ANTIBIOTIC RESISTANCE MECHANISMS IN *SALMONELLA* SPP ISOLATED FROM *RASTRINEOBOLA ARGENTEA* FROM SELECTED MARKETS AND WINAM GULF BEACHES- KENYA

BY

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SCHOOL OF BIOLOGICAL AND PHYSICAL SCIENCES

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DECLARATION

I declare that this work is my original work and has not previously been submitted for a degree in any other University or institution of higher learning. This thesis may not be reproduced or transmitted in any form without written permission from Maseno University or myself.

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DEDICATION

I dedicate this thesis to my dear wife, Everlyne N. Butala daughters Kathleen, Lyne Karen and son Ronny for always being there for me; and to my dear mother Mrs. Catherine Olaka for the invaluable support they gave me throughout this program.

ABSTRACT

Incidence of *Salmonella* resistance to various antibiotic agents, especially to the commonly available antibiotics in Kenya, is rising. Genetic elements such as Class 1 integrons have an important role in development and spread of resistance. In western Kenya, integron-mediated antibiotic resistance was reported in clinical *Salmonella* enteric serovars. However, no similar studies have been done to elucidate the role of class 1 integrons in fish isolates for multidrug resistant *Salmonella* spp. The aims of this study is to determine the levels of microbial contamination of fish, investigate antibiotic resistance mechanisms in *salmonella* spp isolated from *Rastrineobola argentea* obtained from markets (Kibuye, Luanda,Yala and Busia) and selected Winam Gulf beaches (Usenge, Dunga, Port Victoria and Uhanya). A cross sectional study design based on random sampling was used. 96 fish were sampled to determine antimicrobial susceptibility of the isolates, the incidence of resistant as well as screen *Salmonella* spp for the presence of class 1 integrons and characterize resistance genes. *Escherichia coli, Citrobacto*r spp and *Salmonella* spp were isolated on MacConkey and XLD agar plates respectively. Biochemical tests (IMViC) confirmed *E. coli* while TSI, LIA, and urease confirmed the presence of *Salmonella* spp. API 20E further confirmed all the isolates. Susceptibilities of seven antimicrobials were determined by agar disk diffusion method on Mueller Hinton agar. PCR was used to; characterize *Salmonella* spp by *Malic acid dehydrogenase* genes, screen for the presence of resistance genes and class 1 integron. ANOVA was used to determine statistical significance. Mean Aerobic Plate Count of sun-dried *R.argentiae* in markets was statistically higher ($p=0.003$) while that of beaches varied with sampling time (wet, $p=0.023$); (semi-dry, $p=0.03$) (dry,$p=0.02$). By using *chi*-square analysis, classes 1 integrons were not significantly associated with the antibiotics; tetracycline, ampicillin, streptomycine, chloramphenicol, and sulfamethoxazole ($p> 0.05$). *E.coli* was the most frequently isolated 36.66% followed by *Salmonella* spp 8.33% *Citobactor* spp, 5.83% *Klebbsiella* spp and *protieus* spp 1.58% each. Relatively high antibiotics resistance frequencies were found especially for chloramphenical (50%), ampiciline (33.33%).Tetracycline and sulphamethoxazol 20.63% each while naldixic acid streptomycine and ciprofloxacin were 7.93%, 3.17% and 1.85% respectively. For *Salmonella* spp, resistance rates were; 30%, 20% and 10% to chloramphenicol, (tetracycline sulphamethoxazol and ampicilin each) and streptomycin respectively. All isolates tested were susceptible to nalidixic acid and ciprofloxacin. Three isolates (one from market and two from beach) were multidrug resistance with at least three antimicrobials. Tetracycline resistance genes (*tet* A) 20%, ambiciline resistance genes *bla*TEM 20%, and streptomycin resistance genes (*aadA*) 10% were detected. Integron gene was found in two isolates (20%). One was from market and the other from the beach. Fish landing sites and fish markets may be a reservoir of many and different antibiotic resistant genes. Integrons may not be associated with multidrug resistance ($p>0.05$) isolates from fish. Thus presence of alternative resistance mechanisms.

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LIST OF ABBREVIATIONS

***aad*A**	Streptomycine gene
API	Analytical Profile Index
ARGs	Antibiotic Resistance Genes
***bal*PSE-1**	Ampicillin resistance gene
CDC	Center for Disease Control
CFU	Colony Forming Unit
(CS)	Conserved Regions
EU	European Union
FAO	Food and Agriculture Organization
GC	Gene Cassettes
GHP	Good Health Practice
HACCP	Hazard Analysis Critical Control Point
int1	Integron class 1
LVEMP	Lake Victoria Environmental Management Project
Mdh	Malate Dhydogenase Gene
MDR	Multi Drug Resistance
NTS	Non Typhoidal *Sulmonella*
PABA	para-aminobenzoic acid
SGI1	*Salmonella* Genomic Island 1
Sul	Sulphamethoxazol gene
Tet	Tetracyclin gene
US$	United States doller
XLD	Xylose Lysine Deoxycholate

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CHAPTER ONE

INTRODUCTION

1.1 Background

Fish contributes to about 60% of the world supply of protein and 60% of the third world countries derive more than 30% of their animal protein from fish (Emikpe *et al.,* 2011). On a national scale, fish and fish products from Lake Victoria are the most important source of protein, representing 85% of Kenya's fish supply and constitute 25% of total catch from Africa inland fisheries (Gitonga, 2006). Out of these percentages, dagaa (*Rastrineobola argentea* contributes 62.9% (Gitonga, 2006). Fish are generally regarded as safe, nutritious and beneficial, but its natural habitat is extremely prone to pollution that may be from domestic, industrial and agricultural discharges. Consumption of such fish and fish products may cause diseases due to infection or intoxication (Petersen *et al.,* 2002; Adebayo-Tayo *et al.,* 2012a).

Quality assurance and bacterial pathogens tracing studies have reported the presence of potentially pathogenic bacteria including *Salmonella* spp, *Pseudomonas* spp, and *Streptococcus* spp (Germini *et al*., 2009; Emikpe *et al*., 2011) from different fish such as *Rastrineobola argentea*, Nile perch (*Lates niloticus*), tilapia (*Oreochromis niloticus*), (D'Aoust *et al*., 1992; Sifuna *et al*., 2008).

Landing sites along Lake Victoria are major sources of fish and are usually contaminated by activities of adjoining population and partially treated and untreated sewage which finds its way into the lake (Kayambo *et al.,* 2006; Baudart *et al*., 2000). Fish from these sites often contain pathogenic microorganisms (Kayambo *et al.,* 2006). In addition, poor sanitation on fishing vessels and in handling centers including open air markets exacerbates the situation (Kayambo *et al.,* 2006). Bacteriological quality of freshly landed as well as retail market fish is not meeting the quality criteria (Ogwan'g *et al.,* 2005; Sifuna *et al*., 2008; Onyango *et al*., 2009). Unhygienic handling and storage are among major factors that contributes to poor quality of fish in retail markets as it leads to off-smell, physical damage, building up of bacterial load and contamination with dirt and objectionable microorganisms (Sugumar *et al.,* 2004).

Salmonellosis is the most foodborne bacterial disease in the world (PlymForshell and Wierup 2006) and approximately 95% of human cases are associated with the consumption of contaminated foods (Van Nierop *et al.,* 2005; Suresh *et al.,* 2006). Non-typhoidal *Salmonella enterica* is a common cause of a number of different diseases including; gastroenteritis and bacteremia (Gaikwad and Parekh, 1984). In the USA alone, salmonellosis causes an estimated 1.4 million cases of foodborne illness and 600 deaths annually (CDC 2006). While in Kenya, non-typhoidal *Salmonella* species (NTS) are common causes of bacteremia and gastroenteritis among immunocompromised individuals, infants and newborns (Kariuki *et al.,* 2002). Contamination of fish with *Salmonella* is a major public health concern with incidences relatively high in developing countries (Kumar *et al.,* 2003). Human infections by this pathogen are usually through contact with infected fish while handling them, water or other constituents of fish environment (Acha *et al*., 2003).

Antibiotic resistance of bacteria is a growing public health concern as antibiotic resistance determinants found in food and water can be transferred to bacteria of human clinical significance (Blake *et al.,* 2005). Among *Salmonella,* antibiotic resistance has emerged partly in response to selective pressure caused by indiscriminate use and misuse of antimicrobials including antibiotics given to veterinary animals (Bywater, 2004).

Incidence of multi drug resistant *Salmonella* especially to the commonly used antibiotics (sulphamethoxazole, ampicillin, tetracycline, chloramphenicol, nalidixic acid, ciprofloxacin and streptomycin) for treatment and management of diarrheal infections in the region has risen over the last several years posing significant cost (WHO, 2005).

In Kenya, multi drug resistance (MDR) *Salmonella* has become a major problem and that these resistant strains were quickly replacing the sensitive ones (Kariuki *et al*., 2004). In western Kenya, where many communities in the Lake Victoria basin interact with the lake ecosystem on a daily basis in fishing as well as collecting water for domestic and commercial purposes, prevalence of MDR *Salmonella typhimurium* was reportedly high in clinical samples from Maseno and Mukumu Hospitals (Onyango *et al*., 2007). Studies within the Lake Victoria region have also shown that more than 80% of *Shigella* spp. and *Salmonella* spp. isolated from patients attending hospitals were resistant to drugs commonly used (Sifuna *et al.,*

2008). Drug resistant bacteria may naturally occur in aquatic environments from where they make their way to humans and spread drug resistant genes leading to persistence of ill health (Acha and Szyfres, 2003). Sifuna *et al.,* (2008) showed that *E. coli* isolated from sundried fish sold in markets within the Lake Victoria region showed multiple antibiotic resistance, which was not transferable by conjugation. This study did not identify which genes were responsible. Hence it was thus postulated that Lake Victoria may be an important reservoir for antimicrobial resistant genes due to poor water resource management practices within the catchments (World Agroforestry Centre, 2006). This may serve to sustain the resistance genes among the Enterobacteriaceae within the Lake basin, whereas human, fish, and livestock watered from the lake serve to transfer or disperse the genes (World Agroforestry Centre, 2006).

The association between antibiotic resistance and integrons has been well documented (Collis and Hall, 1993). The coexistence of resistance genes with mobile genetic elements such as plasmids, transposons, and integrons facilitates rapid spread of antibiotic resistance genes among bacteria (Sunde *et al.,* 2006). The most encountered genetic element amongst Gram negative microorganisms are genetically inserted gene cassettes (GC) called integrons (Hall and Collis, 1998). These genetic structures are capable of capturing, excising and co-integrating gene cassettes that encode antimicrobial drug resistance determinants and disseminating them among bacteria (Recchia *et al.,* 1997; Mazel, 2006).This compromise therapeutic options like in the case of invasive *Salmonella* infections (Levesque *et al.,* 1994; Fluit, 2005). They are therefore suspected to serve as reservoirs of antimicrobial resistance genes (ARG) within microbial populations (Bass., 1999; Lucey *et al*., 2000; Ochman *et al*., 2000). Their frequencies in clinical samples are as high as 88% (Pan *et al.,* 2006). Relatively high prevalence of integrons in non-human samples have been reported in wastewater treatment plants (Moura *et al*., 2007), irrigation sediments (Roe., 2003), and farm animals (Goldstein *et al.,* 2001; Barlow *et al*.,2004;Yang *et al*., 2010; Karczmarczyk *et al*., 2011). Integrons are likely to be linked to fish habitatas by water flow systems (Rivers, floods), acting as a vehicle for antimicrobial resistance gene carrying bacteria. Onyango *et al*., (2010) demonstrated the presence of class 1 integron mechanism as a means of antibiotic resistance in the clinical *Salmonella enterica* serovars in rural western Kenya. Prevalence of MDR and integrons in isolates obtained from fish is however

lacking as most studies have always used clinical isolates (Sepp *et al.,* 2009), or a combination of animal and human derived specimens (Mazel *et al*., 2000; Cocchi *et al*., 2007). Currently, the single most basic role in dissemination and evolution of antimicrobial resistance in MDR *Salmonella typhimurium* DT104 and many other organisms is attributed to integrons gene expression. These elements potentially account for rapid and efficient transmission of drug resistance (due to their presence in plasmids and transposons) and ability to collect resistance gene cassettes (Recchia *et al.,* 1995). These elements have been described in a wide range of pathogenic organisms (Jones *et al.,* 1997), including *S. typhimurium* and *S. enteritidis* (Rankin *et al*., 1998; Brown *et al*., 2000).

1.2 Problem statement

The risk of fish contamination with pathogenic microorganisms can occur along the food chain including production, processing, distribution and handling at retail markets (Kayambo *et al.,* 2006). The presence of *Salmonella* spp in food usually indicates cross contamination and mishandling of the fish products (Acha *et al*., 2003), while total coliform counts are normally used in food processing as indicators of hygien (Jay, 2000). Environmental degradation of fish landing sites affecting fish and fisher folk has already been reported (Lyhs, 2009). To the present, information on bacterial contamination levels of *R. argentea* during landing, processing and at the markets is still limited.

The presence of *Salmonella* spp and occurrence of MDR *E. coli* were identified as some of the possible health risks that may be associated with *R. argentea* displayed for sale in Kisumu city markets (Sifuna *et al.,* 2008). Previously, high prevalence of antibiotic resistance to tetracycline, ampicillin and co-trimoxazole was reported to occur in human bacterial isolates, including *Salmonella* enterica serovar, *S. typhi* and other diarrhea-causing pathogens like *E. coli* in the lake basin region (Onyango *et al.,* 2008). This could be as aresult of longstanding, unregulated antimicrobial use (Kakai, 2009). However, little has been published about the occurrence of antibiotic-resistant bacteria in freshly landed and dried fish.

Incidence of *Salmonella* resistant to various antibiotic agents especially to the commonly available antibiotics is aided by horizontal gene transfer via mobile genetic elements (transposons and conjugative plasmids). Recently, integrons have been recognized as genetic

elements that have the capacity to contribute to the spread of resistance. Enviromentally, relatively high prevalence of integrons have been reported, (Moura *et al*., 2007) while in clinical samples, frequencies are as high as 88% (Pan *et al.,* 2006). Integrons constitute an efficient means of capturing gene cassettes and allow expression of encoded resistance. In a study of mixed sample set of animal, commensal, human and clinical human isolates, MDR was found to be associated with presence of integron. In western Kenya, integron presence was demonstrated in clinical *Salmonella* isolates (Onyango *et al*., 2010). In this study, *bal*PSE-1, *bla*TEM and *aad*A resistance genes were not identified as part of the chromosomal multiresistance cluster. Sifuna *et al*., (2008) indicated that *E. coli* isolated from sundried fish sold in markets within the Lake Victoria region showed multiple antibiotic resistance which was not transferable by conjugation. Despite the observed increase in resistance among *Salmonella* strains to commonly used antibiotics, data on the occurrence and the role of integrons in the emergence and spread of MDR *Salmonella* in the Lake Basin is still inadequate.

1.3 Justification of the study

The ban of fish and fish products for export to Europe (Spain, Germany and Italy) like in 1998 (Abila *et al*., 1997) due to pathogenic bacterial contamination led to lose of revenue by fisher folk that affected per capital of a developing country like Kenya. In 2003, United Nations and the World Health Organization declared in a joint statement of action by the Food and Agriculture Organization that "Illness due to contaminated food was perhaps the most widespread health problem in the contemporary world," and "an important cause of reduced economic productivity" (Kaferstein, 2003). Onyango *et al*., (2008), showed that Nile tilapias from Lake Victoria were contaminated by Enterobacteriaceae, including *Salmonella.* Since fish can harbor *Salmonella* for a long period without any symptoms, contact with infected fish can lead to human infection. This study contributes to vital data acquisition that is critical in assessing and controlling the risk associated with the presence of *Salmonella* in the acquatic environment that is prone to contamination from pollution.

Morbidity and mortality due to bacterial infections is a major public health problem in developing countries including Kenya (Kariuki *et al*., 2006). This is partly as a result of bacterial resistance to antimicrobial drugs used for therapeutic purposes. Occurrence of antibiotic

resistance in patients at the local hospitals leads to high cost of hospital bills; medical expenses for much higher potent antibiotics that patients may not afford. These situations results into prolonged pathological conditions like diarrhea, bacteremia and gastroenteritis. Diarrhea causes 22% of pediatric deaths worldwide, 40% of which are in Africa. With increasing antibiotic resistance worldwide, there is need for the development of new drug regimen, making it expensive for the drug companies and research organizations. In western Kenya clinical data indicated high rates of MDR *Salmonella typhimurium* and ampicillin resistance gene (bla_{TEM}) was amplified in 90% of the isolates (Onyango *et al*., 2007). MDR bacteria are known to be resistant to primary antibiotic preferred for treatment, requiring the use of secondary and or tertiary drugs instead. These may be less effective and more toxic to the patient (CDC, 2012). A more worrying situation with MDR is the fact that only two new classes of antibiotics have reached the market in the last few decades thus confirming that new discoveries of antimicrobial agents have been few and far between in recent years (Davies, 2007; Coates *et al*., 2011). This information can point to a future where no new antibiotic classes will be introduced (Coates *et al*., 2011). Thus, the threat of a return to the pre-antibiotic era is very real without the continuous introduction of new antibiotics (Coates *et al*., 2011; Davies and Davies, 2010). All these factors contribute to a problem that if not addressed would put more pressure on drug development. The paucity of comprehensive data regarding the extent to which antimicrobial resistant pathogens in the region poses a challenge in developing evidence-based interventions for treatment, control and prevention of diarrhea, gastroenteritis and typhoid fever, conditions caused by *Salmonella* spp is a challenge. Morbidity due to *Salmonella* spp leads to lose of man-working hours by affected patients. If unchecked antimicrobial resistance would lead to halting of cottage industries and our interconnected, high-tech world may find itself back in the dark ages of medicine, before today's miracle drugs ever existed. The environmental reservoir of resistance elements contributing to resistance had not been studied in this area.

