in the MAR index of *E. coli* isolates from food should prompt an immediate investigation even though the number of *E. coli* organisms present may be below the established guideline or standard.

While developing MAR indexing of *E. coli* as a methodology to identify sources of fecal contamination among foods, Krumperman (1983), choose MAR index of 0.200 to differentiate between low- and high-risk contamination. Primary reservoirs for high-MAR *E. coli* such as human, sawage, poultry, swine and dairy cow were observed by Krumperman (1983), to be the major reservoirs for enteric diseases which are transmitted to humans through food and water.

Although, these methods have received significant attention as viable tools for tracking the sources of fecal pollution, antibiotic resistance determinants are often carried on mobile genetic elements e.g. transposons, integrons and plasmids, which can be lost from cells via cultivation and storage or by changes in environmental conditions (Prescott *et al*., 2000). In addition, strains from different locations may show variations in specific sensitivities due to variable antibiotic use among humans and livestock species (Witte, 1998). Nonetheless, the data generated from MAR indexing of *E. coli* can be grouped to provide profiles or indices for a single isolate, sample, environment, food process, or any other designation or grouping thereby providing useful information for the evaluation of a health risk possibly associated with the food.

2.4.1.3 Discriminant Analysis (DA)

Discriminant analysis is a multivariate statistical method designed to separate sets of observations and allocate new observations to previously defined groups (Johnson and Wichern, 1992). Discriminant analysis transforms observations obtained from different populations with overlapping distributions into non-overlapping distributions. This transformation can then be applied to a set of observations from an unknown source population to determine the most probable population that served as the source for the unknown source observation. Discriminant analysis can be used to determine which variables discriminate between two or more naturally occurring groups and then classify cases into the values of categorical dependent groups (Johnson and Wichern, 1992). According to Wiggins (1996), this gives DA an advantage as it generates a classification rule based on all the bacterial isolates, and that the rule is then used to actually classify each individual isolate into one of many possible sources.

According to Kaneene *et al*. (2007) DA of antimicrobial resistance profiles can be used as a valid technique for microbial source identification as long as decision rules generated in the process are developed carefully. Since antimicrobial resistance patterns can differ by location and time due to differences in selection pressure (Parveen *et al*., 1999), it is important that geographic and temporal variations in antimicrobial resistance are taken into consideration when samples are collected for use with DA as these differences may have an effect on the Average Rate of Correct Classification (ARCC) of the DA.

However, Kaneene *et al*. (2007) further suggested that the rates of correct classification by the DA should be viewed in terms of the relative contributions of random chance and the true discriminatory power of the DA and therefore any methods applied to the DA to improve the ARCC should be conducted to specifically increase the true discriminatory power of the DA, rather than simply improving the overall ARCC. Kaneene *et al*. (2007) also proposes that selectively reducing the number of potential species classifications and the number of antimicrobial agents entering the analysis may improve the performance of DA. DA has been found to be very useful and of low-cost screening discrimination approach of antimicrobial resistance profiles among *E. coli* originating from different sources (Guan *et al.*, 2002; Kaneene *et al*., 2007).

2.4.1.4 Immunological Methods

Immunological methods include sero-grouping of microorganisms based on the presence of different somatic antigenic determinants (Crichton and Old, 1979; Gonzalez and Blanco, 1989; Parveen *et al.*, 2001). Sero-typing is one of the classic strain typing techniques that has been used over the years for epidemiological studies of many species of bacteria (Tenover *et al.*, 1997).

Different serotypes of *E. coli* have been associated with different animal sources, however many of these serotypes are also reported to be shared among humans and animals (Bettelheim *et al*., 1976; Orskov and Orskov, 1981). According to Parveen *et al*. (2001) who tested 100 human source and nonhuman source *E. coli* isolates for the presence of various "O" antigens, they successfully typed 77% of the isolates based on immunological method. Human-derived isolates exhibited 19 serotypes, with 48% being classified within 7 serotypes. Animal-derived isolates spanned 26 serotypes, with 36% being classified within 7 of those serotypes. Overlap between

predominant serotypes of human- and animal-derived isolates was not significant, which indicates that serotyping may be useful in discriminating *E.coli* from human and animal sources. The other limitation for this method is maintaining stocks of typing sera, for instance more than 2,000 antisera are required for definitive *Salmonella* typing (Scott *et al*., 2002).

2.4.2 Genotypic Methods used in MST

Genotypic methods are those based on analysis of the genetic composition of the organism and include polymorphisms in DNA restriction patterns based on cleavage of the chromosome by enzymes (Tenover *et al.*, 1997). Genotyping methods are less subjected to natural variation, but they can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites (Tenover *et al.*, 1997).

Although genotypic based MST studies could help significantly in the discriminating microbes, there are a number of problems that need to be addressed (Simpson *et al*., 2002). Some of these problems that have been identified relate to detection limits, temporal and spatial variability of markers, and reproducibility of the assays (Simpson *et al*., 2002; Scott *et al*., 2002). A clear understanding of the role of each variable plays is necessary to obtain robust results and correctly interpret the data. Another relevant issue relates to the stability of the marker used in source tracking, like in with some phenotypic methods, which are often unstable if the selective pressure is not maintained. Nonetheless, genotypic techniques also suffer from the problem of reproducibility of results (Meays *et al*., 2004). Since there is little selective pressure to maintain an intact genome structure among microbes, this genetic instability increases the level of pattern complexity in and outside of the host (Simpson *et al*., 2002).

Pulsed Field Gel electrophoresis (PFGE) is a method of DNA fingerprinting whereby DNA fingerprints are generated after treatment of genomic bacterial DNA with rare-cutting restriction endonucleases (Banatvala *et al*., 1996). Pulsed Field Gel electrophoresis has been a very useful technique in determining bacterial relatedness and epidemiological studies (Kariuki *et al.*, 1999). Simmons *et al*, (2000) used PFGE to match 51% of 439 *E. coli* isolates from a stream in an urban watershed, and classified the majority of isolates as belonging to wildlife (especially raccoons) and dogs. Pulsed Field Gel Electrophoresis, nevertheless suffers from the drawback of being too sensitive to broadly discriminate sources (Meays *et al*., 2004), long assay time, limited simultaneous processing and a database construction is required (Scott *et al*., 2004).

Repetitive element PCR are techniques that employ primers corresponding to interspersed repetitive DNA elements present in various locations within the prokaryotic genome to generate highly specific genomic fingerprints (Dombek *et al*., 2000). Three methods of repetitive sequence analysis have been used, with each targeting a specific family of repetitive element. These methods include repetitive extragenic palindromic sequence PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC- PCR) and PCR with extragenic repeating elements (BOX-PCR). The REP primer set generally generates a lower level of complexity, while the ERIC primer set is more sensitive to suboptimal PCR conditions, such as the presence of contaminants in the DNA preparation (Rademaker *et al.*, 1998). The BOX primer has been used in cases where a detailed characterization is needed. This primer generates robust fingerprints and generally yields a highly complex pattern of amplified fragments (Scott *et al.*, 2002). This method has been used by Versalovic *et al.* (1994) to

differentiate between closely related strains of bacteria. Repetitive element PCR, however has been associated with reproducibility concerns, and being dependent on cell culture (Meays *et al*., 2004). Similarly a large database is required, although variability increases as the database increases (Meays *et al*., 2004).

Ribotying is a method of DNA fingerprinting that involves the identification of highly conserved rRNA genes using oligonucleotide probes after treatment of genomic DNA with restriction endonucleases (Carson *et al*., 2001). The method is labour intensive and involves bacterial culture and identification, DNA extraction, gel electrophoresis, southern blotting and discriminant analysis of the resulting DNA fingerprints (Scott *et al.*, 2002). The technique has been very useful in epidemiological studies for various bacteria, including *E. coli* (Stull *et al.*, 1988), *S. enterica* (Olsen *et al.*, 1992), and *V. cholerae* (Popovic *et al.*, 1993). The approach nonetheless is complex, expensive, labour intensive, geographically specific, and also requires setting up a database (Meays *et al*., 2004).

Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool (Schaberg *et al.*, 1981). The number and size of plasmids present in isolates is used as the basis for strain identification. This approach has been used successfully for analysis of outbreaks of nosocomial infections and community acquired infections (Kariuki *et al.*, 1999; Schaberg *et al.*, 1981). Plasmid fingerprinting nevertheless, suffers the effect of bacterial cells losing plasmids due to selective pressure from the surrounding environment to maintain plasmids outside of the host (Scott *et al*., 2002).

Meays *et al*. (2004) has suggest that a choice of which method or combination of MST methods to use for any given situation, depends on a number of factors namely; specific objectives or expected outcomes of the tracking study, availability of resources (cost of analysis varies depending on technique used, and size of the study site), time constraints and turnaround time, and the ability to access labortories or facilities with expertise to analyze the samples. Moreover comparison studies on source tracking methods are needed in order to determine which method works best for watershed studies (Meays *et al*., 2004). Based on Meays *et al*. (2004) MST considerations, this study considered antimicrobial susceptibility profiles, MAR indexing and Discriminant Analysis (DA), since these techniques are easy to use, affordable and therefore amiable for a developing country like Kenya; and potent the ability to determine sources of diarrheagenic bacteria contaminating fish in Lake Victoria.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site

This study was conducted along Lake Victoria in the Nyanza region in Western Kenya, targeting both the rural and urban communities. The study site was located between latitude 1^{o}South – 0^{o} 20'North and longitudes 34^{o} – 34^{o}53'East. Rainfall in the region occurs in two season, long rains commonly occur from March to June and short rains from October to December, with a temperature range from a minimum average of 14^{o}C to 34^{o}C. The Lake lies within an elevated plateau in the Africas Great Rift Valley and the Kenyan component of the lake is 6% with a shoreline of 550km. Generally, fishing, cattle rearing and subsistence farming are the principal occupation for the rural communities within the study area (Brooks *et al.*, 2003). This study targeted five fish landing beaches from four Counties along Lake Victoria, Kenya. The beaches were Sirongo (Siaya County), Dunga (Kisumu County), Homa Bay, Mbita town (both in Homabay County), and Luanda Konyango (Migori County) (Figure 2).

The region has also one of the highest prevalence of human immunodeficiency virus (HIV) of 15.1% among adults 15 – 64 year old compared to a national average of 5.6% (National AIDS and STI Control Programme, 2013). It has also been reported to have a diarrheal infection prevalence rate of 16% among children according to (Kenya National Bureau of Statistics, 2013). These demographics make the study site suitable for studying distribution of diarrheagenic bacteria in the environment; and how the sources of these bacteria influence fish quality in the region.

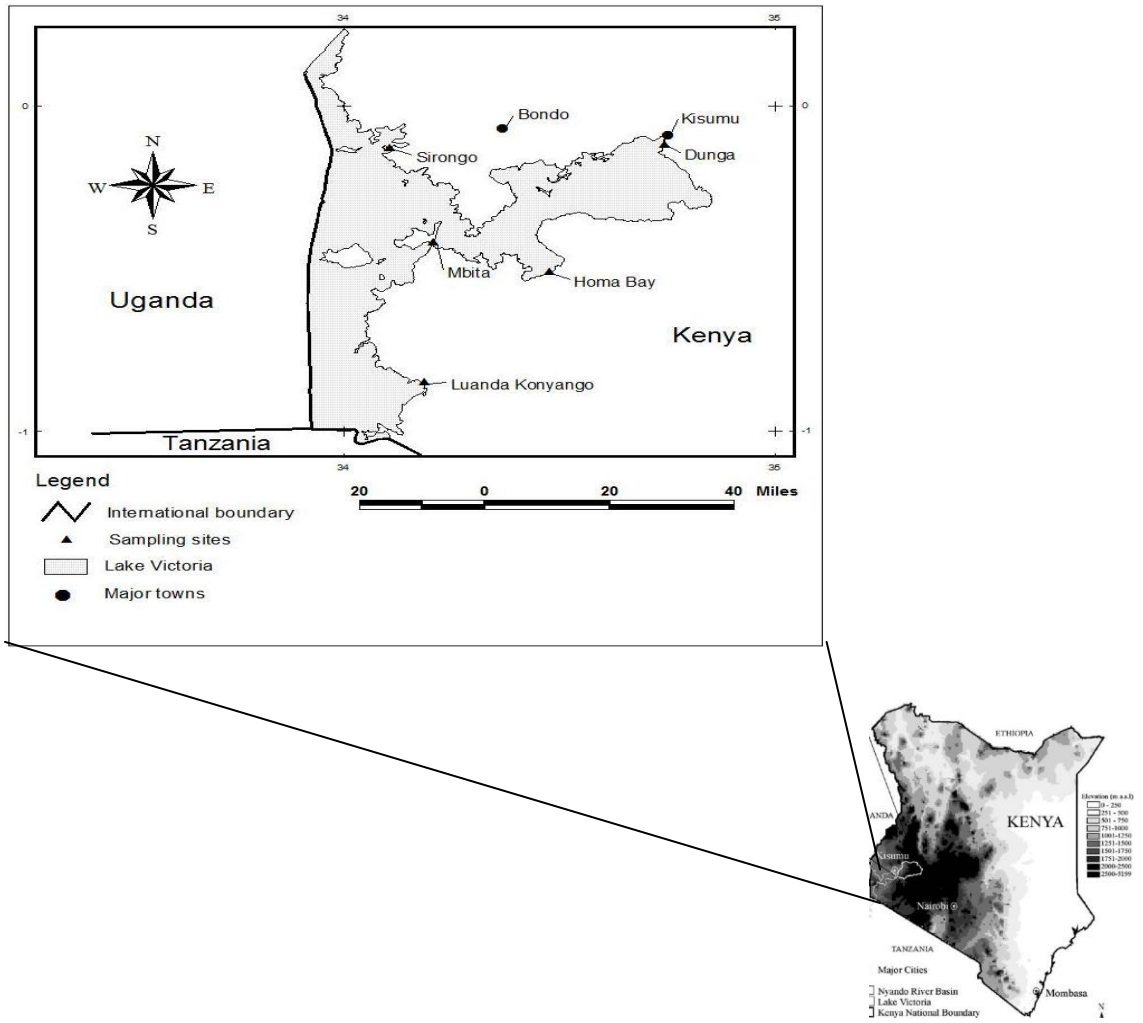

Figure 2: A map of the Nyanza Gulf of Lake Victoria, Kenya showing the sampling sites

3.2 Study Design

This study was based on a repeated sampling within a longitudinal study design. Sampling was repeatedly carried out over a period of two years (2010 and 2011) and samples collected three times in a year (April, August and December). The fecal samples from companion animals -

donkey and farm animals – goats, chicken and cattle were collected; the study also collected samples of Lake Victoria, Kenya water, Lake Victoria fish and soil from the same sites. Human stool samples were collected and analyzed as patients were recruited at the Kisumu East Sub-County hospital (formerly Kisumu East District hospital) for the study. The selection of Kisumu East Sub-County hospital was purposively chosen to act as a control based on its location along Lake Victoria, Kenya and therefore present a picture of the types of diarrheagenic bacteria infecting people within the study site. It was also chosen because of ease of access from Maseno University were bacterial isolations were carried out as sites far away from Maseno may have contributed to logistical and financial challenges in availing human stool specimen within acceptable time durations and conditions.

The five fish landing sites used in this study were chosen based on tonnage of fish production from the fish landing sites (Sirongo, Lunda Konyango and Mbita town), and proximity to an urban town and human activities (Dunga and Homa Bay).

3.3 Sampling Criteria

Animals stool specimens, cattle and donkey, samples were collected at random from animals that come to the lake for watering, whereas chicken and goat stool samples were collected randomly from animals wandering (chicken) or tethered (goats) at the landing site. Only animal samples found within a radius of 500m from the fish landing site were collected for this study.

Fish sampling was based on a systematic sampling plan. Fresh fish were purchased from fishermen as the vessels land based on the schematic sampling procedure described by the Canadian Food inspection Agency (2013). Briefly this was done as described below: -

a. The approximate number of *R. argentea* and Nile perch fishing boats out on the fishing expedition (N).

b. A sample of size n is desired to be collected on the material day

c. k – The sampling interval is determined, where k=N/n (round up).

d. Randomly select a number j between 1 and k.

e. The required systematic sample is then produced by the population (N) units corresponding to the numbers (j, j + k, j + 2k,..., j + (n-1)k).

For example if at a landing site, 10 fishing boats went out for a fishing expedition, then N = 10; and if only 3 samples are to be collected, then n = 3. Therefore: -

k = 10/3 = 3.3 (rounded up to 3)

Therefore; in the case of this study the first boat to land would be sampled and then at intervals of three boats i.e. 4^{th}, 7^{th} etc. For *R. argentea*, the standard measurement of collection of 500g tin (which gives about 100 to 150g of fish) was purchased from the fishermen and the fish traders. Only Nile perch of 500g and above was purchased from the fishermen. The same sampling procedure described above was used to purchase sundried *R. argentea* from market stalls at the landing sites: that is the first stall (chosen at random) and then at internals of three stalls in between, either to the left or right (4^{th}, 7^{th} etc).

Human stool specimen were collected from adult patients (18 years and above) who accepted to sign a consent form after it was explained to them in a language they understood (Appendix 2).