1.4 Significance of this study

This study sought to determine the role of *R. argentea* in the dissemination of *Salmonella* and other pathogenic microbes to people living in the Lake Basin. Previous studies by Onyango *et al*., (2008) indicated that fish from the Winam gulf of Lake Victoria were contaminated by *Salmonella* and were potential reservoirs of these pathogens to human. In addition, integron

mechanism was linked to antibiotic resistance in the clinical *Salmonella* enterica serovars in western Kenya Onyango *et al*., (2010). This study explains fish as a possible environmental reservoir of pathogenic bacteria including *Salmonella.* The information generated can be shared with fisheries department, public health officers and other stakeholders in managing cross species infections through surveillance, prevention, and control of fish-borne salmonellosis so that they can advise fish farmers and consumers on ways to break the cycle of infection from human to fish and back to human (anthropogenic- zoonotic cycle). The study also sought to explain fish as a possible reservoir for the class 1 integrons.

1.5 Null Hypothesis

1. There is no difference in the level of microbial contamination of *R. argentea* by pathogenic *enterobacteriacae* obtained from selected beaches of Lake Victoria and open markets in the region.
2. There is no difference in antibiotic susceptibility test by disk diffusion zone sizes from *E.coli, Citobactor Proteus* spp and *Salmonella* spp isolated from *R. argentea.*
3. Antibiotic resistance genes are not widely distributed in *Salmonella* isolates
4. There is no association between phenotypic antimicrobial resistance profiles of *Salmonella* isolates with class 1 integrons.

1.6 Main objective

To determine antibiotic resistance mechanisms in *salmonella* spp isolated from *Rastrineobola argentea* from selected markets and beaches of Winam gulf in western Kenya.

1.7 Specific objectives

1. To determine microbial contamination of *R. argentea* by pathogenic *enterobacteriacae* obtained from selected beaches of Lake Victoria and open markets in the region.
2. To determine antibiotic susceptibility test and resistant patterns of *E.coli*, *Citobactor* and *Salmonella* spp isolated from *R. argentea.*

3. To determine the distribution of resistance genes in *Salmonella* spp isolates.

4. To determine association of phenotypic antimicrobial resistance profiles of *Salmonella* spp with class 1 integrons.

CHAPTER TWO

LITERATURE REVIEW

2.1. Microbiological contamination of *R. argentea* obtained from selected beaches of Lake Victoria and trading market in the region.

2.1.1 Seafood safety and microbiological standards

Fish is known to be highly perishable and one prone to contamination. Time and temperature are factors if unconsidered can enhance fish spoilage (Adams and Moss, 2008). Every product has a typical spectrum of microbial contaminants reflected by the exposure pattern of the product. As a result of contamination, microbiological standards are recommended for most products (Najiah Musa *et al.*, 2008). Crucial element in assuring the final quality of the product is the proper handling of fish between capture and its delivery to the consumer. Significant factors of quality involved in such cases are standards of sanitation, methods of handling, the time and temperature of holding fish. Apart from a few exceptions, when first caught, fish are assumed to be free of pathogenic bacteria of public health significance. However, this is not the case in most instances since during capture, the fish undergoes physiological stress that exposes it to infestation by pathogenic microbes at respective vital places (gill rake, skin, intestine and the eyes). Hence, when found, these pathogens are generally an indication of poor sanitation along the processing line, and contamination is almost always of human or animal origin (Popovic *et al*., 2010).

One-fourth of the world food supply is lost through microbial activity alone (EEC, 1992). This is according to the US National Research Council Committee (FND/NRC). *Salmonella* in cooked, ready-to-eat products and raw, frozen products leads to rejection of such products (Huss, 2003 cited in Amagllini *et al.*, 2012). In international trade, import refusal of such products is termed as "filthy." This refers to product which appears to consist in whole or in part of a filthy, putrid or decomposed substance majorly due to microbial spoilage. Thus, the need for control of quality of sea foods to avoid high microbial contamination which may lead to antibiotics resistance is well documented and since the rate of seafood borne illnesses is increasing, there is also an urgent means of assuring quality of sea food (Adebayo-Tayo *et al.*, 2012a).

Several studies have been done on *R. argentea* Sifuna *et al*., (2008) on microbial quality of *R. argentea* sold in Kisumu markets, Ogwan'g *et al.,* (2005) on smocking as a preservation method of fish and Owaga *et al.,* (2009) on the effects of 3% sodium chloride salt as a preservation method of *R. argentea* and other Lake Victoria fish including tilapias. However, there is still a dearth of information on the bacterial load in *R. argentea* of Lake Victoria water sampled from markets and beaches.

2. 2 Prevalence of *Salmonella* spp in fish

Bacterial flora of marine fish, sediments and sea water has been studied the world over with a view to explain the spoilage of fish (Yagoub, 2009). The U.S. Food and Drug Administration's (USFDA) data shows that *Salmonella* was the most common contaminant of fish and fishery products (Allshouse *et al.,* 2004). An overall *Salmonella* incidence of 7.2 % in imported and 1.3 % in domestic seafood was reported during an 8-year study (1990–1998) of 11,312 imported and 768 domestic seafood samples (Heinitz *et al.,* 2000). According to World health organization, there are about 17 million cases of acute gastroenteritis or diarrhea due to non typhodal salmonellosis (Rabsch *et al.,* 2001). In USA, *Salmonella* causes an estimated 14 million cases of foodborne illness and 600 deaths annually (Mead *et al.,* 1999). More than150, 000 cases of human salmonellosis were reported by The European Surveillance System during 2007 (European Food Safety Authority, 2009). *Salmonella enteritidis* and *Salmonella typhimurium* are two of the ten most common serotypes confirmed in salmonellosis cases in human, representing 81% of the isolates (European Food Safety Authority, 2009). Relatively high incidences of *Salmonella* contaminations have been reported from developing countries. India and Mexico reported a percentage of up to 10-15 of fish samples positive of *Salmonella.* Huss and Gram (2003) also reported *Salmonella* detection in several crustacean and molluscan products from India and Malaysia. In 1989, more than 8,300 tones of frozen raw prawns were detained by the USFDA due to contamination with *Salmonella*. However Japanese accepted *Salmonella* in raw frozen shrimps (Reilly and Twiddy, 1992 cited in Kumar *et al.,* 2009). Central Pacific and African countries reported highest *Salmonella* incidence in fishery products while it was lower in Europe, and North America (Heinitz *et al.,* 2000). However, Novotny *et al.,* (2004) reported an outbreak of *Salmonella* blockley infections following smoked eel consumption in Germany. Da Silva, (2002) reported *Salmonella paratyphi* B infections associated with consumption of

smoked halibut in Germany. Besides, consumption of dried anchovy was found to be the cause of *Salmonella* infection (Ling *et al.,* 2002). Deep-sea fish are generally *Salmonella* spp. free but are susceptible to contamination post-harvest. Water temperature has been proposed as playing an important role in the long-term survival of *Salmonella* in the environment (FAO, 2010). Raw seafood products mainly from tropical climates have high prevalence of *Salmonella* with low prevalence or absence common in temperate regions (Millard and Rocklif, 2004).

In Africa, reports of *Salmonella* from human and foods of animal origin are well documented. For instance, in Uganda prevalence of *Salmonella* spp was found to be 8.1% in patients with acute diarrhea in Kampala district (Nasinyama *et al.,* 1998) with no mention of origin point source. However, in Kenya, Onyango *et al.,* (2008) reported isolation of *Salmonella* spp from Nile tilapia in the Winam Gulf of Lake Victoria in Western Kenya. In addition, Sifuna *et al.,* (2008) also isolated *Salmonella* in fish (*R. argentea*) sold in six markets in Kisumu city, western Kenya within Winam Gulf. These incidences act as indicator of beach contamination by pathogenic bacterial that are usually associated with untreated municipal sewage, runoff, and storm-water. In another study, no *Salmonella* spp. was reported from all the smoked analyzed fish samples (*Lates niloticus* and tilapiine) from selected beaches and markets within the Winam Gulf of Lake Victoria beaches (Ogwan'g *et al.,* 2005). However, (Onyango *et al.,* 2014), found contamination of *R. argentea* collected from respective markets and beaches in western Kenya an indication of continuous contamination of fish by pathogenic microbes. Fish and shellfish have been reported to be passive carriers of *Salmonella* that demonstrate no clinical disease and can excrete *Salmonella* spp. without apparent symptoms (Fell *et al* ., 2000) and that human contact with *Salmonella* infected fish, or water could result to infection (Newaj-Fyuzul *et al*., 2006; Onyango *et al*., 2008). Their presence in fish and fishery product is therefore seen as a sign of poor standards of procesing, hygiene and sanitation (Dalsgaard, 1998).

2.3 Public health perspectives of *Salmonella*

Salmonella has become one of main causative agents of enteric infections in humans and animals (Tauxe, 1996). Several salmonellosis outbreaks have been documented worldwide due to the consumption of contaminated fish (Flick, 2008). Acute gastroenteritis caused by *Salmonella* spp. remains to be a worldwide public health concern with the predominant serotypes from clinical

cases varying with the geographical region. Conversely, morbidity in turn translates into a significant economic impact. *Salmonella enteric* serovar *enteritidis* is the most common in Europe, Central and South America, while *Salmonella enteric* serovar *typhimurium* is predominant in Oceania, North America and Africa (WHO, 2006). These pathogens are transmitted to humans at both processing and kitchen environment by their respective vehicles which have been identified in various foods particularly of animal origin (Uyttendaele *et al*., 1998). Despite improvement in hygiene, food processing, education of food handlers and information to the consumers, food-borne diseases are still important to public health (Dominguez *et al.,* 2002). By 2007 the percentage of population that suffered from food-borne diseases had reportedly gone up to 30 % (WHO, 2007) and gastroenteritis was the most disease associated with food-borne transmission (Bremer *et al.,* 2003). The control of *Salmonella* is required to decrease the number of organisms that are discharged into the environment. Water and soil are also a part of the epidemiological cycle and can transfer bacteria to vegetables and herbs. The most high risk food categories which cause salmonellosis are raw or undercooked meat, eggs and products containing raw eggs, unpasteurized milk, and juices (Plym and Wierup, 2006). Water and food which is contaminated with feces and an asymptomatic human carrier can be reservoir for *Salmonella*. Fish in our open air markets is usually displayed unwrapped hence exposing the product at ambient temperatures and further contamination from flies and dust. Several reports have indicated that quality of fish sold in domestic markets in western Kenya is poor compared to that of export trade and are mostly contaminated with pathogenic microorganisms (91/493/EEC; KEBS, 1998; EAS, 2014). Among fish food consumers, the group at greatest risk appears to be consumers of raw *R. argentea* because of environmental contamination and naturally occurring vibrios and other coliforms.

2. 4 Status of *Salmonella* in developing countries

According to developing world census, there is a positive population growth resulting into a multifactorial risk of foodborne hazards due to: poor sanitation, inadequate access to portable water, scarce financial resources and low priority on food control issues in public health programs (Henson, 2003b). Besides, health and economic consequences are often overlooked by relevant authorities, governments and even at individual level because most food-borne illnesses are perceived as mild, self-limiting and therefore don’t require medical intervention (Venter,

2000). This result in limited data through which the incidences of particular diseases and trends over time can be assessed. High prevalence of diarrheal diseases is reported in many developing countries, suggesting the presence of major underlying food safety problems. And because aquatic environments are the major reservoirs of *Salmonella,* fish and fishery products have been recognized as a major carrier of food-borne pathogens in many developing countries and even in developed ones (Upadhyay *et al*., 2010). *Salmonella* causes a heavy toll on human health, particularly among infants, children, elderly and other immunocompromised groups. Sifuna *et al.,* (2008) postulated that the presence of *Salmonella* spp and occurrence of MDR *E. coli* as some of the possible health risks that may be associated with *R. argentea* displayed for sale in Kisumu city markets. This poses a real health risk through consumption or directly through contact with the fish products. This study did not include other markets in the region that receive fish from Lake Victoria. Most published work regarding microbial contamination of *R. argentea* in the lake basin region generally concentrates on limited spectra of bacteria, mostly *E.coli* and *vibrios* (particularly *V. cholera*) and other coliforms, but this study provides an insight into the occurrence of *Salmonella* in fish.

2.5 Antimicrobial resistance of *Salmonella* spp isolates

2.5.1 Drug Resistance

Drug Resistance takes two forms; one is inherent or intrinsic resistance, whereby the organism is not normally susceptible to a particular drug (Novak, *et al.,* 1999). This may be due to the inability of the antibacterial agent to enter the bacterial cell and reach its target site, or to a lack of affinity between the antibacterial and its target (site of action), or an absence of the target in the cell (Figure 1). The other is the acquired resistance, where the species is normally susceptible

to a particular drug but certain strains express drug resistance (Hernández, 2005).

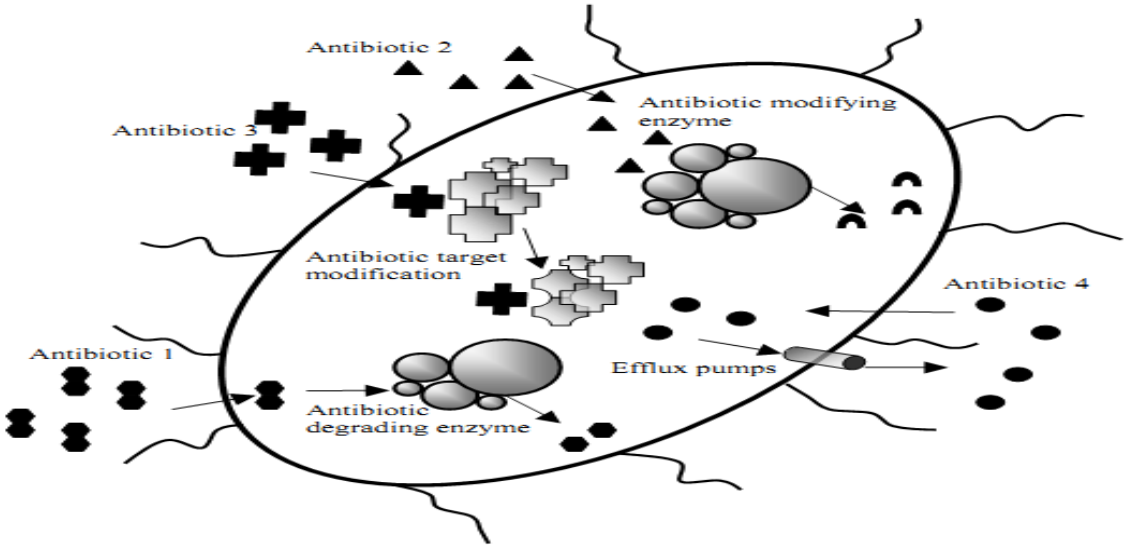

Figure 1: Schematic presentation of the mechanisms of antimicrobial resistance (Image from http://www.chembio.uoguelph.ca/merrill/research/ enzyme mechanisms. (Html accessed on 2nd March 2014).

2.5.2 Mechanisms of antimicrobial resistance in bacteria

2.5.2.1 Alteration of the targeted site of action

This occurs when there is a change in the "targeted site of action" in such a way that the target can perform its function (although perhaps not quite as well) but is not as sensitive to antimicrobial therapy; e.g. resistance to bacterial cell wall inhibitors such as penicillin's.

2.5.2.2. Overproduction of the target

Instead of altering the sequence (nucleic acid or protein) of the antimicrobial target, it is possible to alter (increase) the copy number of the target so that the effective drug concentration required to inhibit a specific process is increased; e.g. resistance of sulfonamides in case of para-aminobenzoic acid (PABA) conversion to dihydropteric acid and over expression of the enzyme dihydropteroate synthase.

2.5.2.3 Production of a new enzyme to bypass the targeted site of action

Some bacteria such as members of Enterobacteriaceae and *Staphylococcus aureus* can synthesize entirely new enzymes, which can carry out the PABA to dihydropteroate conversion. These enzymes decrease the affinity for trimethoprim and sulfonamides.

2.5.2.4 Limiting access of the antibiotic to the targeted site of action (Efflux)

An efflux pump is essentially a channel that actively exports antimicrobial and other compounds out of the cell (Madigan *et al*., 2000). The antimicrobial enters the bacterium through a channel called porin, and then is pumped back out of the bacterium by the efflux pump (Lewis, 1995).
Thus by actively pumping out antimicrobials, the efflux pumps prevent the intracellular accumulation necessary to exert their lethal activity inside the cell (Schlegel, 1995).

2.5.2.5 Modification of antimicrobial agent

This mechanism of action is particularly found in Gram negative bacteria. Changes in the lipid composition of the outer membrane may significantly contribute to resistance as seen in erythromycin resistance. The expression of specific transporters is another way that bacteria limit access of an antimicrobial agent to its site of action e.g. resistance to tetracycline.

2.5.3 Antimicrobial resistance in *Salmonella*

Exposure of micro flora to antibiotics may increase the number of resistant factors which can transfer resistance to pathogenic bacteria (Mc Dermott, 2002). Over the past several years, the prevalence of antimicrobial-resistant *Salmonella* has increased (CDC, 2004). This increase in resistant factors was noted in different parts of the world (Gales *et al.,* 2002). In 1980, in the USA, 13% of *Salmonella* isolated from humans were of serotype *typhimurium*. These isolates were resistant to more than 1 of the many antimicrobial agents by 2001, and this proportion had increased to 51% by (CDC, 2004). In Asia it is believed that the use of antimicrobial agents in aquaculture contributed to the emergence of MDR-DT104 genotype (Davis *et al.,* 1999). In Japan the incidence of MDR-DT104 was detected as 1.9% (Ahmed *et al*., 2005). In Malaysia, fish was implicated as an important human source of *Salmonella* and had high potential to transmit antibiotic resistance genes to other pathogens via plasmids (Hradecka *et al.,* 2008**).** In

sub-Saharan Africa, resistance to ampicillin and trimethoprim/sulfamethoxazole is almost universal, but susceptibility to chloramphenicol and sometimes gentamicin appeared preserved (Bolton *et al.,* 1999). The emergence of world wide multidrug resistant strains of a number of pathogenic bacteria including *Salmonella* in foods has increased human infection through food and animal contact (Threlfall, 2002). A recent estimate in the United States suggests that 24.6 million pounds of antibiotics are given to animals annually as growth promoters at sub-therapeutic amounts in their feed compared to 3 million pounds consumed by humans (White *et al.,* 2001). Resistances to commonly used drugs e.g. quinolone, fluroquinolone, nalidixic acid and ciprofloxacin preferable for treatment of salmonellosis in adults and cephalosporines for pediatrics (Threlfall, *et al*., 1996; Fey *et al.,* 2000) are of particular importance to the medical community. *S. typhimurium* is frequently associated with MDR (Mulvey *et al.,* 2006; Onyango *et al*., 2008), in part due to the worldwide emergence of *S. typhimurium* definitive phage type (DT) 104, which contains the chromosomal *Salmonella* genomic island type I (SGI-1). SGI-1 harbors genes that confer the ACSSuT phenotype (i.e. resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (Bolton *et al.,* 1999; Mulvey *et al.,* 2006). Other reports suggested a link between specific *Salmonella* serovars, phage types, and resistance to quinolones (Fey *et al.,* 2000). This phenomenon shows that non-typhoidal *Salmonella* isolates resistant to nalidixic acid has increased with a concomitant decrease in the level of susceptibility to ciprofloxacin. Other than this penta group of antimicrobials, scientists are vigilant about the ceftriaxone, ciprofloxacin and fluoroquinolones resistance in DT 104 since ceftriaxone is the current drug of choice against DT 104 in children and ciprofloxacin and fluoroquinolones are considered to be the last line of defense against DT 104 in adults (CDC, 2013). Worse still, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism, and are therefore likely to retain their drug-resistant genes even when antimicrobial drugs are no longer in use (WHO, 2005). Although *S. typhimurium* DT104 act as the main example of multi drug resistant strain in *S. enterica*, many antimicrobial resistance genes have been reported also in isolates of other serotypes, for example DT 208 that may have acquired this resistance phenomenon for DT 104 (Michael *et al*., 2006). Alternatively, another *S. enterica* serotype, such as Agona, may be elevated to the status of DT 104 because of horizontal gene transfer from one serovar or genus to the other related serovas or genera, thus suggesting that this phage type can acquire additional resistance to other new and portent drugs like higher

generation cephalosporins. Moreover, Miriagou *et al.,* (2002), reported the emergence of non-typhoid *Salmonella enterica* serotype *typhimurium* strains that are resistant to expanded-spectrum cephalosporins from a pediatric population in Iasi, Romania. This phage accounted for 7% of non-typhoidal *Salmonella* isolates tested in US national public health surveillance (CDC, 2004) whereby antimicrobial resistance resulted in increased morbidity and mortality as well as health care costs, with *S. typhi,* yearly expenditures arising from drug resistance running in the $3 billion range **(**WHO, 2005).

The use of antibiotic agents in humans and animals affect all bacteria in the intestinal tract. People who use antibiotic drugs for unrelated reasons and have *Salmonella* in the intestinal tract are at risk of becoming *Salmonella* resistant to the antibiotic agent. This risk is described as an attribute fraction (WHO, 2005). High incidence of treatment failure and severity of infection can cause extended duration of sickness, increased rates of infection, hospitalizations and death (WHO, 2005).

2.5.4 Resistance pattern of *Salmonella* spp a worldwide picture

After the first generation of antibiotics was introduced in medicine, evidence of bacterial strains resistant to those antibiotics began to surface in just less than a decade (Davies, 2007; Davies and Davies, 2010). What followed next was uncovering of evidence that bacteria were not only capable of developing resistance to one antibiotic, but to multiple antibiotics and that the resistance was also transferable to sensitive strains (Davies, 2007). This resulted in multidrug-resistant (MDR) bacteria due to unscrupulous antibiotic use in both medicine and agriculture in the decades that followed (Barlow *et al.,* 2004, Cocchi *et al.,* 2007). MDR bacteria may be resistant to a couple of antibiotics, several classes of antibiotics, and in some cases every antibiotic (CDC, 2012). A more worrying situation with MDR is the fact that only two new classes of antibiotics have reached the market in the last few decades thus confirming that new discoveries of antimicrobial agents have been few and far between in recent years (Davies, 2007; Coates *et al.,* 2011). Development of antimicrobial resistance causes enormous costs: the costs to bring out a new drug onto the market are estimated at a minimum of US$ 300 million (Byarugaba, 2004). This information can point to a future where no new antibiotic classes will be introduced (Coates *et al.,* 2011).

Most non-typhoidal *Salmonella* infections manifest as potentially self-limiting diarrhea. Antimicrobial resistance is clinically relevant because 3-10% of these infections can progress to life-threatening bacteremia, particularly in young and immuno-compromised patients. In Indonesia, proportion of cases of typhoid fever attributed to multidrug resistance gradually increased since 2001 (Hatta and Ratnawati, 2008). In this report the levels of antibiotic resistance to all three first line drugs, i.e. ampicillin, chloramphenicol and co-trimoxazole in S. *typhi* from clinical cases in South Sulawesi had risen to 6.8% in 2007. *S. enteritidis* isolates were reportedly resistant to most of the antimicrobials tested, with the exceptions of fluoroquinolones. In a comparative study of 95 strains of *Salmonella* weltevreden from different sources in Malaysia, clinical isolates remained drug sensitive, but isolates from vegetable were resistant to at least two antibiotics (Thong *et al*., 2002). Aarestrup *et al.,* (2003) found that 9.5% of the 503 *S.* weltevreden isolates from ten countries were resistant to one or more antimicrobial agents.

One of the studies in Spain reported high percentages of resistance of *Salmonella* isolates to sulfadiazine, neomycin, tetracycline and streptomycin, which might be the result of use of antibiotics as a prophylaxis, growth promoter or treatment (Carraminana *et al.,* 2004). In Alberta Canada, another study indicated high resistance of *Salmonella* isolates from food and food animals to ampicillin, streptomycin, sulfamethoxazole and tetracycline (Johnson *et al*., 2005). Over the past decade in Nepal, increasing antibiotic resistance in *Salmonella enterica* lead to a shift in the antibiotics used against this organism from chloramphenicol and ampicillin to trimethoprim-sulfamethoxazole, fluoroquinolones and ceftriaxone, where only 16-40% positive response to treatment was achieved (Pokharel *et al.,* 2006). In a study of 380 *Salmonella* isolates from animal origin in the US, 82% of the isolates were resistant to at least one antimicrobial, and 70% to three or more antimicrobials. Resistance was also most often observed to tetracycline, followed by streptomycin, sulfamethoxazole, ampicillin, chloramphenicol, kanamycin, amoxicillin/clavulanic acid, and ceftiofur (Zhao *et al.,* 2007). In the USA, between 1999 to 2003, of the 34 411 *Salmonella* isolated from animals, 10.9% were found to be resistant to ceftiofur and only 0.3% were resistant to ceftriaxone, both third generation cephalosporin used in animal and human medicine respectively. However later on, resistance to ceftiofur increased (Frye and Fedorka-Cray, 2007). Increased antibiotic resistance among *Salmonella* is not only in the percentage isolates resistant to a particular antibiotic, but also the development of resistance

against newer antibiotics (Fluit, 2005). In a study in Nepal, 35 multi-drug-resistant strains out of 132 strains of *Salmonella typhi* were observed showing simultaneous resistance to ampicillin, chloramphenicol, and co-trimoxazole. Although there were no isolates resistant to ciprofloxacin, 69.23% of 52 isolates tested for minimum inhibitory concentration of ciprofloxacin showed reduced susceptibility and 76% of 112 strains tested for nalidixic acid were resistant (Khanal *et al.,* 2007). There were reports of *Salmonella* resistant strains isolated from The Netherlands (Duijkeren *et al*., 2003) and Portugal (Antunes *et al*., 2003). Between the year 1999 and 2004, the number of publications reporting *Salmonella* resistant to β-lactams antibiotics increased drastically. In 2004, *Salmonella* resistant to extended spectrum cephalosporins were identified in 43 countries (Arlet *et al*., 2006). Human and food animals *S. typhimurium* strains isolates reportedly conferred antimicrobial resistance genes encoding for β-lactamase (Lee *et al*., 1994). They included TEM-1, TEM-2, OXA-1which have been found out to be the commonest with SHV-1 type predominant in Africa (Wegener *et al.,* 1997). Such a phenomenom is a typical example of a cross resistance between species.

Salmonella isolates resistance to expanded-spectrum oxyimino- cephalosporins are documented to have acquired plasmids encoding various class A extended-spectrum β- lactamases (Bradford *et al.,* 1998). The increasing number of infections with antimicrobial drug-resistant *Salmonella*, including the emergence of multidrug-resistant (MDR) *Salmonella enterica* serotype *typhimurium* phage type DT104 (Threlfall, 2002), extended-spectrum β-lactamase (ESBL)-producing *Salmonella* (Miriagou *et al*., 2004;Fluit.2005) and fluoroquinolone-resistant *Salmonella* strains (Threlfall,2002; Aarestrup *et al*.,2003) deserves special attention.

Fey *et al*., (2000) previously described this phenomenon of production of plasmid-mediated class C β-lactamases by *Salmonella* isolates. *Salmonella* isolates exhibiting this phenomenon can have serious implications because of the limitation of therapeutic choices for patients with invasive salmonellosis and by facilitation of the spread of *bla*TEM genes in the human population.

Increased access to antibiotics in developing countries, without controls on over-the-counter use, has also contributed to some of the highest rates of resistance in the world, as was seen with penicillin resistance in Vietnam. Relatively wealthy countries such as the Republic of Korea and

Japan not only have lax controls but also greater access to funds to purchase antibiotics (Song *et al.,* 1999). However in Korea, over a period of 9 years, (Yoo *et al.,* 2004) reported a mild increase in resistance rate against chloramphenicol with ampicillin, trimethoprim/sulfamethoxazole and nalidixic acid remaining at a similar level in a retrospective study. Majority of human cases of non-typhoidal salmonellosis are acquired through the consumption of contaminated food and water. Data on their prevalence and their resistance patterns in different countries are important for natural and global public health management as food consumption practices vary in different countries. The increase in global travel and food trade will most likely increase the likelihood of acquiring more and new infections from non-domestic sources (Lauderdale *et al.,* 2006).

2.5.5 *Salmonella* antimicrobial resistance and resistance pattern in Kenya

In Kenya, multidrug resistant serovars have been spreading to other parts of the country and are gradually replacing common and fully sensitive strain types. This has resulted in invasive salmonellosis therapeutic management complications (Kariuki *et al.,* 1996; Oundo *et al.,* 2000; Kariuki *et al,* 2005). Furthermore, more than 50% of non-typhoidal *Salmonella* isolates from children in Kenya were multi-drug resistant (Okeke *et al.,* 2005). Similar observations were made in the analysis of NTS serovars from chicken, pigs, and beef cattle in Kenya (Kariuki *et al.,* 2002). Due to the relatively low cost and ready availability of ampicillin, tetracycline and streptomycin, these antimicrobial agents are widely used by farmers for therapeutic and prophylactic applications (Laxminarayan, 2002). Although in Kenya neither chloramphenicol nor its fluorinated analog florfenicol is approved for use in food animals, chloramphenicol resistance was detected in faecal isolates. This observation might be due to the acquisition of resistance genes from other sources, such as bacteria in water contaminated with human sewage, contaminated foods or due to illegal use of chloramphenicol.

Recent reports have shown widespread emergence of reduced-susceptibility and full-resistance to cheaper and commonly used antibiotics including tetracycline, sulphamethoxazole, streptomycin, naldixic acid, chloramphenicol, cotrimoxazole and ampicillin) (Kariuki *et al.,* 1996; Oundo *et al.,* 2000; Kariuki *et al,* 2005). High percentage of cases of diarrhea in Western Kenya may be caused by antimicrobial-resistant bacteria thus illustrating the effect of longstanding, unregulated antimicrobial use (Kakai, 2009). In a study to determine the prevalence and antimicrobial

susceptibility patterns on microorganisms isolated from water and two fish species collected from fish landing beaches and markets in the Lake Victoria Basin, all isolates were found to be sensitive to ciprofloxacin. *E. coli* isolates were resistant to ampicillin, tetracycline, cotrimoxazole, chloramphenicol and gentamicin while *S. typhimurium* isolates exhibited resistance to ampicillin, tetracycline, and cotrimoxazole (Onyango *et al*, 2007). None of the *E. coli, Salmonella,* and *V. cholerae* were resistant to ciprofloxacin. Although it's at the institutional levels (e.g. referral and private hospitals laboratories) of Ministry of Health that antimicrobial sensitivity testing of the *Salmonella* and other isolates is mostly done, these procedures are rather treatment based and the published data only available but sharing of information if any on the same is limited. Hence the actual scale of regional or national antimicrobial drug resistance is not well documented.This is highlited by the fact that in poor settings, the diagnosis and treatment of multidrug resistant infections is difficult based on lack of microbiology services (Oneko *et al*.,2014).

2.6 Classes 1 integrons and their associated genes.

2.6.1 Integrons

Integrons are genetic structures capable of capturing and excising gene cassettes. They usually encode antimicrobial drug resistance determinants. Integron acquisition is considered the major cause of multiple resistances in Gram-negative microorganisms, mainly in enteric bacteria. This is because genes yielding resistance to antibiotics commonly used in the treatment of human infections can be acquired by integron-harbouring strains that may potentiate the possibilities of selection by a variety of different antimicrobials (Rowe-Magnus *et al.,* 2002**;** Leverstein-van *et al*., 2003).

Resistance genes are often located on extrachromosomal genetic elements or in segments inserted within the chromosome that originates from other genomes. The acquisition of a new gene may occur by genetic transformation, but when resistance genes are located on plasmids, e.g. due to mutation, they can be mobilized by F plasmid conjugative transfer. Integrons encode a site-specific recombinase (*Rec* Genes), the integrase, which efficiently promotes the acquisition of exogenous genes (Martinez and de la Cruz, 1990; Hall and Collis, 1995). The *Rec* A protein

promotes genetic exchange (recombination) between a fragment of the donor DNA and the recipient's DNA.

More than nine classes of integrons have been found and characterized according to the sequence of the integrase genes (Hall, 1997; Collis *et al.,* 2002). Class 1 is thought to be a major factor implicated in the dissemination of antibiotic resistance. More than 100 different resistant gene cassettes have been found within integrons and most of them encode for antibiotics resistance.

2.6.2 Class 1 integrons

Since class 1 integron was first described by Strokes and Hall in 1989, integron-mediated resistance to antibiotics has been reported in clinical isolates of various organism including *Klebsiella pneumoniae, Klebsiella oxytoca, Pseudomonas aeruginosa, E. coli, Citrobactor freundii* and *Vibrio cholerae* (Dalsgaard *et al.,*2000). Many studies have also revealed that integron-borne gene cassettes exist among the *Salmonella* (Khan *et al*., 2000).

Class 1 integrons (Figure 2) carrying the (*intl*1) integrase gene, is the most common structure found in bacterial pathogens (Carattoli, 2001) located within transposons that contribute to vertical transmission (Liebert *et al*., 2000). A resistance gene that has emerged on a plasmid, located within a transposon or an integron, may be transferred to other strains and species, enabling it to penetrate into niches not accessible to its original host strain (O'Brien, 2002). Class 1 integrons consist of a variable region bordered by 5' and 3' conserved regions (CSs). The 5' region is made up of the integrase gene of about 1358 bp, (*attI*) a recombinase site which integrates gene cassettes and the promoter part which drives transcription of genes within the variable region. 3'-CS region contains several open reading frames (ORFs). These include qacEΔ1, which confers resistance to quaternary ammonium compounds, often associated with antiseptics, along with a *sul*1 gene expressing resistance to sulphonamide antimicrobial agents. The *sul*1 gene encodes the enzyme hydopteroate synthase. Transcription of the *sul*1 gene begins at a promoter located in the 5'-CS. The latter is also responsible for the transcription of the inserted gene cassette(s) (Hall and Stokes, 1998). Two additional ORFs, (ORF-5 and -6) are located toward the distal end of the 3'-CS. The gene product of ORF 5 appears to share some sequence similarity with puromycinacetyl transferase, and this feature suggests a possible role in antimicrobial resistance. A biological function has yet to be ascribed to ORF-6.

Classes 2 and 3 also contain integrase genes (*intI* 2 and *intI* 3), with the former showing 40% sequence identity to those of class 1, and the latter showing 61% (Hall and Recchia,1995). All three classes of integrons contain similar gene cassettes from the same families, which suggest the existence of a common pool of gene cassette with cross-specificity between the classes (Hall and Recchia, 1995).

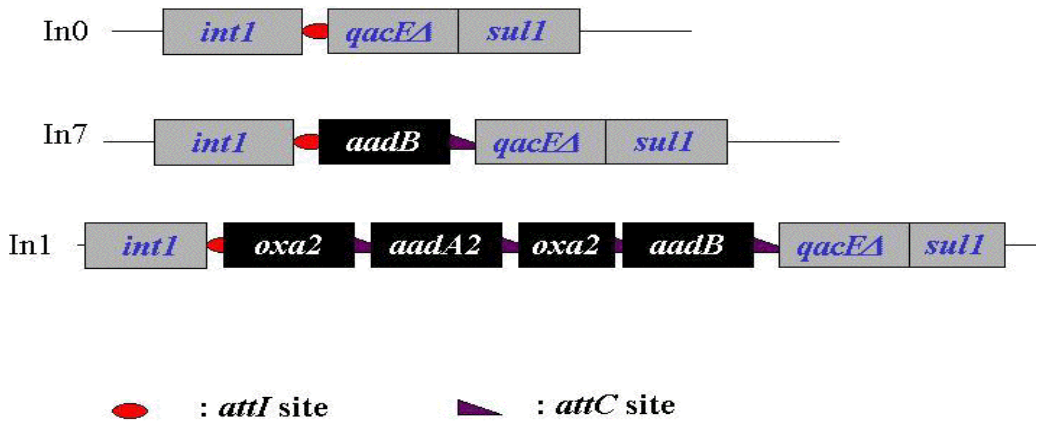

Figure 2: The organization of class 1 integron (Figure courtesy of http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/transposons/integrons/integrons.htm (accessed on 12th July 2013)

Integrons have been described in a wide range of pathogenic organisms including *S. typhimurium* and *S. enteritidis* (Rankin *et al.,* 1998; Brown *et al*., 2000). In *Escherichia coli* and *Salmonella* species, antimicrobial resistance genes are found on large, transferable plasmids. Another type of mobile DNA element, the transposon, also often carries antimicrobial resistance genes. In all, plasmids and transposons, coding multiple drug resistance, often possesses another genetic element, the integron.