Children (3 months -17 years) consent forms were signed by consenting patients or caregivers. Furthermore, for children between the ages of 12 and 17 years assent by the patient to participate in the study was sort, before consent was given by the parents or caregivers. The inclusion criterion for the study was non- restrictive to either in-patients or out-patients. The following inclusion and exclusion criterion for collecting human specimen was however adopted for this study: -

3.3.1 Inclusion criteria

Patients aged three months old to 17 years presenting with the following signs and symptoms:

1. Those presenting with three or more loose, watery, mucoid or bloody diarrhoea in the last 24 hours.
2. Those presenting with abdominal pain / cramps

3.3.2 Exclusion criteria

1. Those presenting with signs other than the ones described in the inclusion criteria.
2. Those declining consent for participation.
3. Those who were below the age of 3 months. Generally children of less than three months have more frequent, loose and watery stool and may not necessary occur as a result of a diarrheal infection.

The patients or guardians were also asked to respond to a questionnaire seeking to identify where they come from, and their source of food and drinking water.

3.3 Sample Size Determination

The population of domesticated animals within the fish landing sites or visiting the fish landing sites was not known. To determine the number of domesticated animals to be sampled at each beach, the approach by Sayah *et al*, (2005) was adopted for this study and the formula described by Smith (1995) for infinate populations was applied to determine sample size.

Sample size (n_{inf}) = log (α)/log(1 - prev)

Where: -

α = probability that none of the sampled animals harbours diarrhegenic bacteria.

prev = expected prevalence of diarrheagenic bacteria.

Assuming that:

a. The expected prevalence of diarrheagenic bacteria among the domesticated animals is 10% as adopted from Sayah *et al.*, (2005).

b. The type I error is 0.05.

Using the equation and assumptions;

(n_{inf}) = log (0.05)/ log (1 – 0.1)

n_{inf} = -1.30/-0.046

n_{inf} = 28.3

The calculated minimum number of animals to be sampled was 29 per landing site sampled.

At least 3 sample types each of fish viz: - sundried *R. agentea*, fresh Nile perch and fresh *R. argentea* were collected at the landing site or a market near the fish landing site.

For clinical specimen, the number of human stool samples collected was based on CDC EPI Info V.3 (2005) at 5% desired precision and 5% expected prevalence, the minimum sample size was calculated at 73 samples as shown in appendix 8. The 5% expected prevalence is based on Brook *et al*, (2006) report of isolation rates of 5% for Salmonellosis infection among human in western Kenya.

Overall 543 samples were collected during the two year study period (170 - fish, 180 - animal feces and cloacal swabs, 90 - lake water, 30 - soil samples and 73 human stool specimen).

3.4 Ethical Considerations

The research proposal was presented to the Nairobi University/Kenyatta National Hospital Ethics committee (Appendix 3) and the National Commission of Science Technology and Innovation (NACOSTI) (Appendix 4) for clearance. Additionally, during field visits permission was also sought from District Fisheries Officer (Homa Bay, Kisumu, Bondo, Suba, and Migori), Medical Superintendent Kisumu County Hospital and the Beach Management Unit (BMU) officials at the respective study sites. Good Laboratory Practices (GLP) was enforced within the laboratories where the study took place. To avoid contamination and infecting persons working within the laboratories, sterilization of the working surface was done before and after work. Pathogenic microbes were stored in the fridge (8^{o}C) well labeled for further analysis.

3.5 Collection of Samples and Processing

3.5.1 Collection of fish samples

A total of 500g of each fish samples; Nile perch, fresh *R. argentea* and sundried *R. argentea* were purchased from fishermen at landing sites, during and after processing or at the nearest markets. The fish were collected aseptically and placed in sterile plastic containers. Fish

products collected for analysis included fresh Nile perch [*Lates niloticus*] and fresh and sundried *omena* [*Rastrineobola argentea*]. No quality or freshness checks were performed during sample collection. However fish purchased was on offer to regular consumers at markets, whereas the freshly landed fish was that on offer to regular fish traders by fishermen.

3.5.2 Collection of water samples

Three water samples were collected from the Lake surface at each sampling site (at the shores (0m), 100m and 150m) by submerging sterile 100ml bottle to a depth of 30cm below the surface and collecting a 100ml sample. The collected water samples were then labeled viz: - W01/0/DG, where W = water; 01 = first sample; 0 = shoreline (0m); DG = Dunga. The code number was then recorded in the sample collection form (Appendix 1), in which the Beach name, date of sampling.

Other codes for the sampling sites include HB = Homabay, DG = Dunga, SR = Sirongo and LK = Luanda Konyango; W01/100/DG - where 100 = 100m off-shore; W01/150/DG – where 150m = off-shore.

The water samples were then placed on ice in a cool box ready for transportation to Maseno University, microbiology laboratory, department of biomedical science. A fishing boat was used to access the water body.

3.5.3 Collection of soil samples

Soil samples were collected aseptically using a sterilized spoon at landing site and put in sterile containers. At least six samples of top loose surface soil 20g each from different points approximately 6 inches apart from each other within the beach were collected and pooled

together to form representative sample for that site as described by Solo-Gabriele *et al.* (2000). The specimens were then labled viz: - S01/LK, where S = soil; 01 = the first sample; LK = Luanda Kotieno. The code number was then recorded in the sample collection form (Appendix 1).

3.5.4 Collection of stool samples from goat, cattle, donkey and chicken

Approximately 40g of freshly defecated faeces from domesticated animals were aseptically collected using a sterilized spoon and put in sterile containers and labled viz: - G01/SR, where G = goat; 01 = the first sample; SR = Sirongo; D = donkey (D01/SR); C = cattle (C01/SR), CH = Chicken (CH01/SR). The code numbers was then recorded in the sample collection form (Appendix 1).

3.5.5 Human stool specimen

The patients were refered to the laboratory by the hospital clinician. At the laboratory they were provided with stool collection containers and given instruction for stool collection by the laboratory personel. On presentation to the laboratory technologist, about 40g was picked and placed immediately in Cary-Blair transport medium (HIMedia Lab. Pvt. Mumbai, India) and stored at 4^0C - 8^0C for preservation. The stool specimens were later transported on Cary Blair medium placed on ice in an insulated cool box.

The stool specimens were collected in sterile plastic disposable stool cups and labeled viz: - H01, where H = Human and 01 = first sample, the code number was then marked on the individuals' consent forms (Appendix 2). Laboratory results were communicated back to the hospital clinican for follow up and referrals.

All the samples were transported on ice in insulated containers to Maseno University, Biomedical Science and Technology laboratory for analysis.

3.6 Determination of Distribution of *Shigella* spps, *Salmonella* spp, *E. coli* and *Vibrio cholerae* in Fish, Water, Soil. Domesticated Animals and Humans Stool along Lake Victoria, Kenya

3.6.1 Recovery of diarrheagenic bacteria from fish products and water

Enumeration of total coliform counts from water was carried out as described by Anazoo and Ibe, (2005) using the pour plate method. Serial dilutions of $10 - 10^5$ of samples were prepared using buffered peptone water (HIMedia Lab. Pvt.Mumbai, India) from which 1 ml was transferred to sterile petri dishes and MacConkey agar (HIMedia Lab. Pvt.Mumbai, India) that had cooled to about 45^oC added. The plates were left to solidify and later inverted and incubated at 37^oC for 48 hr, after which plates with pink/red colonies (lactose fermenters - coliforms) were enumerated and reported as colony forming units per ml (cfu/ml).

Recovery of *Salmonella ssp* in water, and fish samples was determined as described by FAO, (1992). Briefly, 25g or 25ml of fish sample (muscle) or water sample respectively was weighed aseptically and added to 225ml of buffered peptone water (HIMedia Lab. Pvt.Mumbai, India). Fish samples were homogenized using a stomacher 400 circulator (Seward Ltd; England). Water samples were mixed by whirling the mixture in the hand for 30 seconds. The samples were left to stand on the laboratory bench for 1 hr at room temperature for resuscitation of any injured *Salmonella.* This was followed by direct plating on MacConkey agar (HIMedia Lab. Pvt.Mumbai, India) for recovery of *E. coli*. The inoculated MacConkey agar was then incubated at 37^oC for 18 - 24hr. Using a sterile pipette 1ml was picked and added to 10ml selenite cystene broth (HIMedia Lab. Pvt.Mumbai, India) and 10 ml tetrethionate broth (HIMedia Lab.

Pvt.Mumbai, India) for enrichment. The selenite cystene and tetrethionate broths were incubated at 37^{o}C for 18 - 24hr. This was then followed by plating on Xylose Lysine Desoxycholate (XLD) (HIMedia Lab. Pvt.Mumbai, India) and MacConkey agar (HIMedia Lab. Pvt.Mumbai, India) using a sterile wire loop. The plates were inverted and incubated at 37^{o}C for 18 - 24hr, after which characteristic colonies of *Salmonella* spp (colonies of nonlactose fermenters are translucent or colourless on MacConkey; and red with blackened centers or yellow with blackened centers on XLD for i.e. *S. arizonae*) were picked for further identification using biochemical tests and API.

Recovery of *Shigella ssp* in water and fish samples was also carried out as described by FAO, (1992) herein. Selenite cystene broth (HIMedia Lab. Pvt.Mumbai, India) was used for enrichment, 25g or 25 ml of fish sample or water sample respectively was measured aseptically and added to 225ml of selenite cystene broth and homogenized as described above. The samples were then incubated at 37^{o}C for 18 - 24hr, followed by plating on Xylose Lysine Desoxycholate (XLD) (HIMedia Lab. Pvt.Mumbai, India) and MacConkey agar (HIMedia Lab. Pvt.Mumbai, India) using a sterile wire loop. The plates were inverted and incubated at 37^{o}C for 18hr after which characteristic colonies of *Shigella* spp (colonies nonlactose fermenters are translucent or colourless on Mackonkey and red/colourless on XLD) were picked for further identification using biochemical tests and API.

Recovery of *Vibrio cholerae* from fish and water samples was carried out as described by FAO, (1992). Briefly, 25 g or 25ml of water were aseptically added to 225ml of alkaline peptone water (HIMedia Lab. Pvt.Mumbai, India) and then incubated at 37^{o}C for 18 - 24hr, this was then

followed by plating on Thiosulfate citrate bile salts sucrose (TCBS) agar (HIMedia Lab. Pvt.Mumbai, India) using a sterile wire loop. The plates were inverted and incubated at 37^{o}C for 18 - 24hr after which characteristic colonies of *V. cholerae* (colonies that were slightly flattened, yellow colonies with opaque centers and translucent peripheries) were picked for further identification using biochemical tests and API.

3.6.2 Recovery of diarrheagenic bacteria from goat, donkey, chicken, cattle, human stool specimen

Animal and human faecal materials were analyzed for diarrheagenic bacteria as described by Kariuki *et al.* (1999). Recovery of *E. coli*, *Salmonella* spp and *Shigella* spp were done by direct plating on selective media MacConkey and XLD agar (HIMedia Lab. Pvt. Mumbai, India) using a sterile wire loop, followed by incubation at 37^{o}C for 18 - 24hr. Similarly 5g of stool sample was picked using sterile applicator sticks and inoculated in 10ml selenite F broth (HIMedia Lab. Pvt. Mumbai, India) and 10ml tetrathionate broth (HIMedia Lab. Pvt. Mumbai, India); and incubated at 37^{o}C for 18 - 24hr then followed by plating on MacConkey and XLD agar (HIMedia Lab. Pvt. Mumbai, India). The plates were inverted and incubated at 37^{o}C for 18 - 24hr after which characteristic colonies showing *Salmonella* and *Shigella* spps (characteristics as described in 3.6.1) were picked for further identification using biochemical tests and API.

Vibrio cholerae, was recovered by inoculating alkaline peptone water (HIMedia Lab. Pvt. Mumbai, India) with 5g of stool sample using a sterile applicator stick. The inoculated samples were then incubated at 37^{o}C for 18 - 24hr for enrichment. This was followed by plating on TCBS agar (HIMedia Lab. Pvt. Mumbai, India) using a sterile wire loop and incubating it at

37^{o}C for 18 - 24hr, after which characteristic colonies suspected to be *V. cholerae* were picked for further identification using biochemical tests and API.

3.6.3 Recovery of diarrheagenic bacteria from soil

The moisture content of the soils, recovery of *E. coli*, *Shigella*, *Salmonella* and *V. cholerae* was carried out using standard procedures as described by van Elsas and Smalla, (1997). Briefly, 1 – 2 spoonfuls of the pooled soil sample were placed in a pre-sterilized, pre-weighed Whirl Pak bag. An aliquot of the sample was portioned and used for moisture content analysis at 110^{o}C for 16hr in an oven (Binder; Germany). Moisture content of soil was determined to establish if it had influenced survival and distribution of diarrheagenic bacteria in soil along Lake Victoria, Kenya.

The remaining soil samples were used for the recovery of *E. coli*, *Shigella* and *Salmonella* by adding 25ml of sterile phosphate–buffered water. The mixture of phosphate–buffered water and soil was mixed in the bag for 2mins and then filtered through a pre-sterilized 28µm – pore – size nylon filter. The filtration steps were repeated until 100ml of phosphate-buffered water was collected. The 100ml sample was then used to analyze for the presence of *E. coli*, *Shigella* and *Salmonella* as describe above for stool samples. The same procedure was repeated using alkaline peptone water instead of phosphate buffered water for *V. cholerae* analysis. Incubations were at 37^{o}C for 18 - 24hr.

3.7 Identification of Bacterial Isolates

Identification and confirmation of bacterial isolates was performed using standard biochemical techniques as described by Ewing, (1986). Four colonies resembling *E. coli*, *Shigella*, *Salmonella* and *V. cholerae* in each case were randomly picked from selective media and plated

on Nutrient agar (HIMedia Lab. Pvt. Mumbai, India), and then confirmed by biochemical tests and API 20 E strips (BioMerieux; France).

The isolates were then stored at -20^{o}C in tryptic soy broth with 15% glycerol until used in other experiments.

3.7.1 Biochemical test

Triple Sugar Iron (TSI) test: The TSI agar (HIMedia Lab. Pvt.Mumbai, India) tubes were inoculated using a sterile straight inoculating needle, by stabbing the inoculum down through the butt (3/4 way the butt), then pulling the needle out and streak up the slant. The tubes were then incubated at 37^{o}C for 18 - 24hr. The results were interpreted as shown in appendix 7.

Motility Indole Lysine medium (MIL) test: MIL (HIMedia Lab. Pvt.Mumbai, India) tubes were inoculated by stabbing using a sterile needle with growth from an 18 - 24 hr pure culture. The tubes were then incubated at 37^{o}C for 18 - 24 hr. After incubation the tubes were examined for evidence of lysine deaminase, motility, lysine decarboxylase reactions and, after addition of Indole Reagent Kovacs (HIMedia Lab. Pvt.Mumbai, India), indole production. The results were interpreted as shown in appendix 7.

Simmon Citrate test: The Simmon Citrate (HIMedia Lab. Pvt.Mumbai, India) tubes were inoculated as describe above for the TSI test. The tubes were then incubated at 37^{o}C for 4 days

Interpretation

E.coli - negative

Salmonella – positive

Shigella – negative

V. cholerae – variable

Analytical Profile Index (API) test: The API 20E System (BioMerieux, France), are standardized, microbiological identification procedure for members of the *Enterobacteriaceae* family, consists of miniaturized microtube system with capability of 21 conventional biochemical tests. The experiments were performed according to manufactures instructions. Briefly a preparation of bacterial suspension was made by transferring 2 -3 well isolated colonies using a wooden applicator stick into tubes of 0.85% saline, the sticks were rotated to release and suspend the bacteria into solution. Using a 5ml Pasteur pipette the bacterial suspension was introduced into the microtubes by placing the pipette tip against the side of the cupule. The strips were then incubated at 37^{o}C for 18 – 24hr. After incubation, identification of the isolates was achieved by inferring the numerical profile read from the strip combinations to the reading table (Appendix 8). Positive results with ≥89% probabilities were confirmed as *Salmonella* spps, *E. coli* or *Shigella* spps (Figure 3).

Figure 3: API strips

All the media were quality controlled using *E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028 and checked for sterility by incubating un-inoculated plate at 37^0C for 18h.

3.7.2 Serological testing of *Salmonella*

Salmonella isolates were further confirmed by serotyping using commercial antisera "O" somatic and Vi antigen (Denka Seikan; Japan) using slide agglutination tests. Briefly, two separate drops of normal saline (0.85% sodium chloride) were placed on a clean glass slide. A small part of an overnight culture of *Salmonella* suspected colony was mixed thoroughly with both drops of normal saline on the slide to obtain a smooth suspension, after which a one drop of antisera was added to one of the bacterial suspension drops on the slide; to the other (control) one drop of normal saline was added. The antiserum with the bacterial suspension was mixed using a sterile loop and by gently tilting the slide back and forth for one minute. The mixture was then observed for agglutination under normal lighting conditions, using a magnification lens.

The results were interpreted as follows; a distinct agglutination (granular clumping) within 60 seconds, without agglutination in the saline control (auto-agglutination) was regarded as a positive result.