Although the spread of resistant microorganisms is disturbing, the association of resistance determinants with mobile DNA elements; such as, plasmids, transposons, and integrons, is also of concern because they play an important role in the evolution and dissemination of multidrug resistance (Gomez *et al.*, 1997; Liebert *et al.*, 1997; Boyd *et al.*, 2001; Boyd *et al.*, 2002). This is because of rapid dispersion of resistance genes within bacterial species and between different species (Hall 1997; Levesque., 1995).

Recently, integrons have become very important as they can capture more than one antibiotic resistance cassette. Their role in dissemination and evolution of antimicrobial resistance in MDR *S. Typhimurium* DT104 (MDR-DT104) and many other organisms has been documented. They account for rapid and efficient transmission of drug resistance because of their mobility and ability to collect resistance gene cassettes (Recchia *et al*., 1995; Tosin *et al.,* 1998).

Integron mechanism as a means of antibiotic resistance in the clinical *Salmonella* enteric serovars in rural western Kenya was demonstrated by Onyango *et al.,* (2010). Integron class 1 and its role in this cluster or anywhere else for fish *Salmonella* isolates in the study area is not known. Similarly, high prevalence of resistance to tetracycline, ampicillin and co-trimoxazole in *E. coli* in the region has been reported (Onyango *et al.,* 2009).

Molecular analysis of antibiotic resistance genes and antibiotic-resistant mobile elements has shown that identical elements were found in bacteria that colonize both animals and humans, suggesting a role for foods in the dissemination of resistant bacteria and resistance genes to humans via the food chain (O'Brien *et al*., 1982; Plummer *et al*., 1995). Resistance to expanded-spectrum oxyimino- cephalosporins among *Salmonella* strains is mostly due to acquisition of plasmids encoding various classes A and C of extended-spectrum β- lactamases (Bradford *et al*., 1998; Fey *et al.,* 2000). Such resistant strains hamper treatment choice in patients with invasive *Salmonella* thus spreading *bla* genes in the population.

Recent studies and reports are focusing on genetic structure of integrons, thus stressing on their role in acquisition and dissemination of antimicrobial resistance among Gram-negative bacilli (Martinez-Freijo *et al.,* 1998; Leverstein-van Hall *et al.,* 2002a; Nijssen *et al.,* 2005; and Norrby, 2005).

Multi-drug resistance in Enterobacteriaceae is strongly associated with integrons which confer resistance to different antimicrobial agents (Nijssen *et al*., 2005). Several gene cassettes may be present in one integron which leads to multiple drug resistance in bacteria (Collis *et al*., 2002). Classes 1 integrons are the most frequently detected in many bacterial species carrying different arrangements of gene determinants associated with antimicrobial resistance (Cambray *et al.,* 2010).

Integrons are likely to be one of the single most important mechanisms for the acquisition, maintenance, and dissemination of resistance to multiple antibiotics. Therefore, additional studies of integron-mediated antibiotic resistance are urgently needed. The distribution of Class 1 integron in *Salmonella* has been previously studied in China (Hongmei *et al.*, 2004) and Portugal (Patrı´cia *et al.*, 2006). These studies have established a strong association between the presence of class 1 integrons and antimicrobial resistance.

In Western Kenya Onyango *et al.*, (2010) investigated and reported the presence of integrons as a means of antibiotic resistance in the clinical *Salmonella* enteric serovars. However no similar studies have been done to elucidate the role of class 1 integrons obtained from *Salmonella* of fish origin.

2.6.3 Antimicrobial resistance genes

There are several antimicrobial resistance genes observed in Gram negative microorganisms as part of a gene cassette inserted in an integron (Rowe-Magnus *et al.*, 2002). These genes confer resistance to a range of antimicrobial agents including β-lactams, trimethoprim aminoglycosides and chloramphenicals (Hall and Collis, 1998). Most of these antimicrobial, have more than one encording gene for resistance. Data on genes encording resistance to these antibiotics in the study area is limited as resistance is tested at phenotypic level.

2.6.3.1 Ampicillin resistance genes

These are families of genes encoding for enzymes (β-lactamases) and *bla*TEM-1 and are the most prevalent among *Salmonella* isolates. Others such as *bla*PSE-1 also have been found in a number of *Salmonella* isolates and chromosomally located or are often integrated within mobile genetic elements such as transposons or plasmids and can therefore be transferred between bacteria (Li *et al.*, 2007).

2.6.3.2 Streptomycin resistance genes

Genes encoding the enzymes aminoglycoside adenyltransferases are typically designated *aadA* and these genes have been found as part of *Salmonella* integron-borne gene cassettes (Hongmel *et al.*, 2004)

2.6.3.3 Tetracycline resistance genes

There are different genes that confer resistance to tetracycline and oxytetracycline *tet*(A), *tet*(B),*tet*(C), *tet*(D), *tet*(G), and *tet*(H) have been found in *Salmonella* isolates. The most commonly reported of these genes is *tet* (A). *Tet* (A) has been found in *Salmonella* genomic island 1, on integrons, and on transferable plasmids. The *tet* (A) gene has been detected in isolates of *Salmonella* serotypes. These genes appear to be easily transferred and widespread among *Salmonella* isolates. They also tend to be found in isolates that display multidrug resistance, making them an important marker in identifying potentially serious *Salmonella* infections. *tet* (G) is linked to *Salmonella* Genomic Island 1 (SGI1).

2.6.3.4 Sulfamethoxazole resistance genes

Sulfonamide resistance in *Salmonella* isolates has been attributed to the presence of an extra *sul* gene, which expressed an insensitive form of dihydropteroate synthases. Three main *sul* genes have been identified: *sul1, sul2, sul3*. The *sul1* gene has been found in a wide range of *Salmonella* serotypes. This gene is often associated with class 1 integrons that contain other resistance genes. These integron-borne gene cassettes have been found on transferable plasmids and as part of *Salmonella* genomic island variants. Although sometimes found in *Salmonella* isolates also harbouring *sul1, sul2* appears to be associated with plasmids, but not with class I integrons. Isolates of *Salmonella* serotypes *agona, enteritidis, typhimurium* have been reported to carry *sul2*. The *sul3* gene has also been identified only recently in *Salmonella*, and it has been associated with plasmids and class I integrons, suggesting that there may be further dissemination of this gene within *Salmonella* populations (Guerra *et al.,* 2004a). *Sul*1 and *Sul*2 are highly prevalent while *sul3* has only been occasionally detected (Blahna *et al.,* 2006).

2.6.4 Antimicrobial resistance genes in other parts of the world

Antibiotic resistance genes (ARGs), as emerging contaminants, have received increasing attention in China (Pruden *et al.,* 2006 and Pei *et al.*, 2006). Various ARGs, such as genes encoding resistance to tetracycline (Patterson *et al.,* 2007 and Tao *et al.,* 2010) sulfonamides (Srinivasan *et al.,* 2005; Vinue *et al.,* 2010) beta lactams, (Cendejas *et al.,* 2010) and quaternary ammonium compounds (qacED1) (Akinbowale *et al.,* 2007) have been widely detected in

various environmental media. The efflux genes, *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(E) are frequently detected in various environmental compartments, a posiblity of assimilation with fish, including *R. argentea,* surface water (Tao *et al.*, 2010), activated sludge of sewage treatment plants (Roberts, 2005) and fish farms (Akinbowale *et al.*, 2007). The sulfonamide resistance genes *sul*1, *sul*2, *sul*3, and *su*lA, encoding dihydropteroate synthase, have been frequently detected in water and sediment in aquaculture settings, (Agerso and Petersen, 2006 and Akinbowale *et al.*, 2007) surface waters (Zhang *et al.*, 2009), and dairy farms (Srinivasan, 2005).

Sulphonamide is inexpensive antibiotic that have a synergistic effect (Huovinen, 2001). When combined with trimethoprim (co-trimoxazole) they can be used for a wide range of clinical indications including uncomplicated urinary tract infections, enteric bacterial diseases and respiratory tract infections (Huovinen, 2001). Plasmid-mediated resistance to these two drugs is normally due to the acquisition of novel target enzymes that are naturally resistant dihydropteroate synthases (*dfr*) for sulphonamides and dihydrofolate reductases (*dfr*) for trimethoprim (Skold, 2001). S*ul*1, *sul*2 and *sul*3 genes encode dihydropteroate synthases and more than 20 dihydrofolate reductase. Both groups of genes are associated with class 1 integrons residing in plasmids and or the bacterial chromosome (Huovinen, 1995; Skold, 2001 and Mazel, 2006).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study Sites

Lake Victoria is the world's second largest fresh water lake. It has a surface area of about 68,800 Km^2 and a catchment area of 284, 000$Km.^2$ It's shore line is approximately 3,500 Km long. It touches the equator on the North, lies between latitude 0.7^oN- 3^0S and longitude 31.80E-34.80E and in the South of equator between 0° 6' S-0° 32'S and 34° 13'- 34° 52'E with an altitude of 1134m above sea level (KNBS, 2010). The lake is shared by three riparian countries where Tanzania occupies 49%, Uganda 45% and Kenya 6% of the lake (Figure 3). The lake is also a source of the river Nile, providing life saving water to countries along the Nile (Sudan South Sudan and Egypt). The basin supports over 30 million people. The major portion of Kenyan water of L. Victoria is the Nyanza / Winam gulf. It has a catchment area of 3,600 km^2 drained by five major rivers (Nzoia, Kuja, Nyando, Yala, Sondu) through which it contributes approximately 30% of total riperrine inflow into L. Victoria (Njiri., 2005). It receives a mean annual rainfall of about 1,153 mm and experiences a mean annual temperature of 22^0C and a mean annual potential evaporation of 1968 mm. Winam gulf experiences long rains from March to May with peak in April and short rains in August and October. The sample collection for this study was done in four markets and four beaches. The markets were; Luanda market (0° 0' 0" North, 34° 35' 0" East), Kibuye market (0°5'34"N 34°46'6"E), Busia market (0^0 27' 11N", 34^0 7' 30"E) and Yala market (0° 60' 0" North, 34° 32' 0" East) while landing sites were Dunga beach (34^o 47' 00" East 0^o 10' 00" South), Usenge beach (0°4'23.23"N: 34°3'37.12"E), Uhanya beach and Port Victoria beach.

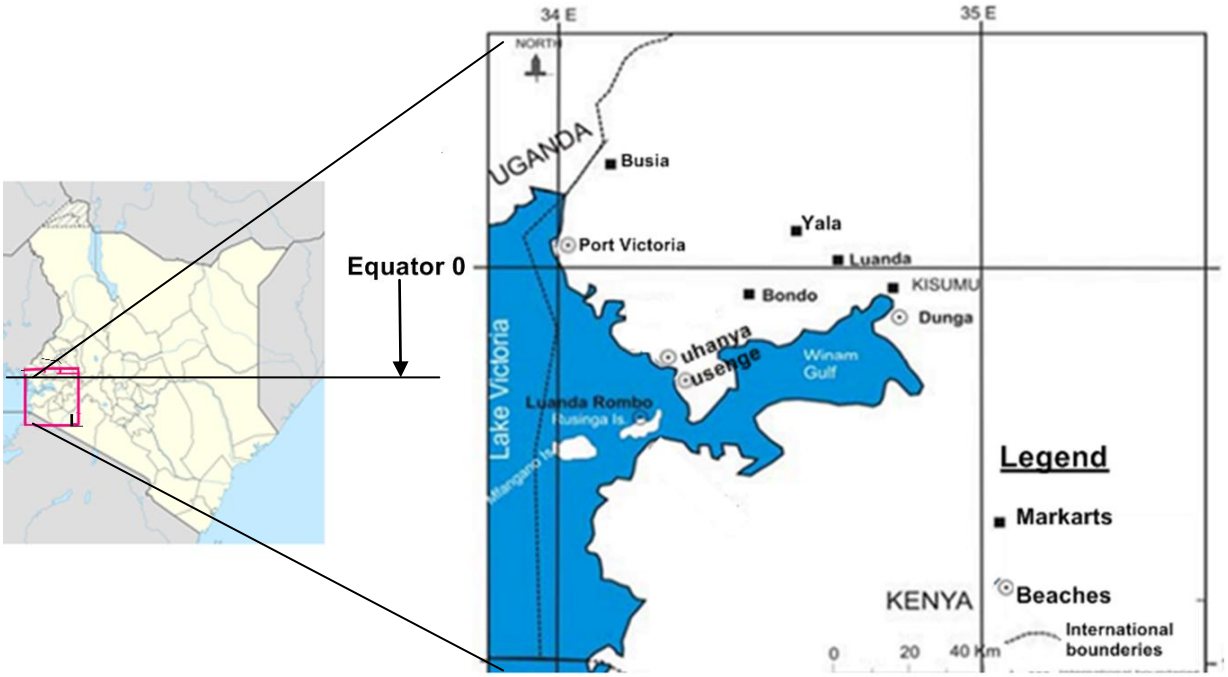

Figure 3: Map of Kenya. Inset is a map of Winam Gulf of Lake Victoria (Kenyan part) showing the sampling market sites and the selected fish landing beaches. (Adapted from knowledge and experiences gained from managing Lake Victoria ecosystem LVEMP 2005, Mallya, G. Wambede, J.Kusewa, M.(Eds).

3.2 Sample size determination

Sample size was determined by the formula according to Glynn *et al.*, (1998). This formula was chosen because according to a study that determined microbiological quality and safety of *R. argentea* retailed in Kisumu town markets (Kenya), 6.67% of samples were contaminated with *Salmonella* (Sifuna *et al.*,2008). This percentage is important because going by the European Economic Commission (EEC, 1998), a Commission decision on protective measures with regard to fishery products from Uganda, Kenya, Tanzania and Mozambique 98/84/EEC, *Salmonella* spp are not naturally present in the marine environment.

At 95% confidence level and a precision of 0.05, the resulting sample size was determined as follows:

$$n_o = \frac{z^2 pq}{e^2}$$

Where n_o = Sample size

Z = desired confidence level

p = Estimated proportion of an attribute present in a population

q = 1 - p

e = Desired level of precision

$$\text{Sample Size} = \frac{(1.96)^2(0,0667)(0.9333)}{(0.05)^2}$$

$$= 95.65 \approx 96$$

This value was divided by 8 and a value of 11.95 ≈ 12 samples fron each site (four markets and four beaches)

3.2.1 Sampling plan

A cross-sectional study design was used to collect market and beach *R. argentea* samples for four months (February-May). A total of 96 *R. argentea* samples were purchased in regular consumer packages of 500 g size tins (quantity approximately 250 - 300g of *R. argentea*). Fish sampling from the fish markets was done randomly from vendors separated by at least 20 meters during market days only in the morning when deliveries were made to the markets. Fish sampling from landing sites was done early in the morning at around 7 00 a.m., the time when fishing boats land their catches. Three fish samples (500g tins) were randomly collected from different fish vendors each day of beach visit at various times, viz; 0, 3 and more than 8 hours. 0 hour for fresh fish sampled from the boat on arrival, 3hour for semi dried fish sampled after 3 hours of drying and 8hour for fish sampled after drying in the sun along the beach for 8 hours or more. The samples were packaged in sterile polythene bags and immediately transported

aseptically in a cooler box with ice packs to Maseno University Zoology Research Laboratory for processing and analysis.

3.3 Determination of aerobic plate count

Microbial determinations were carried out using standard methodologies described in the Bacteriological Analytical Manual (http://www.fda.gov/FoodScience Reseach/Loboratory Methodes/ucm 2006949.htm-Accessed on 20/08/2014). Briefly, 25 grams of each sample was weighed aseptically and ground in a pre-sterilized blender (Moulinex type AR 1043/6WR-2309R). The ground fish was then added into 225 ml of buffered peptone water (HiMedia Lab. Pvt. Mumbai, India). This formed fish slurry from which a series of tests were conducted.

Aerobic plate count (APC), 1 ml of homogenate was added to 9 ml sterilized peptone water to make 10 ml of the mixture followed by serial dilutions from 10^{-2} to 10^{-6}. Using the pour plate method, 1 ml of each dilution was transferred to sterile plates and 15 ml of plate count agar (PCA) was added, mixed and allowed to solidify. Plates were then incubated at 37± $2.^0$C for 48 h, and colonies were counted using colony counter (SC6PLUS colony counter – Bibby Scientific) and reported as cfu/25gm.

3.4 Isolation and confirmation of coliforms and *E. coli* in fish samples

Using the 3 tube Most Probable Number (MPN) method (Appendix 6**)**, total coliforms bacteria as indicator for faecal contamination and *Escherichia coli* was analyzed from the initial 100ml fish slurry. The procedure involved three successive steps namely; **MPN - Presumptive test, MPN - Confirmed test and MPN - Completed test for** *E. coli* (Tharannum, *et al.*, 2009).

In the presumptive test, 10ml of MacConkey broth purple (HiMedia Lab. Pvt. Mumbai, India) was added into 3 sets of 25ml tubes (with inverted Durham's tubes' inserts) and autoclaved. Each set contained three tubes (i.e. 9 tubes in total). A ten-fold difference in the fish samples inoculums volumes, i.e., 0.1ml, 1ml, and 10ml per tube were inoculated and incubated at 37^0C (Gallenkemp, Germany). The tubes were examined and reactions recorded at 24 ± 2 hrs for gas, displacement of medium in Durham tubes or effervescence when tubes are gently agitated. The gas-negative tubes were re-incubated for an additional 24 hrs, examined and reactions recorded again at 48 ± 2 h. Standard Most Probable Number (MPN) table was used to determine the

number of coliforms in the fish samples (APHA, 1998). Confirmation test was performed on presumptive positive (gas) tubes.

In **MPN - Confirmed test for coliforms,** a loopful of suspension from each gassing MacConkey broth purple tube was transferred onto eosin methylene blue agar (EMB agar) (HiMedia Lab. Pvt. Mumbai, India) incubated at 37^0C for 24hrs. Presence of green sheen colonies on EMB streaked from a positive presumptive test was considered positive confirmative test.

The complete test was performed by inoculating a tube of phenol red lactose broth, MacConkey agar and a slant of nutrient agar with green sheen colonies from positive confirmative tests. The culture on the nutrient agar was analyzed by Gram staining, short red rods were subjected to IMViC reactions and API 20E (Biomérieux, France).

Indole production Tubes were inoculated with tryptone broth and incubated for 24 ± 2 h at 35°C. Indole test was performed by adding 0.2-0.3 ml of Kovacs' reagent. Appearance of distinct red color on upper layer was positive.

Voges-Proskauer (VP)-reactive compounds Tubes were inoculated with MR-VP broth and incubate for 48 ± 2 h at 35°C. 1 ml of broth was transferred to 13 x 100 mm tube. 0.6 ml of α-naphthol solution and 0.2 ml 40% KOH, was added and agitated. A few crystals of creatine was added, mixed by shaking and let to stand for 2 h. Test was positive if eosin pink color developed.

Methyl red-reactive compounds After VP test, MR-VP tube was incubated for additional 48 ± 2 h at 35°C. 5 drops of methyl red solution were added to each tube. Distinct red color was positive test while yellow was negative reaction.

Citrate Koser's citrate broth tube was lightly inoculated to avoid detectable turbidity and incubated for 96 h at 35°C. Development of distinct turbidity was a positive reaction.

The confirmed isolates were stored on tryptic soy broth with 15% glycerol at - 20^0C for antimicrobial susceptibility testing.

3.5 Isolation and confirmation of *Salmonella* isolates

This study utilized the conventional methods for the detection of *Salmonella* following the ISO 6579 (2002) standard guidelines; microbiology of food and animal feeding stuffs-horizontal method for detection of *Salmonella* spp. Twenty five grams of freshly obtained *R. argentea* were aseptically weighed and blended aseptically. This was introduced in 225 mL sterile buffered peptone water (Himedia laboratory pvt Ltd Mumbai India) and incubated for 18 hours at 37.^{0}C. After incubation, 0.1 ml of homogenate was aseptically transferred to 10 ml of Selenite F broth (Himedia laboratory pvt Ltd Mumbai India) which was incubated for 24 h at 37.^{0}C (Appendix 7) flow diagram representing the plan of action.

From the overnight Selenite F culture broth, a loop full of broth was obtained and streaked onto Deoxycholate citrate Agar (DCA, Himedia laboratory pvt Ltd Mumbai India), and Xylose Lysine Desoxycholate Agar (Himedia laboratory pvt Ltd Mumbai India). The plates were incubated for 24 h at 37°C. After 24 h incubation, two presumptive *Salmonella*-like colonies were picked from each selective agar and inoculated on Nutrient agar (Himedia Laboratory pvt Ltd Mumbai India) for biochemical confirmation which included Urea broth (Himedia Laboratory pvt Ltd Mumbai India), Triple Sugar Iron agar (TSI) (Himedia laboratory pvt Ltd Mumbai India) and Lysine Iron Agar (LIA) (Himedia laboratory pvt Ltd Mumbai India) and API 20E (Biomérieux, France).

3.6 Biochemical confirmation

3.6.1 Triple sugar iron agar (TSI agar)

The agar slant surface was streaked and the butt stabbed once and incubated at 37^o±1 C for 24 h. The results were observed and recorded after changes in medium were interpreted (Appendix 2) and (Appendix 8).

3.6.2 Urea agar

The agar slant surface was streaked and incubated at 37° C ± 1 for 24 ± 3 hours and examination was done at intervals. In the event that the reaction was positive, urea was split liberating ammonia, which changed the color of phenol red to rose pink, and later to deep cerise (moderate red). The reaction was often apparent after 2 to 4 h.

3.6.3 L-lysine decarboxylation medium

The culture was inoculated just below the surface of the liquid medium and incubated at 37^{o}C ± 1 for 24 h. Turbidity and a purple color after incubation indicate a positive reaction. A yellow color indicated a negative reaction.

3.6.4 Analytical Profile Index (API)

API-20E plastic strips holding twenty mini-test tubes were inoculated with a saline suspension of a pure culture (as per manufacturer's directions). This process also rehydrated the desiccated medium in each tube. The CIT, VP and GEL tubes (Fig. 1), tubes were completely filled. Other tubes (ADH, LDC, ODC, H_2S, and URE) were overlaid with mineral oil such that anaerobic reactions were carried out followed by incubation in humidity chamber for 24 h at 37°C. After incubation, the color reactions were read (Fig. 1) (some with the aid of added reagents), and the reactions (plus the oxidase reaction done separately) were converted to a seven-digit code which is called the Analytical Profile Index (API) from which name the initials "API" are derived. The code was compared with the manufactures data reference book. Positive results with ≥89% probabilities were confirmed as *Salmonella* (Appendix 1)

Salmonella isolates were then transferred to nutrient broth (CDH JO 0003- Himedia laboratories pvt Mumbai India) and preserved at 4^{0}C for later use in genetic analysis.

Plate 1: Typical Salmonella spp reaction of API 20E test kit with twenty reaction tests.

3.7 Antibiotic susceptibility testing

Antimicrobial susceptibility was done according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (2010 M100-S20) using the disk diffusion technique with commercially available discs (Himedia laboratories pvt Mumbai India). Eleven antimicrobials that were tested include; tetracycline (30µg), sulfamethoxazole (25µg), chloramphenicol (30µg), ampicillin (10µg), nalidixic acid (30µg), ciprofloxacin (5µg), streptomycin (10µg), ceftriaxone (30µg), cefuroxime (30µg), cefotaxime (30µg) and augumentine (30µg) (all from HI Media Laboratories Pvt. Ltd Mumbai India). The isolates were inoculated on nutrient agar plates for 16 hours to establish a culture in the logarithmic growth phase then a small colony isolate was suspended in sterile normal saline (0.9% sodium chloride) solution to form turbidity. This was then applied evenly onto the surface of Muller Hinton agar (HI Media Laboratories Pvt. Ltd Mumbai India) in a Petri-plate using sterile cotton tipped swab to make a uniform lawn. Drug disks were then carefully placed onto the agar surface. The plates were inverted and incubated for 18 hours. The inhibition zones were measured in mm using a pair of precision calipers. The results were recorded as sensitive, intermediate and resistant, according to the criteria described by the Clinical and Laboratory Standards Institute (CLSI 2011). A standard reference strain of *E. coli* ATCC 25922 was used as a control.

3.8 Template DNA Preparation and DNA extraction

DNA template was prepared based on the technique described by Mazel *et al*., (2000). Frozen bacterial samples were first streaked for isolation on MacConkey agar and incubated overnight at 37ºC. An isolated colony from the plate was used to inoculate 1 ml of Trypton Soy Broth (TSB) (HIMedia Laboratories Pvt. Ltd Mumbai India), which was then incubated overnight. The overnight growth was transferred to a sterile 1.5 ml Eppendorf tube and centrifuged (Spectrofuge 16M by Labnet Inter EDISON NJ.USA) at 6000 rpm for approximately 1 minute. The supernatant was then discarded, and the bacteria pellet was re-suspended in 0.5 ml sterile distilled water. After briefly vortexing (VORTEX Labnet Inter White Sci USA) the suspension, the tubes were placed on a heat block at 100^0C (TECHINER Dri- Block DB-2D USA) for 10 minutes to lyse the bacteria. The tubes were then centrifuged again at 6000 rpm (Spectrofuge 16M by Labnet Inter EDISON NJ.USA) for 5 minutes to pellet cell debris. The supernatant was

removed and placed into sterile 0.5 ml tubes for use as template DNA. This was stored at -20^{0}C for molecular work.

3.9 Polymerase chain reaction (PCR) assays

3.9.1 PCR for Malic Dehydrogenase gene (*mdh*)

The oligonucleotide primers for Malic Dehydrogenase (*mdh*) gene with respect to annealing temperatures were used (Table 1). These primers were specific for housekeeping gene segments. The Malic Dehydrogenase gene in *Salmonella* isolates were identified by PCR in a 50 µl final volumes containing 34 µl PCR water, 5 µl × 5My Taq buffer, 1 µl forward (5'- TCG CAA CGG AAG TTG AAG TG 3') and reverse 1 µl, (5'- CGC ATT CCA CCA CGC CCT TC -3'), 1 µl My Taq poly Enzyme all from (Bioline UK). Three microliter chromosomal DNA was used as a template. The mixture was thoroughly vortexed and centrifuged at 10,000 rpm for 6 seconds (spectrofuge 16M by labnet inter EDISON NJ. USA). All the amplification reactions were performed by (Arktik Thermocycler 5020 Thermo Fisher Scientific) using the following conditions; 95°C 1 minutes for initial denaturation for 1 cycle, 95°C for 15 seconds, for denaturation for 35 cycles, 60 °C for 15 seconds annealing for 35 cycle, and extension temperature of 72 °C for 10 seconds for 35 cycles, cooling at 72 °C for 2 minutes then 4 °C until removed. The amplification reaction included a negative control, which contained all the PCR master mix reagents except template DNA. The amplified amplicons were then separated by horizontal 1.5% agarose gel (1.5 g agarose powder + 100 ml of 1 × TBE buffer) electrophoresis with a gene marker of 100 bp, for 25 min at 135 V and visualized under ultraviolet (UV) light (UV photo transilluminator (TFP-M/WL VILBER LOURMAT France).

Table 1: Oligonucleotide primers for Malic Dehydrogenase gene (*mdh*)

Target	Primer	Oligonucleotide sequence	Amplicon size (bp)	Annealing Temp (^{0}C)	Reference
Mdh	*Mdh*	5'- TCG CAA CGG AAG TTG AAG TG 3' 5'- CGC ATT CCA CCA CGC CCT TC -3'	216	60^{0}C	Amavitsi *et al.*, 2005

3.9.2 PCR for class 1 integrons and antibiotic resistance genes

Class 1 integron in *Salmonella* isolates was detected using the published conserved segments (5′-CS) and (3′-CS), regions adjacent to the site-specific recombinational insertion sequence (Levesque *et al*., 1995). These primers were specific for class 1 integron conserved segment gene. Class 1 integron gene was identified by PCR in a 50 µl final volumes containing 34 µl PCR water, 5 µl × 5My Taq buffer, 1 µl forward (Int1F 5´GGC ATC CAA GCA GCA AG – 3´) and reverse 1 µl, (Int 2R 5 ´- AAG CAG ACT TGA CCT GA- 3´), 1 µl My Taq poly Enzyme all from (Bioline, UK). Three µl QIAmp chromosomal DNA was used as a template.

All the amplification reactions were performed using (Arktik Thermocycler 5020 thermo Fisher Scientific USA) using the following conditions; 95°C 1 minutes for initial denaturation for 1 cycle, 95°C for 15 seconds, for denaturation for 35 cycles, 60 °C for 15 seconds annealing for 35cycle, and extension temperature of 72 °C for 10 seconds for 35 cycles, cooling at 72° C for 2 minutes then 4° C to infinity. The PCR conditions also applied for the detection of *bla* PSE-1, bla_{TEM}, *tet*A, *tetB tet*G, *aad*A, *sul* 1, and *int* 1 genes using specific respective primers (Table 3). Each amplification reaction included a negative control, which contained all the PCR master mix reagents except 5 µg of targeted DNA segment sequence. The amplified amplicons were then separated by horizontal 1.5% agarose gel (1.5 g agarose powder + 100 ml of 1× TBE buffer) electrophoresis with a gene marker of 100 bp, for 25 min at 135 V and visualized under ultraviolet (UV) light (UV photo transilluminator (TFP-M/WL VILBER LOURMAT France). The oligonucleotide primers for class 1 integrons and antibiotic resistance genes are shown in Table 3, with respect to annealing temperatures and gene bank accession numbers where available.

Table 2: Oligonucliotide primers for class 1 integrons and antibiotic resistance genes

Target	Primer	Oligonucliotide sequence	Amplicon size (bp)	Annealing Temp (C^0)	References
Ampicillin	*bla*$_{TEM}$	F 5'GCACGAGTGGGTTACATCGA-3' R,5'GGTCCTCCGATCGTTGTCAG-3'	310	60	Gebreyes and Altier, 2002
	Bla$_{PES}$	F 5'TTTGCTTCC GCGCTATCTG-3' R,5'TACTCCGAGCACCAAATCCG-3'	150	60	Gebreyes and Altier, 2002
Streptomycin	*aadA*	F 5'GTGGATGGCGGCCTGAAGCC-3' R,5'AATGCCCAGTCGGCAGCG-3'	528	60	Gebreyes and Altier, 2002
Sulfamethoxazole	*Sul*1	F 5'CCT CGA TGA GAG CCG GCG GC 3' 5'GCA AGG CGG AAA CCC GCG CC 3'	437	55	Gebreyes and Altier, 2002
Tetracycline	*tet A* *tet* B *tet G*	F,5'- CCTACATCCTGCTTGCCTTC-3' R,5'-CATAGATCGCCGTGAAGACG-3' F,5'-TTGGTTAGGGCAAGTTTTG -3' R,5'-GTAATGGGCCAATAACACCG-3' F,5'CAGCTTTCGGATTCTTACGG-3' R,5-GATTGGTGAGGCTCGTTAGC-3'	210 659 844	60 55 60	Fonseca *et al.*, 2006 Fonseca *et al.*, 2006 Fonseca *et al.*, 2006
Integron class 1	5´CS-3´CS	*Int*1F 5´GGC ATC CAA GCA GCA AG - 3 *Int* 2R 5´AAG CAG ACT TGA CCT GA- 3	Varied weights	64	Levesque *et al.*, 1995

3.9.3 Data management and analysis

Descriptive analysis was carried out on various microbial isolates from *R. argentea*, including their occurrence and prevalence rates of their resistance to antibiotics.

For Total Plate Count all numeric analyses were made using logarithm bacterial count values in Excel spreadsheets. Counts expressed as colony forming units (cfu/g) was transformed into $\log_{10}$ prior to statistical analysis using MINITAB 16 and one way ANOVA was used to determine significance difference of microbial contamination of *R. argentea* between markets and beaches. P value of <0.05 was considered significant.

Chi-square test was used for analysis on categorical variables also using MINITAB 16. All *p*-values were based on 2-tailed tests of significance with $p< 0.05$ considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Microbial contamination of *R. argentea* obtained by Aerobic Plate Count

In order to determine heterotrophic (aerobic and facultatively anaerobic) bacteria density, Aerobic plate Count (APC) was determined as a measure of quality. The mean APC of sun-dried *R. argentea* sold in commercial markets was found to be significantly higher in three markets (Table 3). Kibuye market had the lowest mean $\log_{10}$ 5.43 cfu/g whereas Luanda market had the highest $\log_{10}$ 5.93 cfu/g followed by Yala $\log_{10}$ 5.87 cfu/g. ANOVA indicated significant difference among the four markets p = 0.003(Appendix 9).

Table 3: Log_{10} mean colony forming units (CFU/gm) of sun-dried *R. argentea* contamination in respective commercial markets

Market	Min	Max	Mean	Standard Deviation
Luanda	5.45	6.3	5.93	0.24
Yala	4.48	6.22	5.87	0.43
Kibuye	4.6	6.3	5.43	0.49
Busia	4.85	6.15	5.64	0.38

Among the landing sites sampled, Dunga beach registered relatively high levels of APC with the highest being after 8 h of the drying process, which showed a mean of $\log_{10}$ 6.11 cfu/gm, followed by Uhanya, whereas Port Victoria recorded the lowest at $\log_{10}$ 5.82cfu/g after 8 h of drying. Taken together, among the three beaches, Uhanya, Usenge and Port Victoria there was a general increase in the levels of APC by the third hour of drying, but by the eight hour the levels had decreased. However this scenario was not replicated for Dunga beach.

When using ANOVA to compare between the beaches with respect to time of fish processing, there was statistically significant difference (p = 0.02) among the microbial levels of fish during

receiving fish from the boat, and for fish sampled after 8 h. There was no statistical significance difference (p = 0.73) (Appendix 9) among APC when fish was sampled just after 3 h of drying (Table 4).

Table 4: Aerobic plate count (APC) values for respective fish landing sites.

Landing site	min	max	mean	Standard Deviation
Dunga				
Boat (0)	5.9	6.33	6.03	0.17
3 h	5.73	6.3	6.03	0.21
8 h	5.99	6.33	6.11	0.15
Usenge				
Boat	5.11	5.95	5.63	0.34
3 h	5.89	6.23	6	0.14
8 h	5.69	6.09	5.87	0.18
Uhanya				
Boat (0)	5.93	6.09	6.01	0.07
3 h	6.01	6.11	6.07	0.04
8 h	5.94	6.12	6.03	0.07
Port Victoria				
Boat (0)	5.3	6.01	5.68	0.26
3 h	5.42	6.3	5.93	0.32
8 h	5.66	5.99	5.82	0.12

Legend: Boat (0 hours) at landing and sample collection; 3 hours after spreading and drying the sample; 8 hours after spreading and drying the sample.

4.1.1 Total coliform counts (MPN/g)

Most Probable Number (MPN) was used to determine the level of coliform contamination of fish at the markets and landing sites. The results show that among markets, Kibuye market had the lowest mean of 0.14 MPN/g whereas Luanda market had the highest 0.71 MPN/g followed by Yala 0.22 MPN/gm. ANOVA shows that there was no statistical significant difference among the markets (p = 0.5) (Table 5) (Appendix 9).

Table 5: Total coliform counts (MPN/g) in the respective markets in the study areas

Markets	min	max	mean	Standard Deviation
Kibuye	0.03	0.61	0.14	0.16
Luanda	0.03	11	0.71	2.5
Yala	0.03	0.62	0.22	0.19
Busia	0.03	0.61	0.18	0.16

In the case of landing sites Dunga beach registered the highest mean at 4.92 MPN/g after 8 hours of processing. Similarly as in the case of APC, there was an increase of total coliform (TC) by the third hour of drying followed by a decrease by the eight hour of processing. When comparing among the beaches and time of sampling no statistical significant difference was observed P > 0.05 (Table 6) (Appendix 9).