3.8 Determination of *E. coli* Pathotypes Present among Fish, Water, Domesticated Animals, Soil and Humans along Lake Victoria, Kenya

Confirmed isolates from the previous cultures as *E. coli* were probed for virulence factors by multiplex PCR as described by Panchalingam *et al*, (2012). A total of 134 *E. coli* isolates were subjected to pathotyping experiments. The PCR primers for EPEC included the intimin (*eae*) and the bundle forming pili A (*bfpA*); ETEC genes included the heat-stable enterotoxin (*estA*)

and heat – labile enterotoxins (*eltB*); whereas EAEC genes included the plasmid encoded (*aatA*) and the chromosomally encoded (*aaiC*). In this regard, DNA from the bacterial isolates was isolated by the boiling method. A whole-cell preparation of bacterial strains was prepared by resuspending 3 bacterial colonies from MacConkey agar in 500µl of sterile DNase – free, RNase – free deionized water (GIBCO). The *E. coli* suspension was then heated on a heating block (Techne; UK) at 100^{o}C for 10 mins. The suspension was then cooled to 4^{o}C for 10 mins and then centrifuged at 14000g for 2 min in a microcentrifuge (Eppendorf Centrifuge 5417R; Germany). The supernatant was then used as a source for DNA template in the experiment. Each test was performed in volume of 20µl containing the following PCR components: 2.0µl of 1.25mM dNTP (Fermentas; Thermo Scientific), 2.5 µl PCR buffer, $MgCl_2$ (final concentration 2mM) and 20 pmol/µl of each primer (Eurofins, UK) (Table 3.1), 0.25 µl of 1.25U of *Taq* DNA polymerase (Bio Labs; New England), and 3µl of DNA template (50ng) and topped up with 7.37µl of H_2O. DNA isolated and purified from reference *E. coli* JM042 (EAEC), H10407 (ETEC) and E 2348/69 (EPEC) were used as controls. A multiplex PCR was performed in Eppendorf Mastercycler (Hamburg) under the following conditions: preheating at 96^{o}C for 4 mins, followed by 35 cycles beginning with 20 sec of denaturation at 95^{o}C, 20 sec of primer annealing at 57^{o}C, and 1min of elongation at 72^{o}C and final extension at 72^{o}C for 7mins. The amplified PCR products (10 µl aliquots) was mixed with 3 µl of loading dye (Bio Rad; USA) was analyzed by electrophoresis in 2% agarose gels (Sigma; USA) in 1 x Tris Borate - EDTA buffer (Sigma Life Science; USA) at 100V. The gels were stained with ethidium bromide (2mg/ml) and photographed on an UV transilluminator. A 100bp molecular ruler (Bio Rad; USA) was used to estimate molecular weights of the PCR products.

Table 3.1: Primer sequences used in this study

Pathogen	target gene (volume)	primer sequence (5' – 3')	Amplicon (bp)	GenBank accession no./Reference
ETEC	*elt*B (0.4 µl)	F: CACACGGAGCTCCTCAGTC	508	Panchalingam
		R: CCCCCAGCCTAGCTTAGTTT		et al (2012)
	*est*A (0.4 µl)	F: GCTAAACCAGTAG/AGGTCTTCAAAA	147	M34916
		R: CCCGGTACAG/AGCAGGATTACAACA		
EPEC	*bfp*A (0.4 µl)	F: GGAAGTCAAATTCATGGGGGTAT	300	Stacy-Phippes
		R: GGAATCAGACGCAGACTGGTAGT		et at (1995)
	eae (0.44 µl)	F: CCCGAATTCGGCACAAGCATAAGC	881	Panchalingam
		R: CCCGGATCCGTCTCGCCAGTATTCG		et al (2012)
EAEC	*aat*A (0.4 µl)	F: CTGGCGAAAGACTGTATCAT	650	Panchalingam
		R: AATGTATAGAAATCCGCTGTT		et al (2012)
	*aai*C (0.4 µl)	F: ATTGTCCTCAGGCATTTCAC	215	Panchalingam
		R: ACGACACCCCTGATAAACAA		et al (2012)

3.9 Determination of Antimicrobial Susceptibility among Diarrheagenic Bacteria

Bacterial isolates obtained were examined for antibiotic resistance using the standard Kirby-Bauer disk diffusion method. The susceptibility panel was chosen on the basis of treatment of infections due to Gram negative bacteria. Six antibiotics namely ampicillin (10 µg), tetracycline (30 µg), cefuroxime (30 µg), nalidixic acid (30 µg), chloramphenicol (30 µg) and gentamicin (10 µg) (Oxoid Inc, UK) were used to test for susceptibility. Bacterial inoculums was prepared by suspending the freshly grown bacteria in 5 ml sterile 0.85% normal saline and the turbidity adjusted to that of a 0.5 McFarland standard (10^8 cfu). Mueller – Hinton medium (HIMedia Lab. Pvt.Mumbai, India) plates were swabbed in replicates for each isolate tested and the 6 commercially prepared antimicrobial agent disks placed on each of the inoculated plates. The plates were then incubated at 37°C for 18 to 20h. The diameters (in millimetres) of clear zones of growth inhibition around the antimicrobial disks, including the 6 mm disk diameter were measured by using precision calipers (Clinical and Laboratory Standards Institute (CLSI), 2008). A standard reference strain of *E. coli* (ATCC 25922) and a plate which lacked the 6 antimicrobials was used as a control. The breakpoints used to categorize isolates as resistant to each antimicrobial agent were those recommended by CLSI, (2008).

3.10 Determination of Origins of *E. coli* Contaminating Fish along Lake Victoria

A source library for determining *E. coli* contaminating fish was developed from antibiotic disc diffusion zones of *E. coli* isolates obtained from human, domesticated animals and environmental samples as described by Guan *et al,* (2002). Discriminant function models were generated for the different species classification groups using Minitab 14. A classification table was generated by Minitab 14 and used to generate the percentage of classified isolates and determine the average rate of correct classification (ARCC) and attribute possible source of *E.*

coli isolated from fish in the study. The table showed source-by-source matrix in which the numbers and percentages of correctly classified isolates are found on the diagonal (Tables 4.14 - 4.16).

3.11 Data Analysis

Generated data in this study was entered in Ms Excel Windows XP professional 2003 and analyzed by Minitab version 14. The data collected was however combined for analysis over the period of study, based on the sources of bacterial isolates and or sampling points (fish landing sites). Levels of total coliform counts were transformed to $\log_{10}$ before analysis. Descriptive statistics were generated to assess the occurrence and distribution of diarrheagenic bacteria based on sources; moisture content, pH, coliform counts and diffusion zones of bacteria among the sources sampled. Analysis of variance (ANOVA) with Tukey's family test used for comparison among groups was used to test for differences in moisture content and coliform counts based on sources and sites. Kruskal-Wallis tests were used to test for diffusion zones across different groups. Discriminate function models were generated for the different species classification groups using Minitab 14.

MAR index was calculated using the formula $a/(b - c)$

Where,

a = the aggregate antibiotic resistance score of all isolates from the sample.

b = the number of antibiotics.

c = the number of isolates from the sample. Source: Krumperman (1983).

Sources of the *E. coli* isolates were then classified based on the MAR indices calculated and the proportion of multiple antibiotic resistance patterns displayed.

CHAPTER FOUR: RESULTS

4.1 Distribution of *Shigella* spps, *Salmonella* spp, *E. coli* and *Vibrio cholerae* in Fish, Water, Domesticated Animals, Soil and Humans along Lake Victoria, Kenya.

A total of 543 samples were collected during the study period, viz: - 170 - fish, 180 - animal feces and cloacal swabs; 90 - lake water, 30 - soil samples and 73 human stool specimen. The study achived a ratio of 1: 1.4 (30:43) male to female ratio for human with the lowest age being 9 months and the oldest being 63 years.

A total of 73 patients were recruited into the study, most of the patients were in the aged between 6 – 10 years representing 26%, followed by 0 – 5 years (23.3%), age stratum 16 – 20 years, (6.8%) had the lowest number of participants (Table 4.1).

The study established that 42.5% of the participants sourced their water from water Kiosk supplied by the local authority water system, whereas 28.8% had piped water supplied by the local authority in thier homes. Only 5.5% of the participants sourced water from private boreholes (Table 4.1).

Table 4.1: Age distribution of patients and types of water sources used by patients who participated in the study

Age range (years)	**frequency (%)**
0 – 5	17 (23.3%)
6 – 10	19 (26.0%)
11 – 15	7 (9.6%)
16 – 20	5 (6.8%)
21 – 25	7 (9.6%)
26 – 30	8 (11%)
> 30	10 (13.7%)
Total	**73 (100%)**
Type of water source	
Home supply (Local authority)	21 (28.8%)
Water Kiosk (Local authority)	31 (42.5%)
Commercial supply (hawkers)	17 (23.2%)
Borehole (private)	4 (5.5%)
Total	**73 (100%)**

Based on the isolates recovered from various source types, *Shigella* spp (2.7%) was only recovered in human specimens, while *E. coli* was recovered from all sources with varying prevalence of 91.8% in human, 90 % in goats,82% in chicken and 80% in donkey as shown in Table 4.2. Fresh fish products namely Nile perch and *R. argentea* had the lowest *E. coli* prevalence rates of 7.3% and 3.5% respectively. Dried *R. argentea* recorded prevalence of 15.5%. Of the 10 sources, *Salmonella* spp in the soil was recorded the highest (16.7%) followed by *R. argentea* (8.6%). However, no *Salmonella* spp was recovered from fresh *R. argentea* and cattle. No *V. cholerae* was recovered from all the specimens investigated in this study.

Table 4.2: Percentage occurrence rates of various pathogens based on sources

		(%) Occurrence			
Source	**n**	***Shigella***	***E. coli***	***Salmonella***	***V. cholerae***
Human	73	2 (2.7%)	67 (91.8%)	5 (6.8%)	0 (0%)
Soil	30	0 (0%)	9 (30%)	5 (16.7%)	0 (0%)
Nile perch (fresh)	55	0 (0%)	4 (7.3%)	2 (3.6%)	0 (0%)
R. argentea (fresh)	57	0 (0%)	2 (3.5%)	0 (0%)	0 (0%)
R. argentea (dried)	58	0 (0%)	9 (15.5%)	5 (8.6%)	0 (0%)
Lake Water	90	0 (0%)	27 (30%)	0 (0%)	0 (0%)
Cattle	62	0 (0%)	43 (69.4%)	0 (0%)	0 (0%)
Donkey	20	0 (0%)	16 (80%)	1 (5%)	0 (0%)
Chicken	50	0 (0%)	41 (82%)	1 (2%)	0 (0%)
Goat	50	0 (0%)	45 (90%)	2 (4%)	0 (0%)
Total	**543**	**2**	**263**	**21**	**0**

Soil and human samples presented diverse *Salmonella* serovars; namely *S. enterica* Arizonae (4.1% in human and 3.3% in soil), *S. enterica* group E (3.3% in soil) and *S. enterica* nontypable forms (10% in soil and 1.4% in human), with *S. enterica* subspacies *pullorum* (1.4%) only recovered from human. *Salmonella enterica* group E (5.2%) was also recovered from sundried *R. argentea*, whereas 3.2% and 3.6% of the nontypable forms were recovered from sundried *R. argentea* and Nile perch respectively. Moreover, *Salmonella enterica* subspecies *arizonae* was recovered from goat (4%), chicken (2%) and donkey (5%), but not in *R. argentea* and Nile perch as shown in Table 4.3.

Table 4.3: *Salmonella* serovars recovered in the study based on sources

		Groups of *Salmonella* (%)				
Source	**n**	***S. arizonae***	***S. pullorum***	**Group E**	**non typable**	**total**
Human	73	3 (4.1%)	1(1.4%)	-	1(1.4%)	5 (6.9%)
*R. argentea**	58	-	-	3 (5.2%)	2 (3.4%)	5 (8.6%)
R. argentea^{+}	57	-	-	-	-	-
Nile perch	55	-	-	-	2 (3.6%)	2 (3.6%)
Soil	30	1(3.3%)	-	1(3.3%)	3 (10%)	5 (16.7%)
Goat	50	2 (4%)	-	-	-	2 (4%)
Chicken	50	1(2%)	-	-	-	1 (2%)
Donkey	20	1(5%)	-	-	-	1(5%)
Total	**543**	**8**	**1**	**4**	**8**	**21**

Key: * Sundried *R. argentea*; $^{+}$ Fresh *R. argentea*

Findings of this study show that *Salmonella* spp infections were widely distributed across the different age groups attending hospital. However, *S. pullorum* (14.3%) infection was reported from one patient in the group range of 21 – 25 years, whereas *Salmonella enterica* (non typable) infection was recorded among group range 0 – 5 years (5.9%) as shown in Table 4.4. Among the age strata 16 – 20 years and 26 – 30 years no *Salmonella* infections was recorded, whereas *Salmonella* infection rate (14.3%) was highest among age strata 11 – 15 years and 21 – 25 years. *Salmonella enterica* sub-species *arizonae* infections were reported among different age groups with varied infection rates 6 -10 years (5.3%), 11 – 15 years (14.3%) and > 30 years (10%). *Shigella* infections were recorded among age strata 11 – 15 years (14.3%) and > 30 years (10%).

Table 4.4: *Salmonella* serovars and *Shigella* spps recovered from human based on the age strata

Age range (years)	n	Pathogen recovered	% infection rate
0 – 5	17	*Salmonella* spp	5.9%
6 – 10	19	*S. arizonae*	5.3%
11 – 15	7	*S. arizonae, Shigella* spp	14.3%*
16 – 20	5	-	0%
21 – 25	7	*S. pullorum*	14.3%
26 – 30	8	-	0%
≥ 30	10	*S. arizonae*, *Shigella* spp	10%*
Total	**73**		

Legend: * *Shigella* spp has similar % infection rate

Findings of this study demonstrate that total coliform counts in lake water reduce progressively away from the shore line (0m) to 150m offshore. The shores had the highest (3.75cfu/ml ± 0.05) mean levels with lowest levels at this point being 3.4cfu/ml and the highest 4.3cfu/ml. The 100m sampling point recorded 2.99cfu/ml ± 0.09 and 150m point 2.71 cfu/ml ± 0.07 as shown in Table 4.5. The study findings indicate statistically significant differences ($p = 0.0001$) among respective sampling points (shore (0m), 100m and 150m). There is statistically significant differences in sampling points and distance from the shore based on ANOVA at shore and 100m from the shore ($p = 0.017$ and $p = 0.04$) respectively. Based on the Tukey's family test, there

were statistically significant differences observed between Homa Bay and Luanda Konyango beaches at shores sampling point and Kisumu and Luanda Konyango beaches at sampling point (100m). There was no statistically significant differences observed at 150m ($p = 0.92$). At shore sampling point, Luanda Konyango recorded the highest mean (4.06± 0.06cfu/ml) with the lowest and highest levels at these points being 3.9cfu/ml and 4.2cfu/ml respectively as shown in Table 4.6. At sampling point 100m, Luanda Konyango also recorded the highest mean colony forming unit 3.44± 0.1cfu/ml, whereas at sampling point 150m, Homa Bay recorded the highest levels 2.85± 0.12cfu/ml.

Table 4.5: Levels of total coliform counts recorded based on the distance from the shore (0m) to 150m into the lake

		$(\log_{10})$ cfu/ml			
Sampling point	**n**	**Mean**	**min**	**max**	**95% CI**
Shore	30	3.75 ± 0.05	3.4	4.3	0.11
100m	30	2.99 ± 0.09	2	3.78	0.18
150m	30	2.71± 0.07	2	3.48	0.15

Table 4.6: Levels of total coliform counts based on sampling points and distance from the shore (0m)

	$(\log_{10})$ cfu/ml			
Sampling point	**Mean**	**min**	**max**	**95% CI**
Shore				
Homa Bay	3.54± 0.1	3.43	3.92	0.26
Dunga	3.69± 0.07	3.48	3.9	0.2
Sirongo	3.74± 0.05	3.65	3.93	0.14
L. Konyango	4.06± 0.06	3.9	4.2	0.16
Mbita	3.7± 0.16	3.4	4.3	0.44
100m				
Homa Bay	2.85± 0.11	2.6	3.26	0.32
Dunga	2.7± 0.26	2	3.6	0.71
Sirongo	3.1± 0.15	2.6	3.48	0.42
L. Konyango	3.44± 0.1	3.18	3.78	0.28
Mbita	2.87± 0.15	2.3	3.18	0.41
150m				
Homa Bay	2.85± 0.12	2.6	3.3	0.35
Dunga	2.69± 0.21	2.3	3.48	0.57
Sirongo	2.7± 0.19	2	3	0.52
L. Konyango	2.67± 0.18	2	3	0.5
Mbita	2.62± 0.18	2	2.95	0.48

Soil moisture content

Soil moisture content was monitored to establish any variation along the study sites and also determine if it influenced survival and recovery of targeted diarrheagenic bacteria in the soils. The study findings show that Homa Bay recorded the highest mean of the soil moisture content at 13.02% ± 4.2 followed by Dunga beach with levels of 13.01% ± 5.2. Luanda Konyango beach had the lowest levels of 1.05% ± 0.3. However there was no statistical significant differences among the sampling sites based on ANOVA ($p = 0.15$). Nonetheless the findings show large standard deviations among Homa Bay and Dunga beach indicating large variations among individual samples collected over the study period as shown in Table 4.7. Luanda Konyango recording the smallest standard error of 0.3.

Table 4.7: Soil moisture content levels based on sampling sites

Sampling site	mean	min	max	95% CI
Homa Bay	13.02 ± 4.2	2.1	26.7	11.05
Dunga	13.01 ± 5.2	2.27	30.7	13.3
Mbita	10.5 ± 3.5	2.7	24.1	8.9
Sirongo	8.9 ± 2.6	2.43	15.7	6.8
L. Konyango	1.05 ± 0.3	0.47	2.4	0.74

The study findings show that *E. coli* was recovered in soils of a wide moisture content range (2.1 – 27.7%), whereas *Salmonella* spp was recovered from soils of moisture content levels of 13.9 – 27.7% as shown in table 4.8.