Table 6: Total coliform counts (MPN/g) of the respective beaches in the study areas

Landing sites	min	max	mean	Standard Deviation
Uhanya				
Boat (0)	0.03	1.5	0.42	0.61
3 h	0.092	2.9	0.72	1.22
8 h	0.03	4.6	1.41	1.98
Port Victoria				
Boat (0)	0.061	0.93	0.27	0.37
3 h	0.062	0.95	0.29	0.38
8 h	0.2	4.6	2.1	1.8
Dunga				
Boat (0)	0.15	2.1	1.2	0.76
3 h	0.15	4.6	2.47	2.02
8 h	0.36	11	4.92	5.56
Usenge				
Boat (0)	0.03	3.6	1.5	1.87
3 h	0.094	3.8	1.98	1.81
8 h	0.21	3.8	1.85	1.58

Legend: Boat (0 hours) at landing and sample collection; 3 hours after spreading and drying the sample; 8 hours after spreading and drying the sample.

4.1.2 Coliform recovered from *R. argentea* processed in different conditions from the Landing sites

Out of all the *R. argentea* samples collected from the four beaches (Port Victoria, Usenge, Uhanya and Dunga), sun-dried (8-10 h) samples at the beach had a higher recovery of *E. coli* (44.7%) followed by wet (31.6%) and then semi-dry (23.7%) respectively. In regard to

Salmonella spp, semi-dried samples (50%) had a higher contamination followed by sun-dry (10%) and wet (0%) samples. *Citrobater* Spp contamination of the samples was highest for semi-dry (42.9%) followed by both sun dried and wet at (28.6%) (Table7).

Table 7: Percentage coliform recovered from *R. argentea* in different processing conditions from the beaches

Bacterial isolate	boat (0 h)	3 h	8 h
E. coli	31.6%	23.7%	44.7%
Salmonella spp	0	50%	10%
Citrobacter spp	28.6%	42.9%	28.6%

Legend: 0h = wet, 3h = semi dry and 8h = dried

R. argentea samples from the selected markets were found to be contaminated with *E. coli*, *Citrobacter* spp, *Salmonella* spp.

Table 8: *Salmonella* spp distribution at beaches with respect to time and Markets (*n*=10)

Beaches	No of *Salmonella* isolates (Time in hrs) 0	3	8	%	Markets	No of *Salmonella* isolates %
Dunga	0	2	1	30	Busia	0 0
Usenge	0	1	0	10	Luanda	0 0
Uhanya	0	0	0	0	Kibuye	3 30
P. Victoria	0	2	0	20	Yala	1 10

From the 10 *Salmonella* spp in this study, six originated from beaches and four from markets (Table 8). Out of six beach *Salmonella* spp isolates, two were *S. arizonae and* four were *Salmonella* spp. All the four market isolates were *Salmonella* spp (Table 8).

4.2 Antibiotic resistance and resistance pattern the isolates (*Citrobacter* spp *E. coli* and *and Proteus* spp and *Salmonella*)

The antibiotic susceptibility profiles for *Enterobacteriaceae* isolated in this study show that 50% isolates were resistant to at least one class of antibiotics. Resistance to at least one drug was 33.33%, two drugs 25.38% and more than three drugs was 15.87% while 25.39% were sensitive.

Table 9: antibiotic profile analysis by disk diffusion *Citrobacter* spp *E. coli proteus spp* and *Salmonella*

	Antimicrobial	Resistance	Intermediate	Susceptible	Total
***E.coli* spp**					
	Na	3(6.8%)	1(2.27%)	40(90.91%)	44(100%)
	T	10(22.73%)	5(11.4%)	29(65.9%)	44(100%)
	A	14(31.8%)	2(4.55%)	36(65.9%)	44(100%)
	S	1(2.27%)	4(9.09%)	39(88.63%)	44(100%)
	CIP	1(2.27%)	0 (0%)	43(97.73%)	44(100%)
	CH	30(68.18%)	10(22.73%)	4 (9.09%)	44(100%)
	SUL	8(18.18%)	4(9.09%)	32(79.5%)	44(100%)
***Citrobactor* spp**					
	Na	1(14.2%)	2(28.5%)	4(57.1%)	7(100%)
	T	0(0%)	2(28.5%)	3(42.8%)	7(100%)
	A	55(71.4%)	4(57.1%)	2(100%)	7(100%)
	S	0(0%)	0(0%)	7(100%)	7(100%)
	CIP	0(0%)	0(0%)	7(93.1%)	7(100%)
	CH	5(71.4%)	2(28.5%)	0(0%)	7(100%)
	SUL	1(14.2%)	1(14.2%)	5(71.4%)	7(100%)

Legend: Na = nalidixic acid, T = tetracycline (30 µg), CIP =ciprofloxacin (5 µg) A = ampicillin (10 µg), CH = chloramphenical (30 µg) S = streptomycin (10 µg), SUL = sulfamethoxazole (300µg)

Ampicillin and chloramphenicol showed equal and common resistance among *Citrobacter* spp. 71.42 % (Table 9), (Appendix 5). *E. coli* isolates showed resistance to chloramphenicol, ampicillin, tetracycline, sulphamethoxazol and nalidixic acid as follows; 68.1%, 31.81%, 22.72%, 18.18% and 6.81% respectively. Resistance to streptomycin and ciprofloxacin was 2.27% respectively.

Table 10: Frequency distribution of antibiogram patterns of *Citrobacter* spp *E. coli* and *and* and *Salmonella*) isolates in the respective market (s) and fish landing beaches

Antibiogram pattern	Market isolates	Landing site isolates
Tet+C	3	-
Amb+Sul	2	-
Amb+C	3	3
Na+Sul	-	1
Na+C	-	1
C+Strep	-	2
Tet+Amb+Sul	2	1
Tet+Amb+C	2	-
Tet+C+Sul	1	-
Na+Tet+C	1	-
C+Strep+Sul	-	1
Na+tet+cipro+C	-	1

Legend: Na= Nalidixic acid, Amp= Ampicillin, C= Chloramphenicol, tet= Tetracycline, Sul= Sulfa- methoxazole, Cipro= Ciprofloxacin, Strep = Streptomycin

Results from the antibiotic susceptibility pattern are shown in (Table 10). The total strains could be grouped into twelve antibiotic resistant patterns. Nine strains were multi-drug resistant strains with three antibiotic agents.

4.2.1 Antibiotic susceptibility test for *Salmonella* isolates

Table 11: *Salmonell*a spp antibiotic profile analysis by disk diffusion

Antimicrobial	Resistance	Intermediate	Susceptible	Total
Na	0 (0%)	1 (10%)	9 (90%)	10 (100%)
T	2 (20%)	2 (20%)	6 (60%)	10 (100%)
A	2 (20%)	4 (40%)	4 (40%)	10 (100%)
S	1 (10%)	0 (0%)	9 (90%)	10 (100%)
CIP	0 (0%)	0 (0%)	10 (10%)	10 (100%)
CH	3 (30%)	2 (20%)	5 (50%)	10 (100%)
SUL	3 (30%)	2 (20%)	5 (50%)	10 (100%)
CRO	0 (0%)	0 (0%)	10 (100%)	10 (100%)
CTX	0 (0%)	0 (0%)	10 (100%)	10 (100%)
AMC	0 (0%)	0 (0%)	10 (100%)	10 (100%)
CXM	0 (0%)	0 (0%)	10 (0%)	10 (100%)

Legend: Na = nalidixic acid, T = tetracycline (30 µg), CIP =ciprofloxacin (5 µg) A = ampicillin (10 µg), CH = chloramphenical (30 µg) S = streptomycin (10 µg), SUL = sulfamethoxazole (300µg), CRO (30µg) = ceftriaxone, CTX (30µg) =cefotaxime, AMC (30µg) =amoxyline, and CXM (30µg) = cefuroxime.

Salmonella spp were 30% resistance to chloramphenicol, tetracycline and sulphamethoxazole. Ampicillin resistance was 20% while streptomycin showed resistance of 10% (Table11). All the isolates were susceptible to ceftriaxone, cefotaxime, amoxyline, cefuroxime, naldixic acid and ciprofloxacin. Results from the antibiotic susceptibility pattern are shown in (Table 12). The total strains could be grouped into 8 antibiotic resistant patterns. Three strains were multi-drug resistant strains. They were resistant to two to three antibiotic agents. Two isolates were MDR with at least three antibiotics (Table 12).

Table 12: Frequency distribution of antibiogram patterns of *Salmonella* isolates in the respective market (s) and fish landing beaches

	Source	
Antibiogram pattern	Market isolates	Beach isolates
Tet+C	1	-
Amp+Sul	2	-
Amp+C	1	-
C+Strep	1	1
Tet+Amp+Sul	1	-
Tet+Amp+C	1	-
Tet+C+Sul	1	-
C+Strep+Sul	1	-

Legend: Na= Nalidixic acid, Amp= Ampicillin, C= Chloramphenicol, Tet= Tetracycline, Sul= Sulfa- methoxazole, Cipro= Ciprofloxacin, Strep = Streptomycin

4.3 Polymerase chain reaction (PCR)

4.3.1 Molecular confermation of *Salmonella* isolates (malate dehydrogenase gene)

Species identification carried out on the isolates using API 20E showed that 20% (2/10) of the isolates were identified as *Salmonella arizonae* (Pv042 and DDW047) and 80% (8/10) were identified as *Salmonella* species. The house keeping *mdh* gene was successfully amplified 100% (10/10) at the expected molecular weight of 216 bp (Plate 2) using specific primers (Table 1).

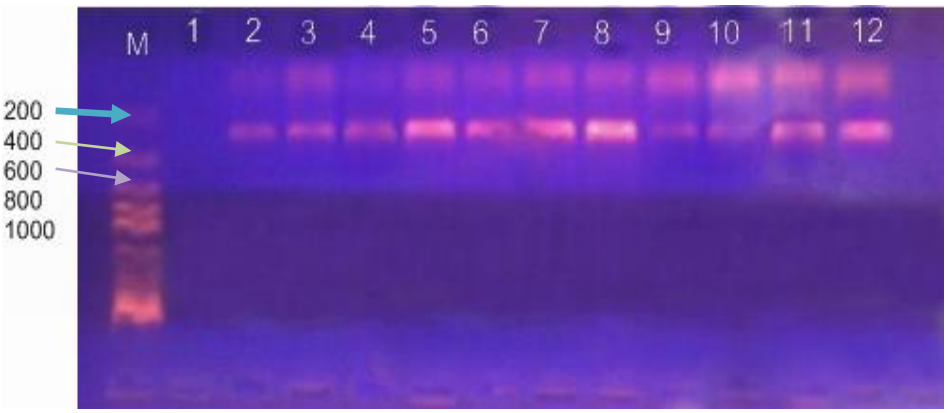

Plate 2: Gel image representative of *mdh* gene amplification. Lanes: M- (1Kb) Hyperladder (Bioline), 1-negative control, 2-positive control (*Salmonella* Typhimirium ATCC 14028), Pv042, 5-DD047, 6-D050, 7-D051, 8-Y063, 9-K065, 10-K066, 11-PO67, 12-K068 and 13-US070.

4.3.2 PCR amplification of the antibiotic resistance gene

Seven PCR primer pairs specific for antibiotic resistant genes were used for screening of the 4 *Salmonella* isolates. The results showed that two of ten isolates (20%) have *tet*A gene, tew of ten isolates (20%) have bla_{TEM} gene, 1 isolate (10%) have *aad*A gene were PCR positive (Plates 3 – 5) (Table 13). $bla_{PES,}$ *tetB* and *Sul 1* genes were not found in the isolates. The sizes of all the PCR products were as expected (Table 2).

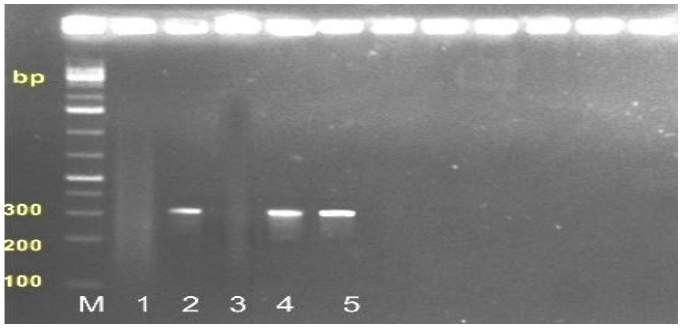

Plate 3: Representative gel image of bla_{TEM} 310 bp gene amplification. Lanes: M 1, 2, 3, 4 and 5 represents – ladder 100 bp, negative control, positive control, DSW047, K065, and K068 respectively. Sample DSW047 is negative for bla_{TEM} gene while K065 and K068 are positive.

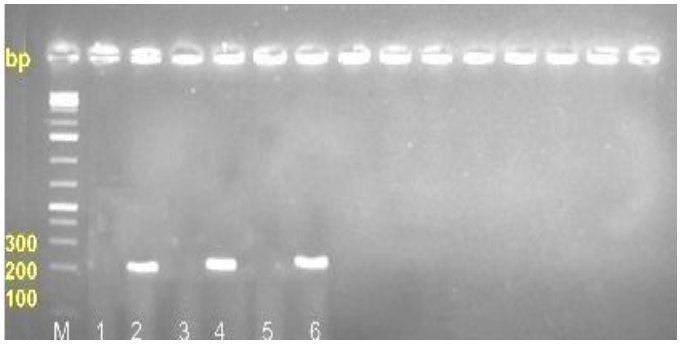

Plate 4: Representative gel image of *tet* A 210 bp gene amplification. Lanes M, 1,2,3,4,5,6, represents 100 bp ladder, negative control, positive control, PvSW042, K065, PvSW067 and K068 respectively. Isolates K065 and K068 are positive for *tet* (A) gene.

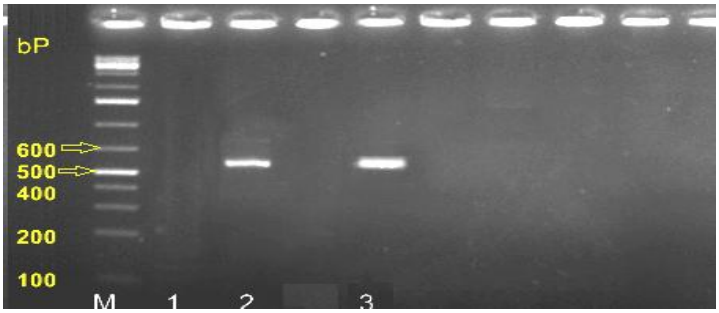

Plate 5: Gel image representative of *aad*A gene amplification. Lanes: M,1, 2, 3, 4 represents: 100bp ladder, negative control, positive control, isolate DSW50 positive for *aad* A gene of 528 bp respectively.

4.3.3 Class 1 integron analysis

Results from the determination of the class 1 integron in the six MDR *Salmonella* isolates showed that two strains (20%) had class 1 integron. The two strains one from the Dunga beach DSW050 and K065 from Kibuye market harbored class 1 integrons. Using 5'CS and 3'CS primers to amplify the integrons gene, PCR products obtained had 800 and 1000 bp (Plate 6).

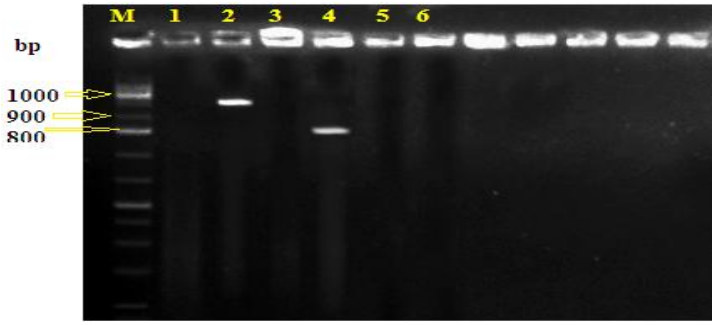

Plate 6: Gel image representative of Class 1 integron gene amplification. Lanes M is 100 bp ladder, lanes 1 negative control, 2-6 phenotyp *Salmonella* isolates resistant to or intermediate resistant to sulphamethoxazol. Only isolates DSW050 (lane No 2) and K065 (lane No 4) amplified approximately 1000kb and 800 bp gene respectively.

Table 13: Percentage resistance genes of *Salmonella* isolates

Resistance genes	No	(%)
tet A	2	(20)
tet B	0	(0)
bla_{TEM}	2	(20)
bla_{PES}	0	(0)
Sul 1	0	(0)
*aad*A	1	(10)
Int 1	2	(20)

Legend: *tet* A / *tet*B=tetracycline gene A or B, *bla*PES and bla_{TEM}=ampicilin resistance gene *Sul* 1= sulphamethoxazol gene and *aad*A= streptomycin resistance gene.

Table 14: Integron class 1 and Genotypic characteristics for antibiotic resistance of *Salmonella* isolates.

Isolate ID No	Integron	*tet*(A)	*tet*(B)	*bla*TEM	*bla*PES	*Sul*1	*aad*A
DSW050	Pos	Pos	Neg	Neg	Neg	Neg	Pos
PSW042	Neg	Neg	Neg	Neg	Neg	Neg	Neg
DSW051	Neg	Neg	Neg	Neg	Neg	Neg	Neg
K065	Pos	Pos	Neg	Pos	Neg	Neg	Neg
K065	Neg	Pos	Neg	Pos	Neg	Neg	Neg

Legend: *tet* A / ***tet*B**=tetracycline gene A or B, ***bla*PES** and bla_{TEM}=ampicilin resistance gene ***Sul* 1**= sulphamethoxazol gene and ***aad*A**= streptomycin resistance gene.

Pos=positive, Neg=negative

Table15: Association between antimicrobial susceptibility and integrons carriage

	Integron positive solates (N= 2)	Integron negative isolates (N= 8)	$\chi 2$	*p*-value
	Resistant, n (%)	Resistant, n (%)		
Tetracyclin	2 (20)	6 (60)	0.064286	0.799846
Ambicilin	2 (20)	4 (40)	0.355556	0.550985
Streptomycin	1 (10)	9 (90)	0.392157	0.531168
Sulphamethoxazol	2 (30)	5 (50)	0.678462	0.410117
Chloramphenical	3 (30)	5 (50)	0.678463	0.410117

Legent "N" indicates number of isolates

In order to assess the effect of integrons carriage on antibiotic susceptibility profile, the percentage of antibiotic resistance and susceptibility among integron-positive and integron-negative isolates were compared. The statistical significance *p*-value was calculated using Pearson $\chi 2$ test. The antibiotic resistant profile was deemed not significantly associated with the presence of integrons as *p*-value was more than 0.05 (Table 15). Fisher exact test was used when at least one cell of the contingency table has an expected cell count smaller than 5.