Table 4.8: Moisture content ranges and the types of bacteria recovered with respect to sampling sites

Organism	% Moisture content levels	Recovery sites
E. coli	2.1 – 27.7%	Mbita, Homa Bay, Sirongo, Dunga
Salmonella spp	13.9 - 27.7%	Sirongo, Mbita and Dunga

The study also recovered other members of the *Enterobacteriaceae* family as shown in Figure 4 and appendix 6, which included *Enterobacter* spp (21.6%), *Citrobacter* spp (6.8%), *Proteus* spp (3.7%), *Klebseilla* spp (6%), *Seriatia* (1.4%), *Providencia* (2.7%), *Pantoea* (0.2%) and *Morganella* spp (0.4%). The study findings show that *Enterobacter* spp were the second most predominant bacteria after *E. coli* among the *Enterobacteriaceae* recovered in this study as shown in Appendix 6. *Seriatia* spp and *Pantoea* spp were only recovered from lake water, whereas *Morganella* spp was only recovered among sundried *R. argentea*. *Escherichia hermannii* was recovered from chicken only.

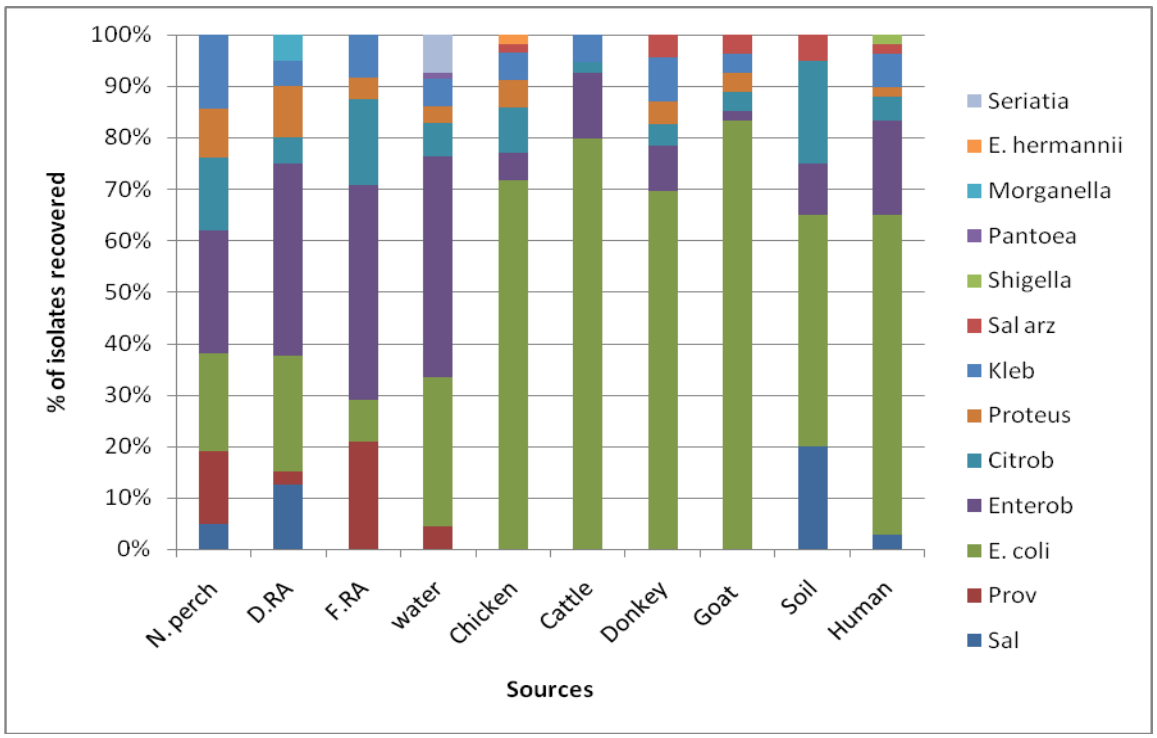

Figure 4: Percentage recovery of members of *Enterobacteriaceae* from the different sources

Key: N = Nile; D.RA = dried *R. argentea*; F. RA = Fresh *R. argentea*; Sal arz = *Salmonella arizonae*; *Klebseilla* spp; Citrob = *Citrobacter* spp; Enterob = *Enterobacter* spp; Prov = *Providencia* spp; Sal = *Salmonella* spp.

4.2 Determination of *E. coli* pathotypes Present in Fish, Water, Domesticated Animals, Soil and Humans along Lake Victoria, Kenya.

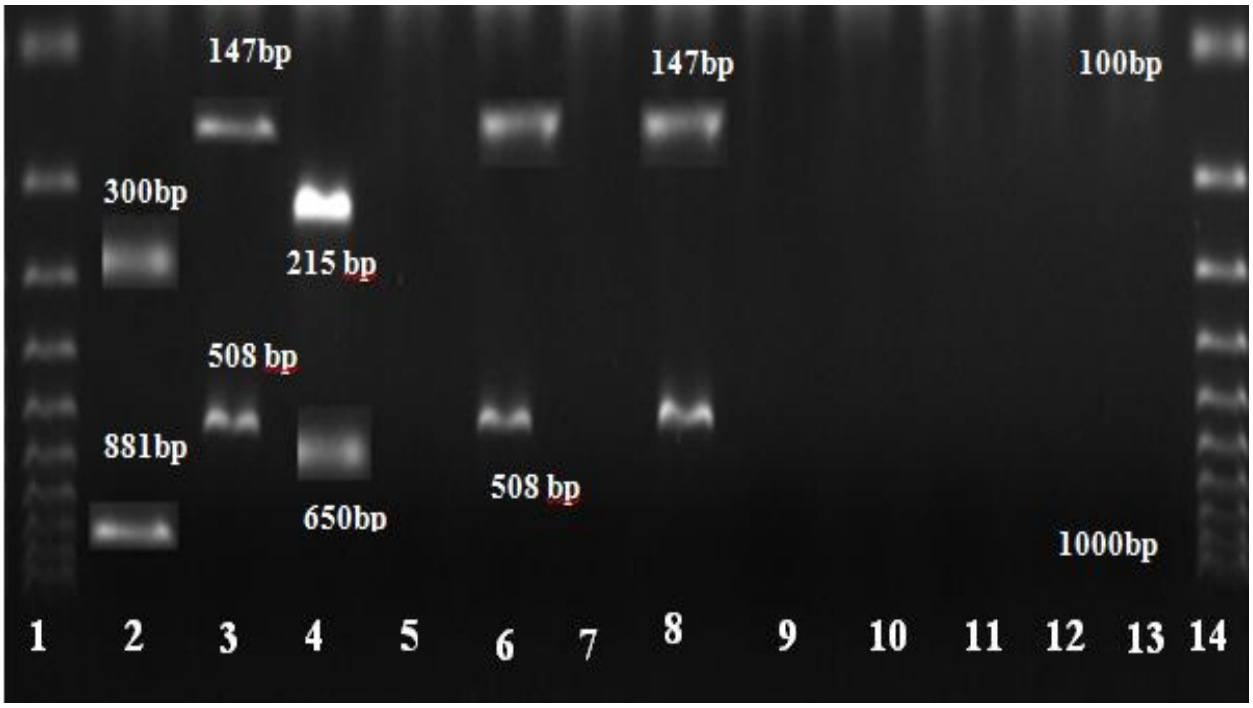

Figure 5: Agarose gel showing multiplex polymerase chain reaction (mPCR) assays for detection of virulence genes for *E. coli* isolates recovered from human

Lane 1 and 14: 100bp molecular marker, lane 2: positive control EPEC (*bfA* [300 bp] and *eae* [881] genes); lane 3: positive control (ETEC) (*eltB* [508 bp] and *estA* [147 bp] genes); and lane 13:positive control EAEC (*aatA* [650 bp] and *aaiC* [215 bp] genes); Lane 5: negative control (water); lanes 6 and 8: *E. coli* strains recovered from children, positive for ST and LT genes (ETEC); lanes 7, 9, 10, 11, 12 and 13: *E. coli* strains recovered from human (negative for all the three pathogenic markers: *aatA* [650 bp], *aaiC* [215 bp], *eltB* [508 bp], *estA* [147 bp], *bfA* [300 bp] and *eae* [881] genes).

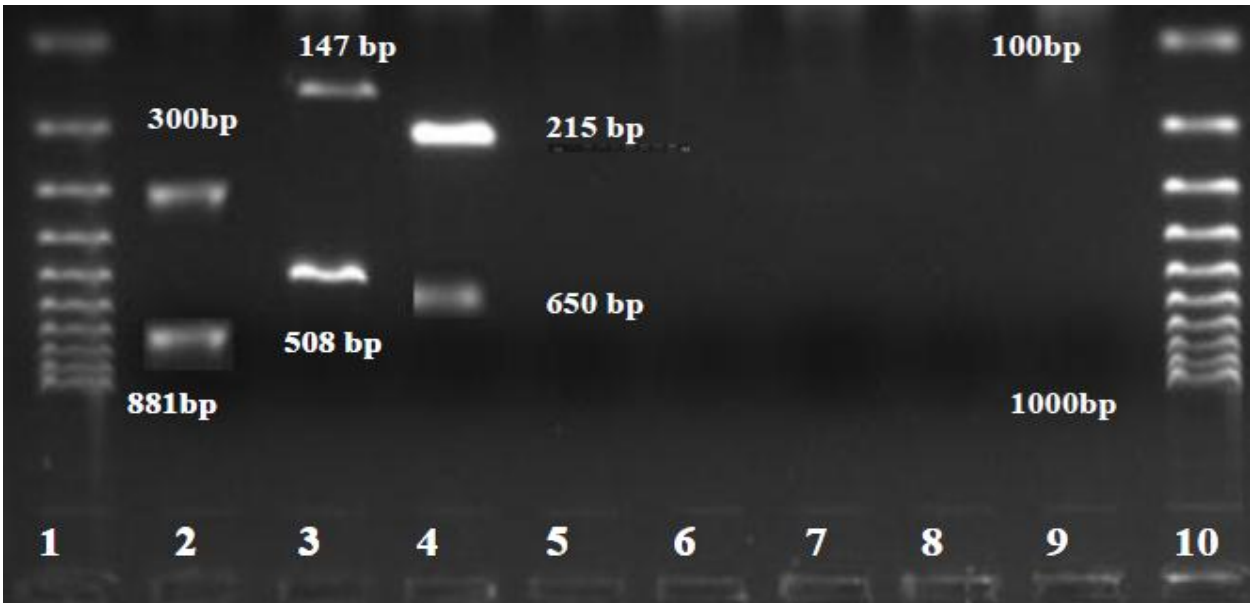

Figure 6: Agarose gel showing multiplex polymerase chain reaction (mPCR) assays for detection of virulence genes of *E. coli* isolates recovered from *R. argentea* and soil

Lane 1 and 10: 100bp molecular marker; Lane 2: positive control EPEC (*bfA* [300 bp] and *eae* [881] genes); Lane 3: positive control ETEC (*eltB* [508 bp] and *estA* [147 bp] genes) and lane 4: positive control EAEC (*aatA* [650 bp] and *aaiC* [215 bp] genes); Lane 5: negative control (water); lanes 6 and 7: *E. coli* strains recovered from *R. argentea* (negative for the three pathogenic markers: *aatA* [650 bp], *aaiC* [215 bp], *eltB* [508 bp], *estA* [147 bp], *bfA* [300 bp] and *eae* [881] genes); lanes 8 and 9: *E. coli* strains recovered from soil (negative for the three pathogenic markers: *aatA* [650 bp], *aaiC* [215 bp], *eltB* [508 bp], *estA* [147 bp], *bfA* [300 bp] and *eae* [881] genes)**.**

The study findings show that ETEC were recovered among children (Figure 5), however ETEC, EPEC and EAEC were absent among cattle, donkey, goat, chicken, fish and the environment

(Figure 6) within the study sites. Among the clinical samples, ETEC was recovered among children with infection rates of 5.9% among children of age ≤ 5 years and 5.3% among children between the ages of 6 to 10 years as shown in Table 4.9. Overall an infection rate of 5.5% was found among children of 0 – 10 years in this study (n = 36).

Table 4.9: Distribution of ETEC infection among human stool samples with respect to age groups

Age range (years)	n	Pathogen recovered	% infection rate
0 – 5	17	ETEC	5.9%
6 – 10	19	ETEC	5.3%
≥11	37	-	-
Total	**73**		

4.3 Determination of Antimicrobial Susceptibility Levels within Diarrheagenic Bacteria Recovered from the Fish, Water, Domesticated Animals, Soil and Humans along Lake Victoria, Kenya

Antimicrobial susceptibility profiles for the isolates in this study showed that 52.2% (n =134) of *E. coli* were resistant to at least one class of antibiotic and 37.3% (n = 134) to more than one antibiotic, whereas 28.6% (n = 21) of *Salmonella* spp were resistant to at least one class of the antibiotics tested, and 9.5% (n = 21) to more than one antibiotic. All the *Shigella* isolates were

resistance to two antibiotics (ampicillin and tetracycline). Overall 49.7% (n = 157) of the isolates recovered in this study were found to be resistant to at least one antibiotic tested.

Among the different sources investigated in this study, isolates from human showed resistance to the highest number of antibiotics tested. *E. coli* Isolates from fish (n = 13) and soil (n = 3) recorded the highest resistance levels at 100% for tetracycline, followed by human *E. coli* isolates at 75% (n = 52). *Salmonella* isolates from chicken, donkey, *R. argentea* and soil were susceptible to all the six antibiotics tested. However human (n = 59) isolates were highly resistant to ampicillin (80%) and tetracycline (60%) as shown in Table 4.10.

Table 4.10: Percentage (%) rates of resistance against individual antibiotics tested based on different sources and types of bacteria

			% rates of resistance					
Type of bacteria	**n**	**Source**	**Tet**	**Amp**	**C**	**Na**	**Gn**	**Cxm**
E. coli (52.2%)* (37.3%)**	13	Chicken	30.8	30.8	-	-	-	-
	23	Cattle	8.7	13.04	-	-	-	-
	5	Donkey	20	20	-	-	-	-
	16	Goat	18.8	12.5	-	-	-	-
	52	Human	75	65.4	36.5	36.5	9.6	15.4
	13	Fish	100	38.5	-	15.4	-	15.4
	3	Soil	100	33.3	33.3	-	-	-
	9	Lake water	44.4	33.3	-	11.1	-	22.2
Salmonella (28.6%)*	1	Goat	-	100	-	-	-	-
(9.5%)**	5	Human	60	80	20	40	20	40
Shigella (0%)* (100%)**	2	Human	100	100	-	-	-	-

Key: Tet = tetracycline; Amp = ampicillin; C = chloramphenicol; Cxm = cefuroxime; Gn = Gentamicin; Na = Nalidixic acid; * = % resistance against at least one antibiotic; ** = % resistance to more than one antibiotic

A total of 18 antibiogram patterns were observed in this study. Tetracycline (14%) had the most common antibiotic resistance pattern among the tested antimicrobials. This was followed by a combination of ampicillin and tetracycline resistance pattern at 10.8% and 7.6% for resistance to ampicillin only (Table 4.11). *Escherichia coli* and *Salmonella* spp displayed different resistance patterns, whereas *Shigella* spp showed only a combined resistance pattern against ampicillin and tetracycline.

Salmonella Arizonae showed 50% resistance to at least one antibiotic. Generally, the study observed that *S.* Arizonae recovered from goat was resistant to only ampicillin, whereas those recovered from other livestock and soil including one recovered from human were susceptible to all the six drugs tested (Tet-Amp-Na-C-Gn-Cxm) Table 4.11. All the *Salmonella* spp isolated from soil and fish samples were susceptible to all the six antimicrobials tested. *Salmonella* Pullorum recovered from human was only resistant to tetracycline. Comparison of mean inhibition zones for *Salmonella* isolates recovered from human and the environment (soil, livestock and fish), indicated that human isolates had the smallest mean inhibition zones for all the tested antimicrobials. Comparing the mean values obtained using the t-test; there was statistical significant difference between the means, $p = 0.01$, with a negative moderate correlation (Pearson correlation = -0.06).

Eighteen distinctive antibiotic resistance patterns were observed altogether within the *E. coli* isolates recovered in this study. The most frequent antimicrobial resistance observed among the isolate was that against tetracycline (21) followed by a combination of tetracycline - ampicillin (15); and ampicillin (9). The other frequent co- resistance was that against tetracycline – ampicillin - nalidixic acid (4) which was common among human isolates. Among the domesticated animals, no resistance to nalidixic acid and cefuroxime was observed. Resistance

to nalidixic acid and cefuroxime was observed frequently among human isolates but also occurred in fish and water isolates at lower frequencies. Resistance to chloromphenicol and gentamicin was also only observed in isolates recovered from human and presented as co-resistance with other antibiotics.

Using the Kruskal Wallis test (Table 4.12) to compare differences among the *E. coli* sources with respect to the six antibiotics, no statistical significant differences were observed among chloromphenicol ($p = 0.075$) and gentamicin ($p = 0.11$). However, there were statistical significant differences among other four antibiotics (tetracycline, ampicillin, nalidixic acid and cefuroxime) $p < 0.05$.

Table 4.11: Antibiogram patterns and their frequency of occurrence among isolates

Type of resistance	frequency n (%)	genera/ species with resistance
Amp	12 (7.6%)	*E. coli* (9), *S. arizonae* (3)
Amp-Tet	17 (10.8%)	*E. coli* (15), *Shigella* spp (2)
Amp-C	2 (1.3%)	*E. coli* (2)
Amp-Na	1 (0.64%)	*E. coli* (1)
Amp-Cxm	1 (0.64%)	*E. coli* (1)
Amp-Na-C	1 (0.64%)	*E. coli* (1)
Tet	22 (14%)	*E. coli* (21), *S. pullorum* (1)
Tet-Na	2 (1.3%)	*E. coli* (2)
Tet-Amp-C	1(0.64%)	*E. coli* (1)
Tet-Amp-Na	4 (2.5%)	*E. coli* (4)
Tet-Amp-Na-C	1 (0.64%)	*E. coli* (1)
Tet-Amp-Na-C-Cxm	2 (1.3%)	*E. coli* (2)
Tet-Amp-Na-Cxm	3 (1.9%)	*E. coli* (2) *Salmonella* spp (1)
Tet-Amp-Na-Gn-Cxm	3 (1.9%)	*E. coli* (3)
Tet-Amp-Na-Gn	1 (0.64%)	*E. coli* (1)
Tet-Na-Cxm	2 (1.3%)	*E. coli* (2)
Tet-Amp-Na-C-Gn-Cxm	2 (1.3%)	*E. coli* (1) *S. arizonae* (1)
Na-Cxm	1 (0.64%)	*E. coli* (1)

Key: Tet = tetracycline; Amp = ampicillin; C = chloramphenicol; Cxm = cefuroxime; Gn = Gentamicin; Na = Nalidixic acid

Table 4.12: Comparison of disk diffusion zones among *E. coli* isolates by sources using Kruskal Wallis test

		Median					
Source	**n**	**Tet**	**Amp**	**Na**	**C**	**Gn**	**Cxm**
Chicken	13	19	18	21	24	18	21
Cattle	23	21	19	22	22	19	21
Donkey	5	20	18	21	24	19	20
Fish	13	6	11	20	20	17	17
Goat	16	16	21	23	20	19	21
Human	52	6	6	17	23	20	19
Soil	3	6	17	22	24	19	19
Water	9	15	16	17	18	19	18
P Value		d	d	d	.075	.11	d

Legend: d = p < .0001, Tet = tetracycline; Amp = ampcillin; C = chloromphenicol; Na = nalidixic acid; Gn = gentamicin; Cxm = cifuroxime

When comparing resistance to antibiotics tested among the 8 sources, *E. coli* isolates originating from fish (n = 13) and soil (n = 3) showed resistance to at least one antibiotic tested and therefore showing 100% resistance as shown in Table 4.13. Isolates originating from humans showed the

highest level of resistance to two or more antibiotics with levels of 78.8% (n = 52). Apart from chicken, isolates from livestock recorded multiple resistance levels below 22%, although isolates recovered from cattle, recorded no multiple resistance. Based on multiple antibiotic resistance patterns (MARP) and MAR indices observed in this study, *E. coli* sources could be classified into 4 groups as shown in Table 4.13. A group with MAR index values of 0.55 and MARP of over 78% represented by human. A group with MAR indices scores of between 0.24 and 0.22 and MARP of between 33% and 38.5% represented by fish and soil. A group with MAR indices of 0.17, 0.1, 0.07 and 0.07; and MARP of 12% to 23.1% represented by water, chicken, donkey and goat. The last group with a MAR index of 0.04 and MARP of 0% represented with cattle, which could not be matched with any other source investigated and therefore classified separately.

Table 4.13: Multiple antibiotic resistance patterns (MARP) and multiple antibiotic resistance (MAR) indices for *E. coli* based on their sources

		% level of resistance		
Source	**n**	**at least one antibiotic**	**more than one antibiotic**	**MAR index**
Human	52	88.5	78.8	0.55
Fish	13	100	38.5	0.24
Soil	3	100	33.3	0.22
Water	9	67	22.2	0.17
Chicken	13	38.5	23.1	0.1
Donkey	5	20	20	0.07
Goat	16	18.8	12.5	0.07
Cattle	23	21.7	0	0.04

4.4 Presumed Origins of *E. coli* contaminating Fish along Lake Victoria, Kenya

By using Discriminate Analysis (DA) with the 134 *E. coli* isolates recovered in this study, four possible sources of contamination of fish could be identified, namely, soil, chicken, water and human. The average rate of correct classification (ARCC) for all isolates was 41% Table 4.14. The ARCC generated was calculated by dividing the number of isolates which were correctly assigned to a given group by the total number of isolates in that group tested and multiplying by

100%. The probability that an isolate would fall into one of the 8 categories by chance alone is 12.5%. Generally, *E. coli* isolates recovered from soil were well classified 100% followed by those from donkey and goat isolates at 60% and 56.3% respectively. However, *E. coli* isolates recovered from chicken, water and fish were classified poorly at 15.4%, 22.2% and 23.0%, respectively (Table 4.14). Among the *E. coli* recovered from fish samples, 38.5% were misclassified as soil isolates, 15.4% as chicken, 15.4% as water isolates and 7.7% as human isolates. Most of the fish isolates were misclassified into the soil categories likely because these groups displayed similar MAR profiles (100% resistance to one antibiotics namely tetracycline)

However, when all isolates were reclassified into five host groups namely livestock, fish, human, soil and water, ARCC improved to 58.2% as shown in table 4.15. However the rates of misclassifications remained the same 38.5% as soil isolates, 15.4% as livestock isolates specifically from chicken, 15.4% as water isolates and 7.7% as human isolates.

When all isolates were reclassified into three host groups namely; human, environment (representing *E. coli* recovered from soil, water and livestock) and fish, ARCC rose to 64.2% as shown in Table 4.16, and similarly the rate of correct classification among the fish improved to 69.2%. Although misclassification to *E. coli* isolates recovered from fish to the human category increased to 15.4%.

Table 4.14: Discriminant analysis of disc diffusion zones of *E. coli* isolates based on individual sources

	(%) of database isolates assigned to each source category							
Source (n)	**Ch(13)**	**Ca(23)**	**D(5)**	**G(16)**	**H(52)**	**S(3)**	**W(9)**	**F(13)**
Chicken	15.4	4.3	0	0	3.9	0	0	15.4
Cattle	15.4	43.6	20	12.5	0	0	11.1	0
Donkey	23.0	13.0	60	12.5	9.6	0	22.2	0
Goat	15.4	26.1	0	56.3	1.9	0	0	0
Human	7.7	0	20	0	44.3	0	11.1	7.7
Soil	7.7	8.7	0	12.5	11.5	100	33.3	38.5
Water	0	0	0	0	9.6	0	22.2	15.4
Fish	15.4	4.3	0	6.2	19.2	0	0	23.0

Key: n = 134; ARCC = 41%; Ch = Chicken, Ca = Cattle, D = Donkey, F = Fish, G = Goat, H = Human, S = Soil, W = Water

Table 4.15: Discriminant analysis of disc diffusion zones of *E. coli* isolates based on classification as livestock, fish, soil, water and human

	No. (%) of database isolates assigned to each source category				
Source (n)	**Livestock(57)**	**Human(52)**	**Soil(3)**	**Water(9)**	**Fish(13)**
Livestock	78.9	11.5	0	22.2	15.4
Human	3.5	46.2	0	11.1	7.7
Soil	10.5	11.5	100	33.3	38.5
Water	0	11.5	0	33.3	15.4
Fish	7.0	19.2	0	0	23.0

Key: n = 134; ARCC = 58.2%

Table 4.16: Discriminant analysis of disc diffusion zones of *E. coli* isolates based on classification of human, environment and fish

	(%) of database isolates assigned to each source category		
Source (n)	**Human (52)**	**Environment (69)**	**Fish (13)`**
Human	55.8	5.8	15.4
Environment	13.5	69.6	15.4
Fish	30.8	24.6	69.2

Key: n = 134; ARCC = 64.2%

CHAPTER FIVE: DISCUSSION

5.1. Distribution of *Shigella* spps, *Salmonella* spp and *E. coli* in Fish, Water, Domesticated Animals, Soil and Humans

5.1.1 Levels and distribution of total coliforms in Lake Victoria waters, Kenya

Coliform bacteria are the most commonly used indicators of fecal pollution in water and food (Harwood *et al*., 2000). In the current study lake surface water was found to have high levels of total coliforms ranging between $\log_{10}$ 2 cfu/ml and $\log_{10}$ 4.3 cfu/ml. The findings compare with those found around Mwanza fish landing site that ranged between $\log_{10}$ 1.6 to $\log_{10}$ 4.0 cfu/ml (Mdegela *et al*, 2010). However Byamukama *et al*, (2005), observed levels ranging between $\log_{10}$ 3.8 to $\log_{10}$ 5.3 cfu/100ml within Lake Victoria shorelines with high human activity influence, and $\log_{10}$ 2.0 to $\log_{10}$ 5.7 cfu/100ml within sites with low human activity influence. The total coliform levels reported in this study (range from $\log_{10}$ 2 cfu/ml and $\log_{10}$ 4.3 cfu/ml) show that waters along the shore of Lake Victoria, Kenya are highly contaminated with coliforms. Total coliform levels reported in this study especially those reported for the shore, exceed the recommended levels published in Environmental Management and Co-ordination Regulations (2006), which directs that total coliform levels for recreational waters should not exceed 500 cfu/100ml ($\log_{10}$ 2.7 cfu/100ml). High total coliforms along shore of water bodies like Lake Victoria, Kenya have been attributed to surface runoff including storm-water which may contain microbes originating from wildlife, agriculture, urban, forestry and beach settlements (Onyango *et al*., 2009). Human activities such as washing of dishes and clothes within the water body, fetching water, boat launching (figure 1) have also been associated to increasing coliforms along shores of water bodies (Pachepsky and Shelton, 2011). These practices stir bottom sediments which are microbial habitats, thereby resuspending bacteria

leading to increased levels of coliforms at the shoreline (Pachepsky and Shelton, 2011). At the shoreline, sediments function as sites of higher nutrient concentrations and provide bacterial attachment surfaces (biofilm) (O'Sullivan *et al*., 2002). Wave action and human chores that take place at these points dislodge and disperse bacteria into surface water and therefore the high levels of coliform counts observed. Since the sites investigated in this study do not meet acceptable levels of total coliform for recreational waters by exceed 500 cfu/100ml (log_{10} 2.7 cfu/100ml) (Environmental Management and Co-ordination Regulations, 2006), the sites monitored therefore may not be conducive for human activities such as bathing and other domestic chores including fishing.

The study also found significant reduction in the levels of total coliform from the shoreline (0m) to 150m offshore. The findings agree with those reported by Mdegela *et al*, (2010), who found that the levels of total coliform contamination along the shores (0m) to 150m offshore along the Mwanza fish landing site of Lake Victoria varied significantly. Decline in total coliforms levels as observed in this study may be attributed to a number of reasons. Mdegela *et al*, (2010), has attributed this to dilution of the microbes along the gradient, probably due to water flow and mixing due to storms and wind (Whitman *et al.,* 2003). Another reason for the decline in total coliforms counts is the possibility of being killed by ultraviolet light (McCambridge and McMeekin, 1981; Whitman *et al.,* 2004). Two possible processes of inactivation of coliforms by sunlight have been described namely: photobiological (DNA damage) and photooxidation (oxidation of cellular components) (Whitman *et al.,* 2003). However, oxygen and organic matter such as lignins and humic acid have been shown to hasten the process of photooxidation damage by producing destructive agents, such as oxygen free radicals (O_2^-) and hydrogen peroxides

(Reeds *et al.*, 2000; Wegelin *et al.*, 1994). Similarly, in large lakes photochemical damage can occur besides photooxidation, because the required ingredients of oxygen and organic matter are available through constant circulation/wave action and surface runoff (Whitman *et al.*, 2003).

Presence of bacterial predators (McCambridge and McMeekin, 1981) can also play a role in survival and reducing coliform loads in lake water and could be enhanced by solar radiation which may harm coliforms thereby, making them more susceptible to the activities of microbial predators (Chamberlin and Mitchell, 1978). Moreover predators such as flagellates are very efficient bacterial grazers of free living cells, but not able to graze on large particles (Chrzanowski and Simelk, 1990) and therefore contribute to the reduced coliform levels in surface water away from the shoreline.

In this study, Luanda Konyango landing site presented generally higher coliform counts on the shoreline compared to the other study sites. The presence of water hyacinth and reeds at the site throughout the study period may have played a critical role in habourage of coliforms. Presence of vegetation in a water body provides suitable bacterial attachment surfaces that lead to formation of glycocalyx or carbohydrate coats bonded to proteins and lipids also referred to as biofilms (Lappin-Scott and Costerton, 1997; O'Sullivan *et al*., 2002). Microcolonies arising from this bacterial colonization then trap organic and inorganic matter within the biofilm, where nutrients can become very concentrated (Lappin-Scott and Costerton, 1997). These microbial biofilms are rich nutrient reservoir and increase the chances for coliforms survival in water bodies (Lappin-Scott and Costerton, 1997). Human activities (Pachepsky and Shelton, 2011) and wave action (Whitman *et al*., 2003) may continuously disturb the bacterial habitat and lead to release of coliform into the water surface. Such action may have contributed to the elevated

levels of total coliform counts observed at Luanda Konyango sampling site. Olapade *et al*, (2006) has shown that fecal indicator bacteria were frequently present at extremely high densities on green algae (*Cladophora*) mats stranded at the shoreline of beach sites and the levels far exceeded the levels acceptable for recreational waters.

5.1.2 Distribution of *E. coli* in fish, water, domesticated animals, soil and human

The observation of high frequency of *E. coli* in chicken and the mammalian hosts in this study is in agreement with findings by Gordon and Cowling, (2003). Gordon and Cowling, (2003) reported the occurrence of high prevalence of *E. coli* among a wide variety of vertebrate hosts especially birds and mammals. A number of factors have been shown to influence colonization of animals with *E. coli*. According to Gordon and FitzGibbon, (1999) host habitat, diet, 'typical' body temperature and gut morphology are important factors that may influence the prevalence of *E. coli* among hosts. In the study by Gordon and Cowling, (2003), *E. coli* has been shown to adapt to mammals with hindgut microbial fermentation chambers and in the absence of a hindgut fermentation chamber; they are more likely to establish a population in 'large' hosts than in 'small' hosts. In addition, environmental contamination by *E. coli* due to the faeces of domestic pets and livestock has also been found to contribute to continuous exposure of animals to *E. coli.*

In this study the high frequency of *E. coli* observed among livestock and human can be attributed to *E. coli* from the environment they inhabit, probably due to elevated levels of environmental contamination by *E. coli* originating from livestock and human faeces (Lankau, 2011). This is evidenced by the fact that in this study, lower levels of *E. coli* were recovered from freshly caught fish which inhabits a different environment from livestock and human investigated in this study. Generally, within the study site, livestock movements and association with human are

close and a previous study has reported that 16% of the rural population in the region lacks access to sanitation facilities and use the bush or fields to ease themselves for nature calls (Kenya National Bureau of Statistics, 2013) and therefore a possibility of high *E. coli* prevalence in the environment.

As in chicken and mammals, occurrence of *E. coli* in fish could also be influenced by feeding habits, and the environment. Generally, coliforms have been found in fish intestines and stomach (Geldreich and Clarke, 1966; Hejkel *et al.*, 1983), although evidence points to them not being part of the permanent microflora in fish, but their occurrence being due to polluted water and feeding habits like ingestion of contaminated food (Guzman *et al.,* 2004; Hansen *et al*, 2008). Apart from the intestinal tract of fish, *E. coli* has also been found on the gills, in muscle and on the skin (Ogbondeminu, 1993) especially among fish recovered from polluted waters. Isolation of *E. coli* from fish has been taken to indicate contamination coming from an external origin such as sewage effluent (Guzman *et al.,* 2004). Other factors such as other bacteria or toxins in digestive tract that may inhibit the presence or growth of bacteria, presence of favourable temperature conditions in digestive tract and retention of feces all may influence baterial survive and multiplication in the intestinal tract of fish (Guzman *et al.,* 2004).

Escherichia coli levels have been shown to be higher among benthic fish species than among pelagic fish species (Geldreich and Clarke, 1966; Hansen *et al.*, 2008). Benthic fish harbour more *E. coli* than pelagic fish because *E. coli* are less abundant in water than in sediments where benthic species often feed (Desmerais *et al.*, 2002; Ishii *et al.*, 2007). Nile perch and *R. argentea* investigated in this study are both pelagic and therefore explain the low levels of *E. coli* recovery

from the freshly landed fish. Sediments function as sites of higher nutrient concentrations that enable *E. coli* survive longer than it could in water. These sediment particles provide suitable environments for high nutrient concentration due to release of organic substances from attached algae. The attached algae also provide biofilm habitat that enables *E. coli* maintain populations despite obvious interspecific pressures from resident organisms, such as periphyton and grazers (Marks and Power 2001). Findings in this study shows *E. coli* among the freshly landed fish was 3.5% and 7.3% for *R. argentea* and Nile perch respectively which compare to those reported by Hansen *et al*, (2008), at 4% occurrence rate among pelagic fish. However the variations observed in these two studies could be associated with the species differences and possibly contamination from fishing equipment and personnel, since fish used in the current study were collected from fishing vessel and the study did not target fish gut content for bacterial recovery.