CHAPTER FIVE

DISCUSSION

5.1. Microbial contamination of *R. argentea* obtained from selected beaches of Lake Victoria and trading market within western Kenya

The study demonstrates that *R. argentea* landed at the beaches have high levels of APC counts ranging from $\log_{10}$5.63 cfu/g to $\log_{10}$6.03cfu/g. The study results also show that there is progressive increase in bacterial levels; APC and TC as the drying process proceeds, however by the eighth hour, there is a general reduction in microbial levels as demonstrated in (Tables 6). This could be attributed to the microbial killing property of UV light as well as handling mode of the fish since coliform counts of recently caught fish was relatively low, but increased considerably during handling. (Mahmoud *et al.,* 2004; Remigiusz *et al.,* 2012). APC levels were generally high after three hours of drying (mean range $\log_{10}$ 5.82 – $\log_{10}$ 6.11 cfu/g) when compared to those recorded when the fish was landed. Human activities could be important in this respect since the shores are inhabited and human activities could be responsible for the increase in the levels of coliform loads.

There was however a marked reduction in the APC levels ($\log_{10}$ 5.43 to $\log_{10}$ 5.93 cfu/g) as the fish gets to the markets as shown in Table 5. The same was observed for total coliforms (Table 5 and 6). It is extrapolated that the reduction in total coliforms in this fish could be due to the difference in moisture content of the products at the landing site at 72.83-76.90% (Ogonda *et al.,* 2014) compared to that recorded at markets 14.9% (Onyuka *et al.,* 2014) since moisture is one of the basic requirements for microbes to thrive. The APC levels reported did not comply with the locally approved safety standards, which specifies TPC values of not more than 100,000 cfu/g ($\log_{10}$ 5cfu/g), zero coliform counts with maximum limits being total viable count of 10^5/g (ISO 4833), *E. coli* / g absent (ISO 7251) and *Salmonella* in 25 g absent (ISO 6579), (KEBS, 1998; EAS, 2014). Moisture content has been reported to be an important factor that determines survival and growth of microbes in food products (September *et al.,* 2006).

The study found that *E.coli, Citrobacter and Salmonella spp* were recovered from *R. argentea* samples. This demonstrates that *R. argentea* may form important reservoirs for human pathogenic bacteria. From this study it can be postulated that the process of drying fish (*R.*

argentea) could be responsible for contamination of the fish with *Salmonella* spp. Generally, *R. argentea* is dried on top of fishing nets spread directly on the ground at fish landing sites (Abila and Jensen, 1997; Huss *et al.,* 2003; Yagoub, 2009; FAO, 2010). In this manner, the fish is exposed to both domesticated animals (e.g. cats, chicken and dogs) and wildlife (birds, lizards among others). These animals are known to be important carries of pathogens and other microorganisms (Olsen and Hammack 2000, Urban and Broce 2000). This lead to about 30% loss of fish in landed beaches by microbial flora activities that are of human and veterinary origin supported by environmental contamination (Geldreichi and Clarke, 1966; Ghaly *et al.,* 2010).

The isolation of enteric pathogenic bacteria from fish that might be transmitted to humans after the handling or consumption of fish was studied in Nile tilapia and 39.5% were Shigella sp.; 11.1% were *Salmonella typhi*; 25.4% were *Escherichia coli*. Ten fishes collected from open-air markets revealed *E. coli* (50%) and *S. typhi* (20%) (Onyango *et al*., 2009). Other studies have also confirmed the occurrence of pathogens in fish sourced from Lake Victoria (Sifuna *et al.,* 2008) affirming the level of the lake contamination. In another study on smoked fish, no *Salmonella* was isolated from a total of 89 tilapiine and *Lates niloticus* fish samples (Ogwan'g *et al*., 2005). The differences in isolation rates may be due to differences in isolation methods, fish species, processing time, sampling methods used and seasonal characteristics of the region when the studies were done.

This study showed a higher prevalence of *Salmonella* spp at the landing sites 60% compared to the markets at 40% (Table 7). And with respect to time, 5 out of 6 *Salmonella* isolates were found on semi dried samples, thus an increase by eighth hour of drying. The increase in *Salmonella* by eighth hour could be attributed to enough moistuer content a basic requirements for microbes to thrive and tempretuer during drying (Ogonda *et al.,* 2014). A decline in moistuer at eighth hour or after, reduced the organims in the fish. In addition, UV light from the sun enhanced the decline by killing the *Salmonella* isolates.

Reduction in contamination levels from the beaches to the markets could be attributed to reduced moisture content in the dried fish at the markets (Wafaa *et al*., 2011). These results are in agreement with an earlier study by Onyango *et al.,* (2009) whereby fish from Dunga had higher number of bacteria isolates (33.8%) than Kisumu market (9.9%). This was attributed to transportation of fish in dirty fishing boats and dirty packaging baskets by the fisher folks

(Onyango *et al.,* 2009) and the use of contaminated water collected directly from the shores of Lake Victoria by local artisanal fish processors as a result of lack of piped water (FAO, 2010).

5.2. Antimicrobial sensitivity profile of all the isolates by Ager Disc Diffusion method

(a) Antimicrobial resistance *E. coli* isolates

In this study, overall multidrug resistance patterns were observed with tetracycline and ampicillin and tetracycline and chloramphenicol. This is in agreement with (Onyango *et al.,* 2010). Most of the isolates were susceptible to two antibiotics, ciprofloxacin and naldixic acid with exception of four isolates. Resistance to tetracycline and ampicillin might be related to their overuse as opposed to naldixic acid and ciprofloxacin which are not used for treating enteric infections. Resistance to tetracycline and ampicillin in *E. coli* in the region has also been reported by (Sifuna *et al.,* 2008). Resistance to these two drugs may be attributed to their use in mass prophylaxis during cholera outbreaks in the past (Shapiro *et al.,* 1999; Sack, 2001). Ampicillin or tetracycline resistant *E. coli* from humans may reach fish through contamination by activities of adjoining population and partially treated and untreated sewage which may find its way into the lake (Kayambo *et al.,* 2006). Due to their relatively low cost and readly availability for sale 'over the counter', these drugs are widely used by farmers for vetenary therapeutic and prophylactic applications (Kariuki *et al.,* 2002).

E. coli isolates in this study were resistant to chloramphenicol, ampicillin and tetracycline as follows; 52.27%, 31.81% and 22.72% respectively. In contrast to this results (Sifuna *et al.,* 2008) reported resistance to chloramphenicol, ampicillin, tetracycline as 0%, 20% and 12% respectively. The prominence of chloramphenicol, ampicillin and tetracycline resistance among *E. coli* isolates in this study demonstrates the similarities in the development of resistance among the isolates and *E. coli* serovas of clinical importance. It can therefore be postulated that this exposure to antibiotics might be of a human origin. This is further supported by the presence of resistance to chloramphenicol, antibiotic generally used in humans only but not in animals feeds.

Antimicrobial resistance patterns in this study demonstrate contamination at both landing sites and markets differ. The results show differences among the antibiograms observed among markets and landing sites. The difference in antibiogram patterns could be indicative of possible contamination of fish at the markets attributable to wild life, domestic animals, insects and poor practices by human handling the products (Ogwan'g *et al.,* 2005).

The presence of antimicrobial resistance among bacteria recovered from *R. argentea* from markets and fish landing sites is indicative of public health risks. This makes the fish a health risk since this resistance could be passed to human and livestock when the fish product is used for livestock feed formulation.

(b) Antimicrobial resistance in *Salmonella* isolates

In Kenya, easy over the counter accessibility of antibiotics at a cheaper price and their extensive use largely by farmers for therapeutic and prophylactic application is responsible for the high resistance leveles (Kariuki *et al.,* 2002). Another major setback might be the quality and potency of locally manufactured or cheap drugs imports. For example, the presence of different brands of the fluoroquinolone and ciprofloxacin in India and Kenya (Hart and Kariuki, 1998), which has lead to widespread availability and uncontrolled use of antibiotics. More disturbing though is the habit self diagnosis that leads to patient home misuse of drug treatments. In salmonellosis, antibiotics are recommended only in serious illness because they do not shorten the illness but significantly prolong the fecal excretion of the organisms and increase the number of antibiotic resistant strains (Alvseike *et al.,* 2002).

In this study, highest resistance was observed in chloramphenicol at 30%, followed by ampicillin and tetracycline at 20%. The finding of this study is in contrast with what was reported by Onyango *et al.,* (2008) whereby *Salmonella* isolates from fish along Winam beaches showed 13% resistance to ampicillin and 12% for tetracycline. Brook *et al*., (2003) on the other hand reported high resistance levels of 90% and 80% for tetracycline and ampicillin respectively for *Salmonella* spp among clinical isolates within the Lake Victoria basin. There is an inter-link of AMR between human and wider environment. Antimicrobial resistance in humans is inter-linked with AMR in other populations like farm animals, and in the wider environment. The multiple links allow movement of the bacteria, mobile genetic elements and the drugs themselves (Woolhouse and Ward, 2013). This finding could imply that the use of antimicrobial agents in clinical situations may be an important factor contributing towards resistance in the Lake basin. Resistance to ampicillin in this study may indicate wide use of this antimicrobial in recent years. It is not a first line antimicrobial in treatment of invasive salmonelosis in Kenya, hence the low resistance level compared to studies by Kariuki *et al.,* (2002), Cabrera *et al.,* (2004) and Chiu *et al.,* (2002). Nalidixic acid resistance has been on the

rise in various parts of the world (WHO, 2000). In this study, resistance to nalidixic acid was not observed in the isolates, compared to 11% in a clinical study by Kariuki *et al.,* (2002). However, the finding of this study is in agreement with (Mengo *et al.,* 2010) where all the tested *Salmonella* isolates from patient's blood in a Nairobi hospital were sensitive to ciprofloxacin and nalidixic acid. This could be attributed to these drugs not being used for enteric diseases treatment (Hooton, 2003). Overally, the differences in levels of resistance and resistance patterns could be due to levels of exposure to the agents or other factors that may have increased or decreased the likelihood of the development and conservation of resistant bacteria. *R. argentea* is not exposed directly to antibiotics but may be contaminated with antibiotic resistant bacteria through personnel, polluted fishing grounds or animal droppings.

5.3 Class 1 integrons and genes associated with antimicrobial resistence in *Salmonella*

Detection of resistance genes was based on their resistance phenotypes. Antimicrobial resistance to the aminoglycosides was coded by *aad*A gene (10%) (Plate 5) (Table13). Tetracycline resistance genes were detected among all 2 phenotypic tetracycline-resistant isolates (Plate 4) (one intermediate). However, only *tet*A gene (20%) was amplified. The two isolates K065 and K068 were from the Kibuye market. They may have shared the gene in a horizontal way through conjugation, transduction and transformation. Since these were environmental isolates the *tet* agene could only have come from either soil or human faecal contamination (Mathew *et al.,* 2007; Davies and Davies, 2010). Tetracycline resistance is encoded by the *tet* genes (French and Schwarz, 2000; Hur *et al.,* 2011; Randall *et al.,* 2004). The (*tet* A) gene is located frequently on transposons such as Tn1721, and the gene has been found to be widespread among Gram negative bacteria including *Salmonella* (Gebreyes and Altier 2002; Pasquali *et al.,* 2005). Efflux pumb is responsible for resistance for *tetA.* Efflux pump actively exports antimicrobial and other compounds out of the cell thus limiting access of an antimicrobial agent to its site of action (Madigan *et al.,* 2000).

The *bla*TEM gene account for resistance to penicillins and cephalosphorins. Resistance to ampicillin in *Salmonella* is usually mediated by TEM type β-lactamases (Randall *et al.,* 2004; Hur *et al.,* 2011). The β-lactamase-related mechanism implicated in antimicrobial phenotype is the production of the enzymes TEM-1, as previously reported in other studieis **(**Guerri *et al.*,

2004). The two ampicillin resistant isolates contined *bla*$_{TEM}$ gene (Plate 3). None of the isolates was positive for *bla*PSE-1. More than one β-lactamase gene at the same time in the same isolate was not detected in this study. This is because the isolates used in this study being environmental may not hane been exposed to various resistant genes unlike clinical ones. This is in contrast with data from other studies (Güerri *et al.,* 2004; Biendo *et al.,* 2005) who reported all *Salmonella* spp isolated from patients carried both *bla*$_{TEM}$ and a second gene encoding beta lactamase either pes-1 or oxa-1.

Resistance to third generation cephalosporins (ceftriaxone, cetotaxime, amoxyline and cefuroxime) was not detected in isolates of this study. This could be due to less exposure of these isolates to the antibiotics unlike in clinical samples. Although antimicrobials are not used in the treatment and as growth promoters in capture fish like *R. argentea,* resistance to various antimicrobial agents including the presence of genes responsible for resistance was detected in the fish isolates. This observation might be due to the acquisition of resistance genes from other sources, such as bacteria in water contaminated with human sewage and other environmental exposures like soil during the process of drying fish (Rysz and Alvarez, 2004).

Resistance genes to commonly used drugs like chloramphenicol, ampicilin, tetracycline, streptomycin and sulphamethoxazol have often been associated with either multiresistance conjugative transposons or plasmid-borne multiresistance integrons which are mobile genetic elements detected in a variety of enterobacterial species (Pai *et al.,*2006). *Salmonella* resistance to fluoroquinolones in most cases involve mutations in the quinolone resistance-determining regions of the DNA genes, active efflux (AcrAB efflux), and decreased outer membrane permeability strains (Lindgren *et al.,* 2009). In this study, all the isolates were sensitive to ciprofloxacin and naldixic acid. This is in agreement with (Onyango *et al.,* 2008) who reported all clinical *Salmonella* isolates sensitive to ciprofloxacin. This suggests that these isolates did not harbor *gyrA, gyrB, parC,* or *parE* genes which occur as a result of point mutation in fluoroquinolones. Resistance in ciprofloxacin has been shown to be anchored in mutations that give rise to the substitution of phenylalanine for serine at position 83 and asparagine for aspartic acid at position 87 in *gyr*A (Yen *et al.,* 2005; Antunes *et al.,* 2007). *Sul* 1 gene was not observed in any *Salmonella* isolates in this study despite two isolates having class 1 integron gene. This could be due to other *sul* genes (*sul*2 or *sul*3) which can be found in integrons that lack the *qacE*D1 and *sul*1 genes. If such integrons lack the *qacE*D1 and *sul*1 genes, the *sul*2 or *sul*3 genes

can confer the sulphonamide resistance (Patrı´cia *et al.,* 2006). This is in agreement with Onyango *et al*., (2010) who reported absence of *Sul* 1 resistance gene in clinical isolates. This observation could also be explained by the fact that the sulfonamide resistance in an isolate may not be as a result from *sul1* gene but the presence of *dfr* gene cassettes which are always found in class 1 integrons may also play a part in determining the resistance (Leverstein-van Hall *et al.*, 2003).

Class 1 integron was found in only two of ten *Salmonella* isolates. A rate of 20% in this study is comparable with (Thi *at al*., 2007) who reported 13% (3 of 23) *Salmonella* isolates from meat and shellfish to be positive for class 1 integron. This low frequency is thought to be due to lower exposure to selective pressure of the antibiotic than for the clinical isolates. This result is also in consistence with Hongmel *et al.,* (2004) who reported 4 out of 23 strains of *Salmonella* isolated from healthy humans were positive for class 1integron gene. However, this result is in contrast with Park *et al.,* (2003), who reported 26% of Gram-negative bacteria in an estuarine environment contained class 1 integron. Also in contrast with this result is Roe *et al*., (2003) who reported 16% of Gram-negative bacteria in irrigation water and sediments contained class 1 integron. In addition, this rate is much lower than that reported by Lin *et al.,* (2005), who showed that 58% of multiresistant isolates from aquatic environment contained class 1 integron. Class I integrons consist of a 5'-conserved segment (CS) and a 3'-conserved segment (SC). The 5'CS contain integrase gene *(int1) while* 3'CS contains *qacE*Δ and *sul1* genes, conferring resistance to quaternary ammonium compounds and sulfonamides, respectively (Hall and Stokes, 1998). Interestingly in this study, three isolates resistant to sulphamethoxazol, (one resistant and two intermediate) did not express integron class 1 gene suggesting the presence of alternative resistance mechanisms for this resistance, transposons, plasmid or presence of other integron classes. These isolates may also harbor other genes such as the *sul*A or *dfr* genes encoding dihydrofolate reductase (Roberts *et al.,* 2005) of which is outside the scope of this study. The two class 1 integron positive were also MDR *Salmonella* isolates with phenotypic antibiotic resistance pattern of Chloramphenicol-Sulfamethoxaeole-Tetracycline (Intermediate)-Streptomycin for isolate DSW050 and Ampicillin-Chloramphenicol-Sulfamethoxaeole-Tetracycline for isolate K065 which amplified approximately 1000bp and 800 bp integron gene respectively by PCR (Plate 6).This is in agreement with Ming *et al.,* (2012) who reported single MDR isolate tested positive for class 1 integron (1 kb).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. This study provided evidence for contamination (e*nterobacteriaceae*) along the landing sites. The sundrying as a method of processing does not arrest bacterial growth but instead provide an enabling environment for them to proliferate although the population slightly goes down a factor that could be attributed to direct effect of UV light.
2. The study findings also showed the occurrence of antimicrobial resistant e*nterobacteriaceae* for example *Salmonella*, *E. coli* and *Citrobactor* spp in *R. argentea* at beaches and markets in western Kenya. This implies that fish is a potential source of single and multiple antimicrobial-resistanc to commonly used antimicrobials including ampicillin, chloramphenicol, streptomycin and tetracycline.
3. Resistance genes were identified in *Salmonella* isolates from *R. argentea* in this study. These genes are not class 1 integron mediated but can be hypothesized to be through plasmid or transposons. The presence of class 1 integron in fish isolates support the notion that integron genes are ubiquitous in several bacterial populations and in this case, the *Salmonella* spp.