In this study relatively lower levels of *E. coli* were recovered from soil (30%) and water (30%) sources compared to their natural hosts (chicken and mammals). The survival of *E. coli* in environments like soil and water is strongly influenced by abiotic (salinity, sunlight, and temperature) and biotic (predation and competition) factors (Whitman *et al.*, 2004; Winfield and Groisman, 2003). *Escherichia coli* reside in the lower intestine of warm animals (Selander *et al.*, 1987; Smith, 1965), an environment that provides a vast supply of nutrients such as high concentrations of free amino acids and sugars for bacterial growth (Winfield and Groisman, 2003; Savageau, 1983; 1974). Survival of *E. coli* in the secondary habitat such as soils and water requires its ability to overcome low nutrient availability and wide temperature fluctuations as the harsh ecological conditions prevent *E. coli* from sustaining a dividing population outside the animal host (Winfield and Groisman, 2003). Winfield and Groisman, (2003) have shown that

the survival of *E. coli* in nonhost environments is very low and that its presence in such locations results from excretion of waste by animal hosts a fact on which the logic behind the use of *E. coli* as an indicator organism for environmental fecal contamination is based. However, in the tropics like the Lake Victoria ecosystems, nutrients in such systems can be maintained at high concentrations by continous loading from sawage drainage points; human and animal wastes, and together with constant warm air, soil, and water temperatures, provide an ideal habitat for survival, growth, and proliferation of *E. coli* outside the animal host (Winfield and Groisman, 2003), therefore explain the recovery of *E. coli* from these sources through out the study period.

Overall, findings in this study point to domesticated animals and human as important reservoirs of *E. coli* and may therefore be playing an important role in sustaining *E. coli* within the environment in which fish is handled and processed along Lake Victoria, Kenya. This is further evidenced by the increased prevalence levels of *E. coli* among fish that had been processed within the study sites. Previous studies by Mungai *et al*, (2002) and Ogwang' *et al*, (2005) have also reported that poor fish handling practices expose Lake Victoria fish to contamination.

5.1.3 Distribution of *Salmonella* spp in fish, water, domesticated animals, soil and human

The study shows that along Lake Victoria, Kenya soil, fish, livestock and human form important reservoirs, for *Salmonella* spp; an indication that *Salmonella* spps in the region may be lacking special host adaptations and / or are capable of colonizing a wide variety of organisms. In Africa, little is known about environmental reservoirs and predominant modes of transmission, although various sources such as farm animals, pets and reptiles have been implicated in the transmission of *Salmonella* between animals and humans (Wall *et al*., 1996). Generally, *Salmonella* has been reported to persist in same animals, such as chicken (Van Immerseel *et al*.,

2002; Sadeyen *et al*., 2004); and in same small ruminants, such as sheep and goats (Alvseike and Skjerve, 2002) and therefore the possibility for its presence in livestock was investigated in this study.

Salmonella have been noted to be resilient microorganisms with complex genomic systems that make the organism able to react to different harsh environmental conditions such as extremely low or high temperatures and pH; and in the gastrointestinal tract (Andino and Hanning, 2015) and therefore enabling it survive different environments as evidenced in this study. *Salmonella* has been reported to respond to different stress factors beyond their normal growth range namely: temperature (35^{o}C to 43^{o}C), pH (7 – 7.5), osmotic shifts (0.5 – 7.5%), and low water activity (0.99) (International Commission on Microbiological Specifications for Foods (ICMSF), 1996; Andino and Hanning, 2015). This ubiquitous nature of *Salmonella* has been associated to facilitating a cyclic lifestyle consisting of passage through a host into the environment and back into a new host (Thomason, 1977). This ability of being able to survive in nonhost environments for long times gives *Salmonella* an advantage over *E. coli* in long term survival in secondary habitats ensuring its passage to the next host (Winfield and Groisman, 2003).

This study finding show that all *Salmonella enterica* serogroups recovered were of the Non typhoidal Salmonellae (NTS). This finding is in agreement with recent studies in Africa, which have also shown that the NTS are the most common cause of infections among human, food animals and wildlife (Kariuki *et al.*, 2002; Gordon and Graham, 2008; Dione *et al*., 2011; Kagambega *et al*., 2013). In this study the prevalence rates of *Salmonella* spps varied among the different sources investigated with soil, fish and human sources presenting high prevalence

rates as compared to commonly documented sources of *Salmonella* (Dione *et al*., 2011; Kagambega *et al*., 2013). Generally, livestock especially chicken have been known to be the most important reservoir of NTS and thus thought to be the major source of transmission to humans (Braden, 2006), but in this study livestock record relatively low prevalence rates. This observation represents the continuously changing epidemiology of NTS in Africa, a phenomenon also reported by Dione *et al*, (2011) in The Gambia. This study reports the occurrence of *Salmonella arizonae* among livestock and human within the study area. In countries where cases of *S. arizonae* have been reported, transmissions have been associated with direct or indirect contacts with reptiles or by ingestion of snake – based products such as meat and traditional medicine preparations (Schneider *et al*., 2009).

Salmonella serotypes have been shown to differ in their pathogenic potential for humans and serotype distributions often have varied vastly between human and animal populations as well as among different animal populations in the same geographic area (Hoelzer *et al.*, 2011). Reptiles and amphibians have been associated with approximately 40% *Salmonella* serotypes (Hoelzer *et al.*, 2011). Reptiles have also been implicated as important reservoirs of *S. arizonae* and recovery from domesticated animals and human has also been reported in three other studies (Mahajan *et al.*, 2003; Bauwens *et al.*, 2006; Di Bella *et al.*, 2011). In Kenya there has been no documented report on *S. arizonae* infections among humans or occurrence in animals. Probably due to the unique characteristics displayed by *S. arizonae* in standard microbiological techniques used to recover *Salmonella* in health and research facilities in Kenya. The *S. arizonae* can be differentiated from other *Salmonella* subgroups by virtue of its ability to utilize malonate and liquefy gelatin, its ability to grow in the presence of KCN, and its frequent ability to ferment

lactose (Weiss *et al.*, 1986). This study therefore postulated that the livestock husbandry practices in the region that allow animals to wonder freely or tethered in the bushes, could expose them to *Salmonella arizonae* contaminated pastures. However, establishment of *Salmonella* within the animal hosts has been noted to depend upon the hosts physiology, health status and environmental stresses the animal may be subjected to (White *et al*., 1997).

Studies in Kenya investigating diarrheal infections have in many cases focused on children of less than 5 years of age. This could be motivated by the view that diarrheal infections are a major cause of high morbidity and mortality among children (Black *et al.*, 2003; Brook *et al.*, 2006). Previous studies have recorded relatively lower *Salmonella* prevalence rates of 3.5% to 7.3%, among children of less than 5 years (Sang *et al.*, 1997; Brook *et al.*, 2006; Sang *et al.*, 2012), which are also in agreement with findings in this study. Kariuki *et al,* (2006b) has associated infections among this age group to underlying factors such as poor background with reduced sanitary conditions in homes and the environment in which the children live and play, particularly in the poor urban residential areas. Findings in the current study however point to higher prevalence levels among persons of more than 10 years of age with prevalence rates ranging above 10%. This observation points to a possibility of persons aged > 5 years being important reservoirs or potential sources of human – to – human transmission of salmonellosis. In Africa NTS has consistently been associated with other diseases among immuno-compromised persons, infants and newborns (Bryce *et al.*, 2005; Morpeth *et al.*, 2009). Other factors identified include malnutrition, and possibly malaria (Graham *et al.*, 2000; Archibald *et al.*, 2000; Jacob *et al.*, 2009; Mtove *et al.*, 2010). Although the association of these factors with salmonellois prevalence among human in the study site was not monitored, however previous

studies within the study area have demonstrated their involvement (Brook *et al.*, 2006; Kenya National Bureau of Statistics, 2007; National AIDS and STI Control Programme, 2013).

Among livestock, the current study recorded relatively low prevalence rates compared to other studies conducted in Africa, which have reported higher rates ranging from 4.8% to over 60% (Dione *et al*., 2011; Kagambega *et al*., 2013). These differences could be due to many factors that determine the susceptibility of Livestock to *Salmonella* colonization as described by White *et al*, (1997). These factors include the age of the animal, the survival ability of the pathogen through gut, effective competition of *Salmonella* with other bacteria, ability of the *Salmonella* to locate a hospitable colonization site, nature of the diet the animal feeds on, physiological status of the animal, health and disease status of the animal, environmental stresses and the host genetic background.

Among fish, *Salmonella* was recovered from sundried *R. argentea* an indication of possible post harvest contamination since no recovery of *Salmonella* was made from freshly landed *R. argentea* sampled from the fishing vessels. *R. argentea* a long Lake Victoria is dried at landing sites on top of fishing nets as shown in Figure 1. The practice of drying *R. argentea* on the ground, coupled with other poor hygiene practices such as cleaning the fish at the lake shore may be responsible for contamination of the fish and therefore the relatively high prevalence (8.6%) of *Salmonella* in dried *R. argentea*. In this study there appears to be a close relationship between *Salmonella* serogroups recovered from soil and those recovered from fish. Sero-typing of *Salmonella* has previously been used to attribute sources of infections by serotype Derby (Chau *et al.*, 1977; Barrel *et al.*, 1987) and serotype Mbandaka (Fantasia *et al.*, 1989). The study therefore postulates that soil could be an important source for *Salmonella* contaminating fish

sourced from Lake Victoria, Kenya. Other than soil; environmental sources and poor hygiene practices could also be responsible for contaminating sundried *R. argentea* with *Salmonella* (Raufu *et al.,* 2014). Raufu *et al,* (2014) has attributed the contamination of fish with *Salmonella* to be associated with terrestrial sources such as the unorthodox utilization of cattle and poultry feces as manure/fertilizer on farmland located close to the river. Other factors such as poor sewage disposal allowing untreated sewage to enter lakes or ponds either through runoff or storm-water, transportation of fish in dirty fishing boats, packaging of fish in dirty containers and displaying the fish uncovered in the open markets may have also played a role in the contamination of fish (Raufu *et al,* 2014). This study did not monitor fish contact surfaces such as fishing boats; however Raufu *et al,* (2014) has identified them as important sources of contamination of fish with *Salmonella*. Sediment have also been reported to provide suitable conditions for microbial growth and survival (Meghan *et al.,* 2012; Ishii *et al.,* 2007; Desmerais *et al.,* 2002; Lim and Flint, 1989) and therefore may be another important source for *Salmonella* spp contaminating Lake Victoria fish.

Among the serogroups recovered from *R. argentea* in this study 3 were *Salmonella enterica* Group E and 2 were nontypable strains. Previous studies have reported recovery of *Salmonella* serovars Entertidis, Typhimurum, Typhi and Paratyphi B from fish sourced from Lake Victoria, Kenya (Sifuna *et al.*, 2008; Onyango *et al.*, 2009; Onyuka *et al*., 2011) but none of these serovars were recovered in the current study. This observation points to a possibility of a high diversity of NTS clones circulating in the study site and therefore could be leading to mixed infection or carriage within the reservoir populations. It is possible that this situation could result in an extensive genetic diversity and variability because of the frequent intraspecific recombination

that may occur, as is the case of *Helicobacter pylori* reported by Suerbaum and Achtman, (2004). This could therefore lead to a wider range of clones and thus make it more difficult to control NTS infections in human and in food.

5.1.4 Distribution of *Shigella* spp in fish, water, domesticated animals, soil and human

In the current study an occurrence rate of 2.7% *Shigella* among human is reported and none among other sources investigated herein. Generally, *Shigella* is typically an inhabitant of the intestinal tract of humans and other primates (Strockbine and Maurelli, 2005; WHO, 2008). This could indicate the reason why no *Shigella* spp was recovered from other sources other than human in this study. Prevealence rates reported in this study are comparable to those reported by Sang *et al*, (2012), who repeported rates of 2% among children. However, in a previous study by Brooks *et al*, (2006) prevalence rate of 16% of infections was attributable to *Shigella* spp. Typically *Shigella* is transmitted by fecal-contaminated drinking water or food or by direct contact with an infected person (Wang *et al.*, 1997). Therefore, the reduced prevalence rated of *Shigella* reported in the current study and that by Sang *et al*. (2012) suggests a possibility of enhanced primary preventive measures such as ensuring proper human waste and sewerage management and also availability of safe drinking water within the study site.

In this study however, no *Shigella* was reported among fish, although a previous study by Onyango *et al*. (2009) has reported its occurrence in fish. Onyango *et al*. (2009) has attributed the contamination of Lake Victoria fish with *Shigella* to being related to pollution of Lake Victoria by direct discharge of raw sewage and poor sanitation due to lack of adequate toilet facilities along Lake Victoria. A study has also shown that *Shigella* is capable of entering the viable but non-culturable (VBNC) state when exposed to unfavourable conditions (Islam *et al*.,

2001). The VBNC state perpetuates the organism after exposure to conditions less than optimal for cell growth and division (Roszak and Colwell, 1987). This orbservation could explain why *Shigella* may not have been recovered from other sources including fish investigated in this study, as if *Shigella* enters the VBNC state it may be impossible to recover it from environments with unfavourable conditions for its habitation.

5.2 *E.coli* Pathotypes Identity in the Various Sources

Diarrheagenic *E. coli* has been identified as an important cause of childhood diarrhea in western Kenya, however the pathotypes involved and incidences are varied. Brooks *et al.* (2006) identified diarrhoeagenic *E. coli* in 20% of specimens in which a bacterial pathogen was detected. In a recent study, pathogenic *E. coli* represented 11.2% of isolates recovered among children with diarrhea, of which EAEC represented 8.9%, ETEC (1.2%), EIEC (0.6%), and EHEC (0.5%) (Sang *et al.*, 2012). In another study, Sang *et al*. (1997) reported EPEC (8.0%), EAEC (0.5%), ETEC (5%) and EHEC (0.1%) among children of ≤5 years. In this study however, no EAEC or EIEC pathotypes were recovered from any of the sources investigated. Only ETEC was identified among *E. coli* isolates recovered from human representing prevalence rates of 2.7%, however all other sources investigated in this study were negative. Among human, the ETEC isolates were recovered from children within the age group of ≤ 10 years. Enterotoxigenic *E. coli* (ETEC) have previously been reported as a common cause of childhood diarrhea in the developing world (Nessa *et al.*, 2007); this finding therefore, provides further evidence that ETEC is an important cause of diarrhea among children in developing countries.

Enterotoxigenic *E. coli* have also been implicated as cause of diarrhea in travelers to developing countries (Qadri *et al.*, 2005). Pathogenesis of ETEC is mainly mediated by the presence of

enterotoxins and colonization factors (CFs). Heat-stable toxin (ST) and heat-labile toxin (LT) are the main virulence factors associated with ETEC diarrhea, and detection of one or both toxins is used to diagnose the infection (Sjöling *et al.*, 2007). In this study the ST and LT toxins were identified among the two ETEC isolates recovered. ETEC are believed to be primarily transmitted via contaminated food and water (Brook *et al.*, 2003; Gonzales *et al.*, 2013). ETEC strains have been recovered from river water in Kenya (Simiyu *et al.*, 1998). Improving water supplies, sanitation and frequency of hand-washing with soap could be an important step in preventing the acquisition and spread of such pathogenic *E. coli* in most parts of Africa (Mensah *et al.*, 2002; Admassu *et al.*, 2003).

Studies in Africa have shown that ETEC, EPEC and STEC are much frequently recovered from foods, food animals and water (Simiyu *et al.*, 1998; Kagambega *et al.*, 2012; Caine *et al.*, 2014; Odwar *et al.*, 2014). Studies, in developed countries have also shown that live chicken and other food animals are important reservoirs of these pathogenic *E. coli* (Johnson *et al.*, 2007; Cortes *et al.*, 2010; Bergeron *et al.*, 2012). In this study, none of the virulence genes tested was identified among all the *E. coli* isolates recovered from animals and environmental source. The absence of virulence genes among *E. coli* isolates in this study could probably be attributed to choice of virulence genes tested, loss of virulence factors due to bacterial recovery from specimen and isolate storage. Some of the genes investigated in this study were plasmid encoded (Panchalingam *et al.*, 2012) and could easily be lost due to changes in growth condition. Species of *E. coli* have a large number of virulence genes located on mobile elements such as plasmids, phages, transposons, integrons or pathogenicity islands, and can easily be lost or gained depending on the host or environmental conditions, therefore new pathotypes with new

combinations have constantly emerged (Kuhnert *et al.*, 2000; Matthew and Finlay, 2010). As defined previously by Gordon and Cowling. (2003) in this study the chance of recovering *E. coli* carrying virulence genes from a specimen is a fraction of hosts in which pathogenic *E. coli* was a dominant member of the *E. coli* community in that host.

Nonetheless, this study points to possibility of *E. coli* contaminating Lake Victoria fish not being carriers of virulence factors namely: EAEC (*aatA* and *aaiC* genes), ETEC (*eltB* and *estA* genes) and EPEC (*bfA* and *eae* genes). However, it is important to note that *E. coli* strains occurring in the environment may possess other virulence gene patterns of the extraintestinal *E. coli* type as reported by Muhldorfer *et al*. (1996) among *E. coli* recovered from surface water. In Muhldorfer *et al*, (1996) study, approximately 40% of *E. coli* strains isolated from surface water were shown to contain at least one virulence gene specific for extraintestinal *E. coli*. However according to Kuhnert *et al*. (2000) the combinations or patterns of virulence genes found in *E. coli* which are isolated from environmental samples do vary significantly and therefore in order to assess the potential risk which such strains constitute for humans and animals, like for this study, a complete analysis for the presence or absence of all virulence genes may be essential to avoid any doubts.

5.3 Antimicrobial Resistance Response among the Isolates

In Africa antibiotic resistance among enteric bacteria originating from human, domesticated animals or environment are varied but range from less than 10% to over 85% (Kariuki *et al*., 1999; Kagambèga *et al*., 2013; Odwar *et al*., 2014). This current study recorded levels of 49.7%, which falls within the range reported in the previous studies. The variations among these studies however can be attributed to differences in study sites, sources of the enteric bacteria and

types of antibiotics used in the study. The use of antimicrobial agents has been identified as an important factor for the development of antimicrobial agent resistance among microbes (Witte, 1998). Therefore the patterns of antimicrobial agent resistance expressed by enteric bacteria among different animal or human populations varies according to the types and quantities of agents used or exposed to and thus influencing the emergence, selection, and dissemination of antimicrobial agent-resistance determinants (Neu, 1992; Witte, 1998).

Four mechanisms to explain the development of resistance to antimicrobial agents have been described; and are regulated by the action of specific genes: enzymatic inactivation or modification of antimicrobial agents, impermeability of the bacteria cell wall or membrane, active expulsion of the drug by the cell efflux pump, and alteration in target receptors (Prescott *et al*., 2000). However, bacteria may gain antimicrobial agent resistance genes through mobile elements, such as plasmids, transposons, and integrons (Rubens *et al*., 1979; Prescott *et al*., 2000), which result in mutations in genes responsible for antimicrobial agent uptake, or binding sites (Spratt, 1994) or activation of portions of bacterial chromosomes (Hachler *et al*., 1991; Alekshun and Levy, 1999). Once these genes are acquired, resistance can further be transferred between bacteria, and some enteric bacteria such as *E. coli* have been shown to possess ability to transfer antimicrobial drug resistance (Kariuki *et al*., 1999).

In this study the three different genera of bacteria investigated had varied levels of susceptibility to antimicrobial tested with *E. coli* having the highest resistance levels. These variations among the bacterial resistance pattern suggest difference in their ecology despite the close genetic relationship as members of the *Enterobacteriaceae* family (Sharp, 1991). *Escherichia coli* and *S.*

enterica ecology outside the host environment has been noted previously to differ appreciably (Winfield and Groisman, 2003). These variations may further be attributed to ‘‘dose–response’’ effect of increasing severity of resistance for populations in closer proximity to the source of exposure. Previous studies have supported that animal populations proximate to human or livestock sources of resistant bacteria often harbor elevated resistance levels relative to populations more distant from potential contamination (Krumperman, 1983; Sayah *et al*., 2005). Thus, both proximity to and degree of exposure may produce a gradient effect in resistance levels, as has been observed in this study. Generally the routine practice of giving antimicrobial agents to animals for different purposes such as growth promotion, prophylaxis, or therapeutics and also the indiscriminate use among human is an important factor in the emergence of antimicrobial resistant bacteria in communities (Fey *et al*., 2000). Such practices lead to possibilities that bacterial resistance may develop in some of the food animals and be transferred to the environment and human population thus posing a risk for public health by spreading of the resistance and resistance genes (Ungemach *et al*., 2006).

Escherichia coli isolates recovered from soil, fish and lake water showed relatively higher resistance levels than those observed among domesticated animals in this study. Among these isolates, the highest resistance rates were observed among tetracycline and ampicillin. Based on this observation it appears that soil and Lake Victoria water select for resistance to the two antibiotics (tetracycline and ampicillin) and therefore account for their higher rates of resistance as compared to those recovered in animals. The occurrence and stability of antibiotic, such as tetracycline and sulfamethoxazole, in the environment has been reported previously (Halling-Sorensen *et al*., 1998; Kummerer, 2004) and therefore may explain the maintenance of resistant

bacteria in soil and Lake Victoria water. However, further studies may be needed to provide some insights into this issue. Conversely, it has been shown that antimicrobial agents can be retained in soil by its association with soil chemicals, for instance the presence of humic substances; in both dissolved and mineral-bound forms was found to increase environmental mobility of antibiotics according to Gu and Karthikeyan, (2008). Binding to soil particles has also been found to delay antibiotic biodegradation and therefore could bring about the long term effects of these drugs in the environment (Baquero *et al.*, 2008). Similarly, soil particles also have been observed to have capability to remove antibiotics from water by sorption (Baquero *et al.*, 2008). Studies have shown that very low concentrations of antibiotics can act as signaling agents in microbial environments by inducing biofim formation and regulating the homeostasis of microbial communities; and therefore beneficial for the behavior of susceptible bacteria in natural environments (Linares *et al.*, 2006; Fajardo and Martı´nez, 2008; Fajardo *et al.*, 2008) leading to development of resistance to antimicrobial agents. Therefore, it appears that in soil and Lake Victoria, Kenya water, diarrheagenic bacteria from different origins (human, animal and the environment) are able to mix, and possibly antimicrobial resistance evolves or is sustained as a consequence of promiscuous exchange and shuffling of genes. At the same time, antibiotics, disinfectants, and heavy metals released in the water might exert selective activities, as well as ecological damage in water communities, resulting in antibiotic resistance maintenance among bacteria colonizing Lake Victoria, Kenya.

This study further shows that resistance to tetracycline and ampicillin among diarrheagenic bacteria colonizing human, fish, Lake Victoria water and soil along Lake Victoria increases the risk of becoming resistant to additional antimicrobial agents. Sayah *et al.* (2005), has also

observed that antibiotic resistance against some antibiotics such as tetracycline could be conservation in the bacterial populations over time regardless of selection pressure. Secondly, exposures to environmental pollutants and changes in nutrient composition have been found to lead to selective pressures favouring certain bacteria or genotypes within water bodies (Lin *et al.*, 2004). Poor water resource management practices, such as release of industrial waste and use of agrichemicals within the Lake Victoria catchments have been documented previously (World Agroforestry Centre, 2006), and may serve to sustain the resistance genes among the *Enterobacteriaceae* along Lake Victoria, whereas human, fish, and livestock watered from the lake may serve to transfer or disperse the genes. Ash *et al.* (2002) has also reported a similar situation for several rivers in the United States of America as being reservoirs for antibiotic resistant microbes. However, other studies have suggested that resistance to ampicillin and tetracycline are almost ubiquitous in *Enterobacteriaceae*, with the exception for the Salmonellae (Livermore, 1995; 1996). Therefore many factors not considered in this study may be playing an important role in the selection and maintenance of resistance genes among the bacteria responsible for diarrheal infections in the basin.

Multiple antibiotic resistance (MAR) analysis has allowed for differentiating bacteria like *E. coli* or fecal streptococci from different sources using antibiotics commonly associated with human and animal therapy (Wiggins, 1996; Harwood *et al*., 2000). Harwood *et al*. (2000) used MAR test to identifying the sources of fecal streptococcal contamination in water. Parveen *et al*. (1997) on the other hand used MAR to distinguishing between *E. coli* strains from specific point sources, such as industrial and municipal effluents, and strains from nonpoint sources, such as land runoff. Basically, the MAR patterns observed in this study reflect the selective pressure

imposed on the *E. coli* recovered from the different sources investigated and is subject to the different types, concentrations, and frequencies of antibiotics the bacteria may have been exposed to. The MARP variation among the *E. coli* isolates in this study reflect specific "fingerprints" of antibiotic resistance the isolates possess with respect to their specific sources.

According to Krumperman, (1983), using MAR indexing, *E. coli* isolate can be categorized into two groups based on risks and public health concerns. Sources with MAR indices with 0.2 and above can be classified as high risk food sources whereas those below 0.2 as low risk food sources. In this study however, guided by Krumperman, (1983) principles, it is possible to generate four different groups' bases on MAR index and MARP. The first group consists of human isolates, which posses the highest levels of resistance 0.55. This group consists of isolates with high levels of MARP of over 40%. A second group that consisted of fish and soil isolates with MAR indices of 0.24 and 0.22, respectively, the group had MARP of between 30 to 39%. The third group consisted of water, chicken, goat and donkey isolates with MAR indices of 0.17 and 0.1 for water and chicken sources and 0.07 for the latter two. These sources have MARP ranging between 12.5 to 23.1%. The last group was that of cattle isolates with MAR indices of 0.04; this group did not record any multiple antibiotic resistances. Although the choice of an MAR index of 0.200 to differentiate between low- and high-risk contamination by Krumperman, (1983) could have been arbitrary, however it is apparent that the primary reservoirs for high-MAR *E. coli* are also the major reservoirs for enteric pathogens which are transmitted to humans through food and water. Another aspect achievable by MAR *E. coli* is the capability to distinguish *E. coli* sources. Krumperman, (1983) was able to identify and distinguished *E. coli* from humans, commercial poultry farms, swine, dairy cattle, and vector animals associated with these environments from *E. coli* isolates originating from other sources.

Compared to other studies conducted in developed countries by Krumperman, (1983) and Sayah *et al*. (2005) this study recorded low levels of MAR indices among livestock. The low antimicrobial resistance profile observed among isolates recovered from cattle could be due to the fact that cattle farmers in the study site do not practice intensive farming. Therefore the animals are not exposed to antibiotics directly as growth promoters. The intermediate MAR indices observed among fish and soil in this study could be due to possibly fish contamination originating from soil, and secondly since soil is the major receptacle of animal waste, it is possible that it provides suitable environment for recruitment and maintenance of resistant strains to antibiotics and especially tetracycline. The difference among the members of the livestock group sampled in this study could be attributed to the different environments where the animals find food. Goats, chicken and donkey feed around the human settlements, whereas cattle graze away in the fields not close to human settlements.

The finding also point to Lake Victoria, Kenya fish being classified as high risk food source. Similarly, using the MAR indices, serves to augment earlier postulations in this study that soil is an important source of bacterial contaminants of fish products within the basin. In addition, findings by Abila and Jensen, (1997) indicated that poor fish handling practices such as drying fish on top of soil, and absence of acceptable sanitary condition along the fish landing ports along Lake Victoria, Kenya may be responsible for high fish post harvest losses.

5.4 Possible Contamination Sources of Lake Victoria, Kenya Fish

This study deployed DA to discriminate among *E. coli* isolates obtained from sources suspected to contaminate fish originating from Lake Victoria, Kenya. Discriminant analysis has previously been used successfully to classify the source species for fecal streptococcus, fecal coliforms, and *E. coli* isolates obtained from surface water samples by Kasper and Burgess, (1990) and Wiggins *et al*. (1999). In these studies DA was used as a tool for microbial source tracking applying antimicrobial resistance profiles from databases of fecal bacteria isolates obtained from known sources. In the current study, known source library made up of *E. coli* isolates from livestock, water, soil and human were used to generate a classification scheme (decision rule) that were later used to classify isolates recovered from Lake Victoria, Kenya. Discriminant analysis was used to transform antibiotic inhibition zones obtained from *E. coli* isolates recovered from the different sources investigated. As described by Kaneene *et al*. (2007), the purpose of using the known-source samples was to determine the representative *E. coli* population within the species group but not to determine the representative *E. coli* population within each fecal or environmental sample.

When the 8 sources investigated in this study were used to classify the 134 *E. coli* isolates, the average rate of correct classification (ARCC) for all isolates was 41%. The ARCC is the accuracy of the decision rule for the classification scheme. In the scheme with an ARCC of 41%, among the eight sources, chicken, water and fish were classified poorly (15.4%, 22.2% and 23.0%, respectively). According to Guan *et al*. (2002) and Wiggins *et al*. (1999) such misclassification arises due to similarities in disk diffusion zones distribution, which may have been influenced by the environmental interactions with the host groups. In this study most of the

fish isolates were misclassified into soil category because these groups displayed similar MAR profiles and antimicrobial susceptibility inhibition zones, further supporting the argument in this study that soil may be responsible of fish contamination.

The classification scheme represents an ARCC of 41%, which may be considered to possess a low rate of correct classification however, it represents higher than would occur as a result of random classification into one of the eight groups investigated in this study (12.5%). Stoeckel and Harwood, (2007) have identified three aspects for such investigation that may probably contribute to the low ARCC levels. One is related to the library size, with small libraries having low ARCC. The second being the challenge isolates collected in at different time frame than the library isolates and lastly the fundamental assumption that *E. coli* subtypes being host specific not being upheld. However, Wiggins *et al*. (2003) has suggested that library size is most commonly addressed in attempts to enhance classification accuracy, although insufficient library size alone may not explain the high error rate observed in library dependent methods. In a study by Moore *et al*. (2005) low correct classification rates of 27 to 28% for *E. coli* isolates (116 to 159 isolates per host species) did not increase the classification accuracy even when the size of the library was increased from 380 to 690 isolates per host species.

In the current study, the variation in classification success of individual sources from set to set could be due to changes in the populations from which the samples were taken. Samples were collected over a 2 years period from five different locations, and so this possibility cannot be excluded. If the resistance patterns represented in terms of disc inhibition zone changed over time or from location to location, the database used to classify samples could therefore be

composed of varied collections. Secondly, only one *E. coli* isolate was selected from each sample, so that repetitious selection of the same clone of *E. coli* was avoided. Guan *et al*, (2002) also selected only one *E. coli* isolate from each animal fecal sample and concluded that by so doing a more diversified and representative collection of *E. coli* isolates was utilized and therefore such a sampling protocol could produce a more heterogeneous collection of bacterial isolates than other protocols described like those by Wiggins, (1996).

When all isolates were reclassified into five groups by grouping all the domesticated animals as livestock, the ARCC improved to 58.2% with cross validation. However, fish and water were misclassified (38.1% and 33.5%). When classified into three groups namely human, environmental and fish the ARCC improved to 64.2%. The study demonstrates that the average rate of correct classification increased with reduction of number of groups to be considered for discriminant analysis. Guan *et al*. (2002) and Wiggins *et al*. (1999) have also demonstrated that ARCC improved by reducing the numbers of species/group classifications and antimicrobial agents. However, Kaneene *et al.* (2007) advises that users of the system need to weigh the benefits of improved model performance (ARCC) versus loss of source specificity, given the low rates of species-specific correct classification. In this study as observed by Kaneene *et al.* (2007) misclassification of fish into human isolates increased from 7.7% to 15.5% when classification groups were reduced from eight to three groups respectively demonstrating loss of source specificity. Findings in this study however, demonstrate that discriminant analysis can be used to classify *E. coli* and attribute possible sources of contamination of Lake Victoria, Kenya fish and increasing size of the *E. coli* library increases the rate of ARCC.

CHARPTER SIX: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

This study has demonstrated that human and domesticated animals along Lake Victoria, Kenya are important sources for *E. coli*, whereas human, soil and sundried *R. argentea* are for *Salmonella* spp. The study also shows that *E. coli* contaminating Lake Victoria, Kenya fish does not carry virulence genes although human may be important carriers for enterotoxigenic *E. coli*. The findings further show multiple antibiotic resistance levels of over 78% among human *E. coli* isolates, whereas soil and fish isolates showed over 33%, and less that 24% for domesticated animals and water. The study further demonstrates the capability of Discriminant Analysis to discriminate *E. coli* isolates from human, livestock, fish and environmental sources, from which the study concludes that soil could be a major source for *Salmonella* and *E. coli* contaminating Lake Victoria fish.

6.2 Conclusion

1. Arising from the results, the shores of Lake Victoria, Kenya are highly contaminated with coliforms. However, the frequency, distribution and occurrence of the *Salmonella* spps and *E. coli* in this study vary among the different sources investigated. Humans were identified as the primary reservoirs of *Shigella* within Lake Victoria, Kenya and *Salmonella arizonae* is an emerging pathogen.

2. In this study, only *E. coli* carrying the ST and LT genes (ETEC) were detected out of the three pathotypes investigated. Enterotoxicogenic *E. coli* are common among children, but absent among *E. coli* colonizing domesticated animals gut, the environment and Lake Victoria, Kenya fish.

3. Based on MAR indices human are major carriers of multiple antibiotic resistance bacteria isolates, whereas cattle show no multiple antibiotic resistance. Antimicrobial susceptibility patterns displayed in the study vary depending of the bacterial species and their sources.

4. The Discriminant analysis (DA) of antibiotic disc inhibition zones of *E. coli* points to soil as one of the most important possible source of *E. coli* contaminating Lake Victoria, Kenya fish.

6.3 Recommendations

From the results of this study the following recommendations are made:

1. Sinces, sundried *R. argentea* and fresh Nile perch sourced from Lake Victoria, Kenya are contaminated with *Salmonella* and multiple antibiotic resistant *E. coli* posing a public health risk, fisheries operatives and regulatory agencies should therefore put in place measures to minimize fish from coming in contact with soil and animals. For immediate action restricting contact with soil may reduce microbial contamination and therefore improve the quality and safety of Lake Victoria, Kenya fish.

2. Human in Lake Victoria may carry pathogenic *E. coli* carrying the ST and LT genes (ETEC) and therefore should exhibit good hygene practice while handling fish to avoid any contamination.

3. Drying of fish on the ground is hazardous since soils are reservoirs of *Salmonella* and *E. coli* within the basin.

The following are recommendations for further investigations: -

4. There is a need for further research to understand factors and mechanisms that influence the distribution and survival of pathogenic *E. coli* in the region.

5. There is further a need for research to understand factors and mechanisms that promote the evolution and development of antibiotic resistance traits among the different diarrheagenic bacteria including their sources within the study site.

6. Microbial source tracking approaches using discriminate analysis (DA) is a good tools for investigating sources of contamination, and can be used for monitoring contamination among water and food products (fish, food animals and vegetable) locally. However since the study focused on use of phenotypic approaches to investigate sources of contamination of fish products with diarrheagenic bacteria, there is need for research using genotypic approaches for comparesion with this findings.

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APPENDICES

Appendix 1: Sample collection form

NAME OF BEACH……..

DATE OF SAMPLING……..

TEMPERATURE……

……

……

pH VALUES FOR WATER

……

……

……

……

SAMPLE CODES

Code	Sample	Code	Sample

COMMENTS AND OR OBSERVATION

……

……

……

……

……

……

Appendix 2: Patient Consent form

Sources of diarrhoeagenic bacteria contaminating fish in the Lake Victoria basin, Kenya

Section A

Information to volunteers/patients:

Bacterial diarrhoea has been identified to cause substantial morbidity and mortality in rural sub-Saharan Africa. In western Kenya the burden of diarrhoeal infections among the community has been shown to be enormous and *Shigella*, *Vibrio cholerae*, *Salmonella*, diarrhoeagenic *Escherichia coli*, and *Campylobacter* have been associated to diarrhoeal infection. Drinking water from Lake Victoria (the major source of water for all use in the community under study) has been found to increased risk diarrhoea. Fish products supplied and consumed by communities within the basin have also been shown to be contaminated similar bacteria. The study intends to find out if the bacteria contaminating fish, lake water, domesticated animals and the environment are related to those causing diarrhea in humans. The findings will also provide information on the distribution of diarrhoeagenic bacteria in the environment.

Procedure

You will collect freshly voided feaces and put in a stool collection container provided to you by the laboratory technologist. From it we will try and isolate diarrhoeagenic bacteria. You will also be asked simple questions like your age, occupation, residence etc.

Benefits of the study

The study will generate information on the prevalence of various diarrhoeagenic bacteria among humans within the selected hospitals. The findings will be useful for health planning and food safety policy development.

Confidentiality

Information obtained from you and your medical records will not be disclosed to any other party. Your name and identity will not be required, hence you will neither be linked to the results nor your identity be made public in any form whatsoever.

Medical problems

We don't expect this investigation to be connected to any medical problems.

Section B

Patient consent seeking form

Obtaining additional information

You are encouraged to ask any questions that occur to you at this time or any other time in the course of your contact with the investigators. You will be given a copy of this agreement. If you require more information at a later date you may call Anthony Sifuna on Tel. Nos. 0733715611 or 0724733328

Basis of participation

You are free to accept or refuse to participate in this study. You are also fee to withdraw your consent to participate in this study at any time you choose. If you choose to refuse / withdraw, your rights to be attended to in this health facility now or in future will not be affected.

I have read the information stated above and have had the opportunity to ask questions all of which were answered satisfactorily.

I hereby give consent for me / my child's participation as explained to me.

Study No: _____

Date: _____

Sex: _____ Age: _____

Residence:_____

Signature of consenter: _____

Questionnaire:

1. Where do you source your food from:

……

……

2. Where do you source your water from:

……

……

3. Comments by the clinician/laboratory technologist:

……

……

……

I the undersigned have fully explained the relevant details of this study to the patient named above. I am qualified to perform the role of principal investigator in this study.

Signature: _____ Name: _____ Date: _____

(Investigator)

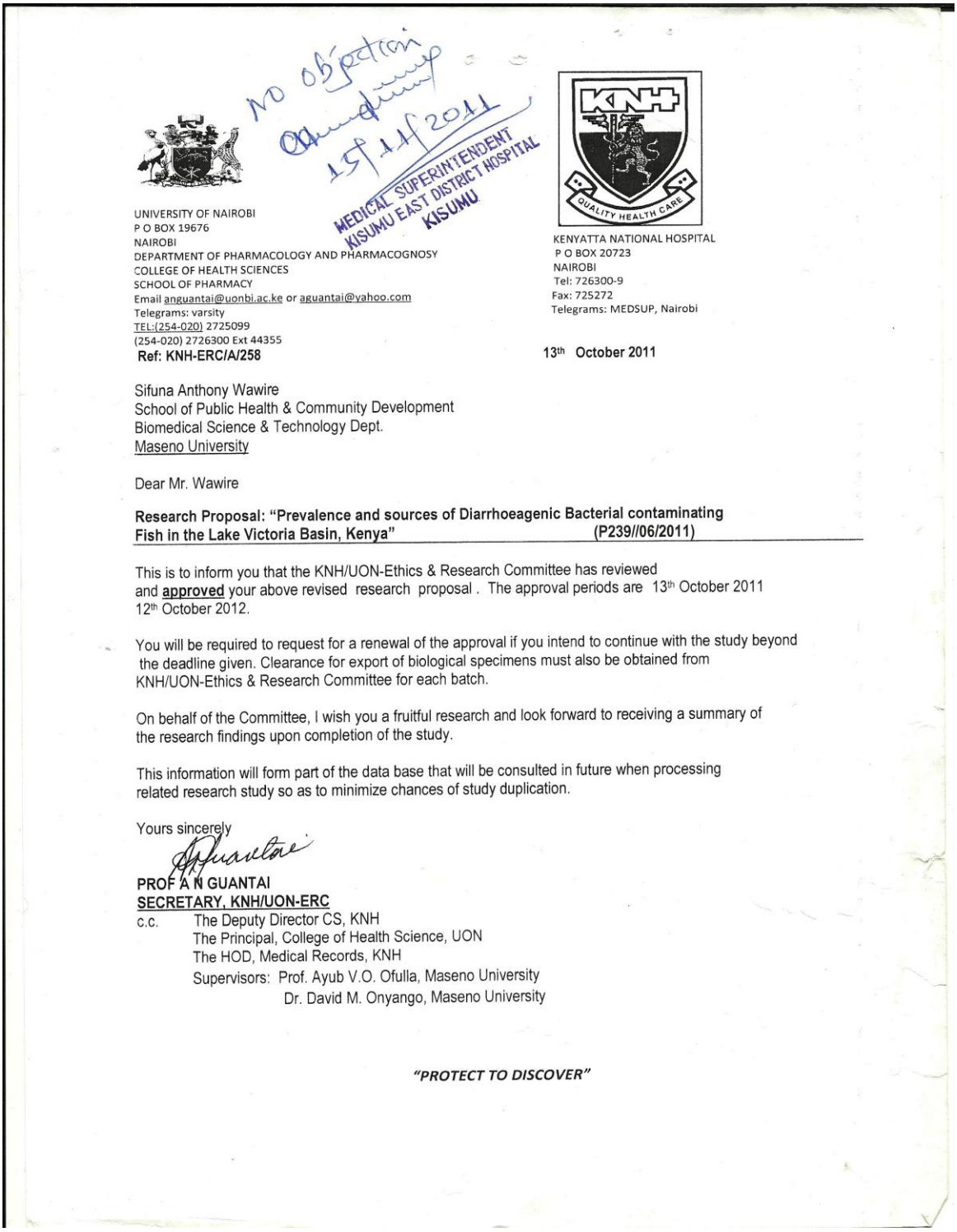
Signature: _____ Name: _____ Date: _____

[Witness]

Address of witness: _____

This work was carried out as part of the proposal Number……. titled; Prevalence and sources of diarrhoeagenic bacteria contaminating fish in the Lake Victoria basin, Kenya

Appendix 3: Research ethical review committee approval



No objection
15/11/2011
MEDICAL SUPERINTENDENT
KISUMU EAST DISTRICT HOSPITAL
KISUMU

UNIVERSITY OF NAIROBI
P O BOX 19676
NAIROBI
DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF PHARMACY
Email anguantai@uonbi.ac.ke or aguantai@yahoo.com
Telegrams: varsity
TEL:(254-020) 2725099
(254-020) 2726300 Ext 44355

KENYATTA NATIONAL HOSPITAL
P O BOX 20723
NAIROBI
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref: KNH-ERC/A/258

13th October 2011

Sifuna Anthony Wawire
School of Public Health & Community Development
Biomedical Science & Technology Dept.
Maseno University

Dear Mr. Wawire

Research Proposal: "Prevalence and sources of Diarrhoeagenic Bacterial contaminating Fish in the Lake Victoria Basin, Kenya" (P239//06/2011)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and **approved** your above revised research proposal . The approval periods are 13th October 2011 12th October 2012.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

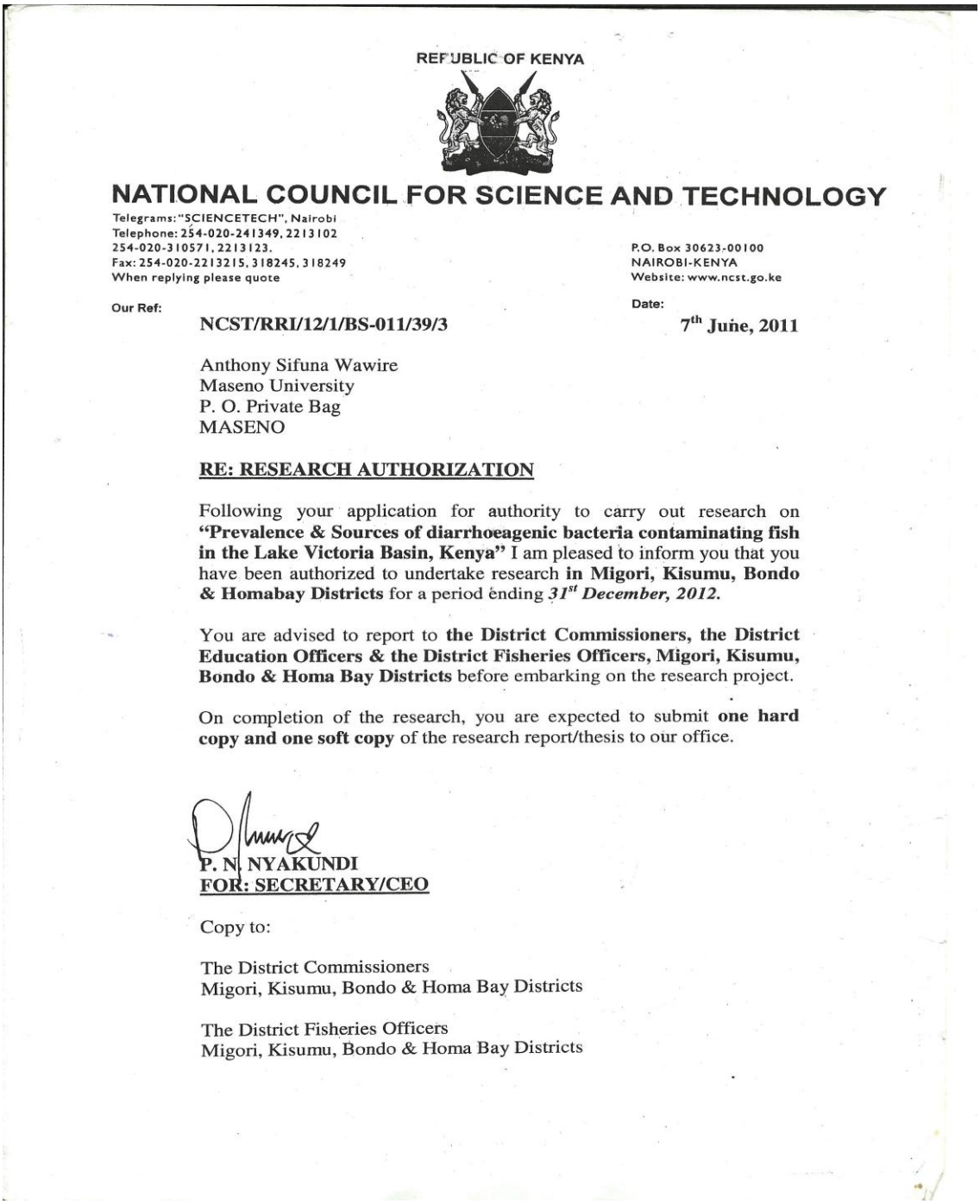
Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH/UON-ERC

c.c. The Deputy Director CS, KNH
The Principal, College of Health Science, UON
The HOD, Medical Records, KNH
Supervisors: Prof. Ayub V.O. Ofulla, Maseno University
Dr. David M. Onyango, Maseno University

"PROTECT TO DISCOVER"

Appendix 4: Research approval from the National Commission for Science Technology and Innovation



REPUBLIC OF KENYA

NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

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P.O. Box 30623-00100
NAIROBI-KENYA
Website: www.ncst.go.ke

Our Ref: **NCST/RRI/12/1/BS-011/39/3**

Date: **7th June, 2011**

Anthony Sifuna Wawire
Maseno University
P. O. Private Bag
MASENO

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on **"Prevalence & Sources of diarrhoeagenic bacteria contaminating fish in the Lake Victoria Basin, Kenya"** I am pleased to inform you that you have been authorized to undertake research **in Migori, Kisumu, Bondo & Homabay Districts** for a period ending ***31st December, 2012.***

You are advised to report to **the District Commissioners, the District Education Officers & the District Fisheries Officers, Migori, Kisumu, Bondo & Homa Bay Districts** before embarking on the research project.

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

P. N. NYAKUNDI
FOR: SECRETARY/CEO

Copy to:

The District Commissioners
Migori, Kisumu, Bondo & Homa Bay Districts

The District Fisheries Officers
Migori, Kisumu, Bondo & Homa Bay Districts

Appendix 5: Species of *Enterobacteriaceae* recovered from sources sampled from the Lake Victoria Basin

Enterobacteriaceae family	Number of individuals
Escherichia coli	263
Escherichia hermannii	1
***Proteus* spp.**	
P. vulgaris	14
P. muribilis	4
***Enterobacter* spp.**	
E. cloacae	81
E. sakazakii	24
***Klebseilla* spp.**	
K. pneumonia	11
K. oxyalocan	18
***Salmonella* spp.**	
S. arizonae	7
Salmonella other species	14
***Seriatia* spp.**	
S. liqueficans	5

S. fricaria	2
***Citrobacter* spp.**	
C. freundii	13
C. youngae	9
C. breekii	5
C. koseri	6
Providencia struattii	13
Morganella morganii	2
Pantoea spp.	1
Total	487

Appendix 6: Interpretation of biochemical tests performed on the isolates

Triple Sugar Iorn Agar (TSI)

1 2 3 4 5 6

Interpretation

1. A/K; with Hydrogen sulfide

2. A/K; with Hydrogen sulfide and gas

3. A/A; with Hydrogen sulfide and gas

4. K/A; with gas:

5. A/A; with gas:

Organism	TSI Profile
Salmonella	A/K; with H_2S and gas
S. arizonae	A/K; with H_2S and gas or A/A; with H_2S and gas
Shigella	K/A; without gas
E. coli	K/A; with gas or A/A; with gas

Motility Indole Lysine (MIL) medium

1 2 3 4

Interpretation

1. Motility positive, lysine decarboxylase positive and Indole positive

2. Motility positive, lysine decarboxylase positive and Indole negative

3. Lysine decarboxylase negative

4. Not inoculated

Organism	lysine decarboxylase	motility	lysine deaminase	indole production
Salmonella	+	+	-	-
Shigella	-/+	-	-	-
E. coli	+	-/+	-	-

Appendix 7: CDC calculations based on EPI Info V.3 (2005) for human samples

Sample Size for Frequency in a Population

Population size (for finite population correction factor or fpc) (*N*): 1000000

Hypothesized % frequency of outcome factor in the population (*p*): 5% +/-5

Confidence limits as % of 100(absolute+/- %) (*d*): 5%

Design effect (for cluster surveys - *DEFF*) 1

Sample size (n) for various confidence levels

Confidence level (%)	**Sample size**
95%	73
80%	32
90%	52
97%	90
99%	127
99.9%	206

Equation: Sample size $\boldsymbol{n = [DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)]}$

Results from OpenEpi, Version 3, open source calculator - SSPropor

Appendix 8: API 20 E reading table

WASTE DISPOSAL

It is the responsibility of each laboratory to handle waste and effluents produced according to their type and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

WARRANTY

bioMérieux disclaims all warranties, express or implied, including any implied warranties of MERCHANTABILITY AND FITNESS FOR A PARTICULAR USE. bioMérieux shall not be liable for any incidental or consequential damages. IN NO EVENT SHALL BIOMERIEUX'S LIABLITY TO CUSTOMER UNDER ANY CLAIM EXCEED A REFUND OF THE AMOUNT PAID TO BIOMERIEUX FOR THE PRODUCT OR SERVICE WHICH IS THE SUBJECT OF THE CLAIM.

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow (1)
ADH	L-arginine	1.9	Arginine DiHydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.756	CITrate utilization	pale green / yellow	blue-green / blue (3)
H_2S	sodium thiosulfate	0.075	H_2S production	colorless / greyish	black deposit / thin line
URE	urea	0.76	UREase	yellow	red / orange (2)
TDA	L-tryptophane	0.38	Tryptophane DeAminase	TDA / immediate: yellow	TDA / immediate: reddish brown
IND	L-tryptophane	0.19	INDole production	JAMES / immediate: colorless pale green / yellow	JAMES / immediate: pink
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / 10 min: colorless	VP 1 + VP 2 / 10 min: pink / red (5)
GEL	Gelatin (bovine origin)	0.6	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation / oxidation (GLUcose) (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	1.9	fermentation / oxidation (MANnitol) (4)	blue / blue-green	yellow
INO	inositol	1.9	fermentation / oxidation (INOsitol) (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation / oxidation (SORbitol) (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation / oxidation (RHAmnose) (4)	blue / blue-green	yellow
SAC	D-sucrose	1.9	fermentation / oxidation (SACcharose) (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation / oxidation (MELibiose) (4)	blue / blue-green	yellow
AMY	amygdalin	0.57	fermentation / oxidation (AMYgdalin) (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation / oxidation (ARAbinose) (4)	blue / blue-green	yellow
OX	(see oxidase test package insert)		cytochrome-OXidase	(see oxidase test package insert)	

(1) A very pale yellow should also be considered positive.
(2) An orange color after 36-48 hours incubation must be considered negative.
(3) Reading made in the cupule (aerobic).
(4) Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.
(5) A slightly pink color after 10 minutes should be considered negative.

- The quantities indicated may be adjusted depending on the titer of the raw materials used.
- Certain cupules contain products of animal origin, notably peptones.