6.2 Recommendations

1. Need for public health education for fish traders and consumers on good health practices and hygiene standards
2. The frequency of resistance to antimicrobials in *Salmonella* spp from *R. argentea* isolates is relatively low. Continuous, future epidemiological investigation and surveillance to monitor emergence of antibiotic resistance strains need to be 1maintained through screening of DNA elements as part of efforts aimed at characterizing antimicrobial resistance among *Salmonella.* Surveillance of fluoroquinolones and third generation cephalosporins resistance should be enhanced as they had minimal resistance in this study. They may be the antibacterial agents of the future (Emmerson *et al.,* 2003).

6.2.1 Recommendation for future studies

More research on other classes of integrons which may be involved in *Salmonella* spp resistance and isolates resistant to sulphamethoxazol but do not express integron class1 gene should be carried out, as this suggest the presence of alternative resistance mechanisms for this resistance, either transposons or plasmid.

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APPENDICES

APPENDIX 1

API chemical reaction

Strain	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	Computer Analysis
Conrtol	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal* spp.(99.9%)
PvSW042	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal arizonae*.(89.4%)
DDW047	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal* arizonae.(99.9%)
DSW050	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	*Sal spp*.(89.4%)
DSW051	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	*Sal* arizonae (89.4%)
Y063	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal* spp.(99.9%)
K065	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	*Sal* spp.(89.4%)
K066	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal* spp.(99.9%)
K068	-	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	-	+	-	+	*Sal* spp.(89.4%)
UsSW070	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal* spp.(99.9%)
PvSW067	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	-	+	-	+	*Sal* spp.(99.9%)

ONPG: β -galactosidase; **ADH:** ***arginine dihydrolase*****; LDC:** lysine decarboxylase**; ODC:** ornithine decarboxylase**; CIT:** citrate utilization**; H2S:** H2S production**; URE:** urea hydrolysis; **TDA:** deaminase**; IND**: indole production**; VP:** acetoin production**; GEL:** gelatinase; **GLU:** glucose-; **MAN:** mannitol-; **INO**: inositol-; **SOR**: sorbitol-**; RHA:** rhamnose-; **SAC:** sucrose-; **MEL:** melibiose-; **AMY:** amygdalin-; **ARA:** arabinose-fermentation/oxidation**. *Control Strain:** *S.* Typhimurium ATTC 14028.

APPENDIX 2

Salmonella isolation and Biochemical Identification

									LIA			TSI			
	Sample	**SSA**	**XLD**	**INDOLE**	**MR**	**VP**	**SC**	**Butt**	**Gas**	**H_2S**	**Butt**	**Slant**	**Gas**	**H_2S**	**Inference**
1	PvSW042	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	-	+	D	*Salmonella*
2	DD047	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	+	+	+	*Salmonella*
3	PvSW067	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	+	+	+	*Salmonella*
4	DSW050	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	+	+	+	*Salmonella*
5	DSW051	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	+	+	D	*Salmonella*
6	Y063	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	+	+	+	*Salmonella*
7	K065	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	D	+	D	*Salmonella*
8	K066	Black Center	Alkaline, Black Center	+	+	-	+	-	-	+	+	+	+	+	*Salmonella*
9	K068	Black Center	Alkaline, Black Center	-	+	-	+	-	-	+	+	D	+	D	*Salmonella*
10	UsSW070	Black Center	Alkaline, Black Center	-	+	-	+	-	-	-	+	-	+	-	*Salmonella*

Legend: The media used are SSA= *Salmonella Shigella* agar, XLD = xylose lysine deoxycholate, TSI = triple sugar iron. Tests done are MR= methyl red test, VP = Voges Proskauer test.

APPENDIX 3

***Salmonella* spp isolates antimicrobial susceptibility**

Antibiotics(ug)	**DSW050**	**PSW042**	**DSW051**	**DD047**	**Y063**	**K065**	**K066**	**PSW067**	**K068**	**UsSW070**
Na (30)	I	S	S	S	S	S	S	S	S	S
TeT(30)	I	I	S	S	S	R	S	S	R	S
CIP (5)	S	S	S	S	S	S	S	S	S	S
A (10)	S	S	S	I	S	R	I	I	R	I
CH (30)	R	S	R	S	S	R	I	S	I	S
S (10)	R	S	S	S	S	S	S	S	S	S
SXL (300)	R	I	I	S	S	R	S	S	R	S
CRO (30)	S	S	S	S	S	S	S	S	S	S
CTX (30)	S	S	S	S	S	S	S	S	S	S
AMC (30)	S	S	S	S	S	S	S	S	S	S
CXM (30)	S	S	S	S	S	S	S	S	S	S

APPENDIX 4

A comparison of antimicrobial agent disc diffusion zone sizes for *E.coli*

Antimicrobial agent	**Antimicrobial distribution patterns of *E.coli* isolates**	
	Mean	**Standard Deviation**
NA	23.705	6.479
T	20.045	7.557
CF	27.477	5.622
A	17.023	7.479
C	23.659	6.291
S	18.659	3.766
SXL	16.591	4.597

A **comparison of antimicrobial agent disc diffusion zone sizes for *Salmonella***

Antimicrobial agent	Antimicrobial distribution patterns of *Salmonella* spp isolates	
	Mean	Standard Deviation
NA	22.400	4.033
T	17.800	5.073
CF	29.200	2.658
A	18.000	8.287
C	24.100	8.736
S	18.600	3.658
SXL	18.000	5.908
CRO	24.000	2.567
CTX	30.000	2.449
CXM	20.000	1.155
AMC	21.000	5.908

A comparison of antimicrobial agent disc diffusion zone sizes for *Citrobactor* spp

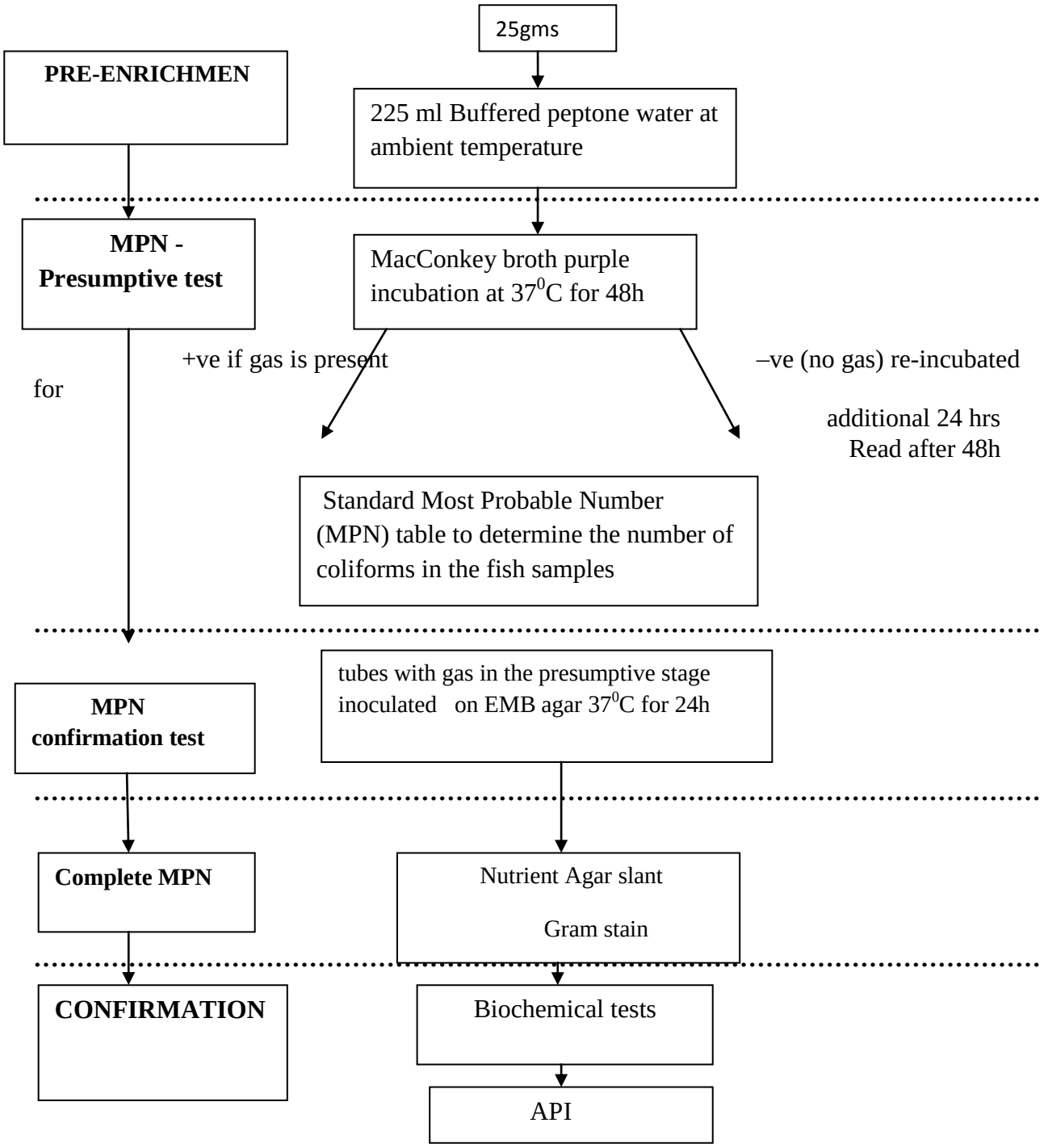
Antimicrobial agent	Antimicrobial distribution patterns of *Citrobactor* spp isolates	
	Mean	Standard Deviation
NA	18.429	4.198
T	18.571	3.780
CF	26.000	1.915
A	16.000	6.583
C	24.000	2.944
S	20.429	2.149
SXL	18.000	5.260

APPENDIX 5

Antimicrobial resistance of respective isolates

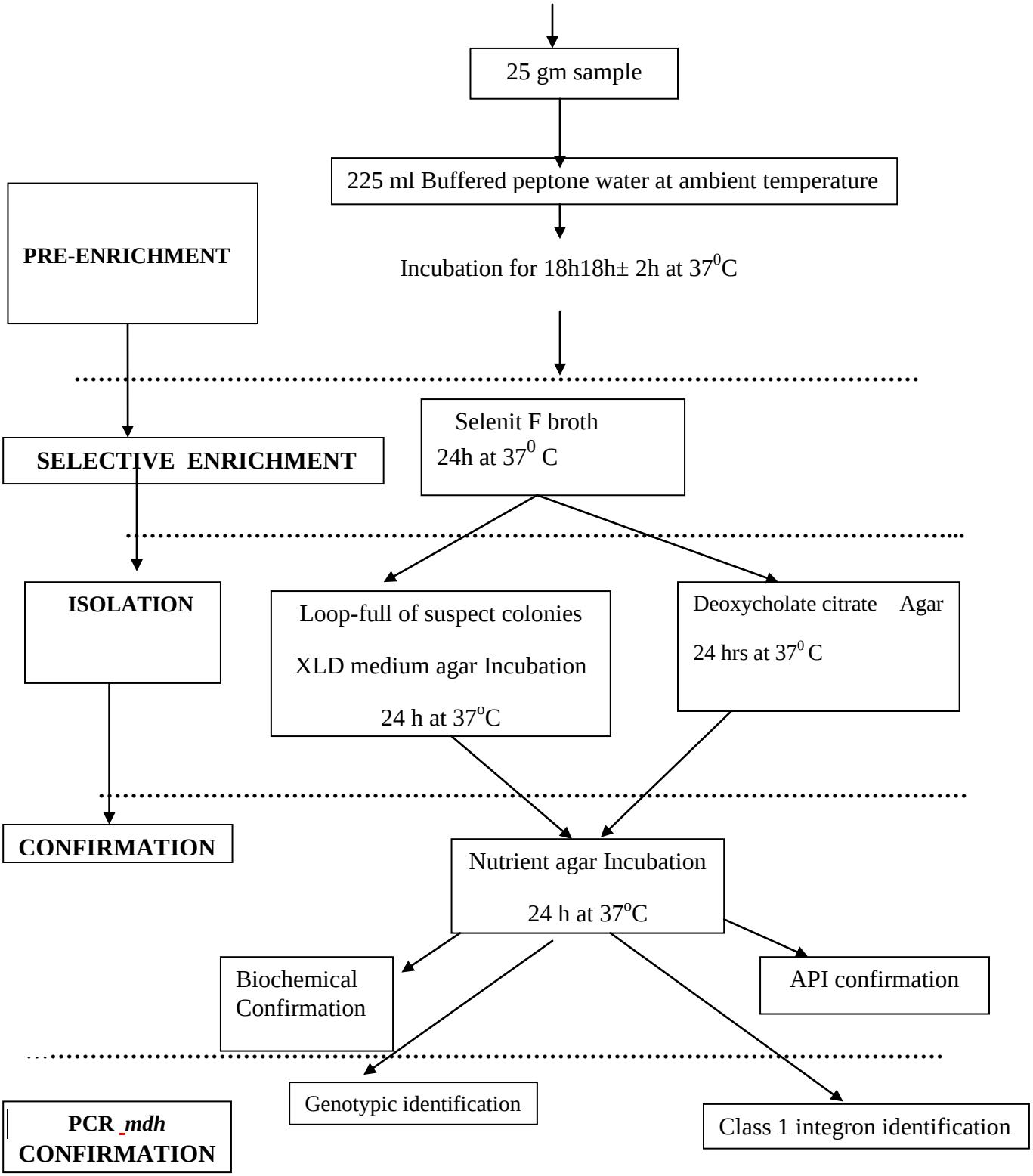
Isolates	% antimicrobial resistance of respective isolates																				
	NA (30µg)			**Tet (30µg)**			**CF (5µg)**			**Amp (10µg)**			**Chlor (30µg)**			**S (10µg)**			**SXL (300µg)**		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
E. coli (n=44)	3	0	40	10	5	29	1	1	41	14	3	29	23	11	8	1	5	37	8	4	32
Citrobacter Spp (n=7)	1	2	4	0	4	3	0	0	7	5	0	2	5	2	0	0	0	7	1	1	5
Klebsiella Spp (n=1)	1	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	1	1	0	0
Proteus Spp (n=1)	0	0	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0

APPENDIX 6



Flow diagram showing procedure for isolation and confirmation of coliforms and *E. coli*

APPENDIX 7



Flow diagram showing procedure for isolation of *Salmonella* spp. (ISO 6579: 2002)

APPENDIX 8

Typical biochemical reaction of *Salmonella* culture on TSI agar

Reaction area	Results	Interpretation
Slant surface	Yellow	Lactose and/or sucrose positive (lactose and/or sucrose used)
	Red or unchanged	Lactose and sucrose negative (neither lactose nor sucrose used)
Butt	*Yellow*	Glucose positive (glucose used)
	Red or unchanged	Glucose negative (glucose not used)
	Black	Formation of hydrogen sulfide
	Bubbles or cracks	Gas formation from glucose

APPENDIX 9

ANOVA RESULTS

Aerobic plate count (APC) values for respective fish landing sites.

Anova: Single Factor α 0.05

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
DUNGA	14	81.67747	5.834105	0.032065
USENGE	6	34.72817	5.788028	0.036745
UHANYA	15	88.25578	5.883719	0.021023
PORT VIC	7	40.60045	5.800064	0.020592

LSD	0.125263
HSD	0.16622
Scheffe	0.376752

Post Hoc	DUNGA	USENGE	UHANYA
USENGE	0.046077		
UHANYA	0.049614	0.095691	
PORT VIC	0.034041	0.012036	0.083655

Colored cells have signficant mean differences

ANOVA Cannot Reject Null Hypothesis because p > 0.05 (Means are the same)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between Groups	0.056519	3	0.01884	0.702939	0.556	2.851741
Within Groups	1.018451	38	0.026801			
Total	1.07497	41				

Log_{10} mean colony forming units (CFU/gm) of sun-dried *R. argentea* contamination in respective commercial markets

Anova: Single Factor α 0.05

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
LUANDA	15	88.97885	5.931924	0.057342
YALA	15	88.12187	5.874791	0.184829
KIBUYE	15	81.42577	5.428384	0.236972
BUSIA	15	84.59595	5.63973	0.147848

LSD	0.289603
HSD	0.384773
Scheffe	0.90018

Post Hoc	LUANDA	YALA	KIBUYE
YALA	0.057132		
KIBUYE	0.503539	0.446407	
BUSIA	0.292194	0.235061	0.211345

Colored cells have signficant mean differences

ANOVA Reject Null Hypothesis because p < 0.05 (Means are Different)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between Groups	2.405221	3	0.80174	5.114842	0.003	2.769431
Within Groups	8.777879	56	0.156748			

Total coliform counts (MPN/g) in the respective markets in the study areas

Anova: Single Factor α 0.05

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
LUANDA	19	13.444	0.707579	6.238571
YALA	16	3.907	0.244188	0.036624
BUSIA	15	2.794	0.186267	0.026631
KIBUYE	15	2.444	0.162933	0.027583

LSD	0.885352
HSD	1.169972
Scheffe	3.118189

Post Hoc	LUANDA	YALA	BUSIA
YALA	0.463391		
BUSIA	0.521312	0.057921	
KIBUYE	0.544646	0.081254	0.023333

Colored cells have signficant mean differences

ANOVA Cannot Reject Null Hypothesis because p > 0.05 (Means are the same)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between Groups	3.535171	3	1.17839	0.632748	0.597	2.755481
Within Groups	113.6026	61	1.862338			
Total	117.1378	64				

Aerobic plate count (APC) values for respective fish landing sites at 0 hour.

Anova: Single Factor α 0.05

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
DUNGA 0HRS	5	30.16571	6.033142	0.029828
USENGE 0HRS	5	28.16587	5.633174	0.114692
UHANYA 0HRS	5	30.06905	6.013809	0.00444
PORT VICTORIA 0HRS	5	28.3969	5.679379	0.06886

LSD	0.312871
HSD	0.422241
Scheffe	0.531222

Post Hoc	DUNGA 0H	USENGE 0	UHANYA 0HRS
USENGE 0	0.399968		
UHANYA 0	0.019332	0.380635	
PORT VICT	0.353762	0.046205	0.33443

Colored cells have signficant mean differences

ANOVA Reject Null Hypothesis because p < 0.05 (Means are Different)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between Groups	0.680446	3	0.226815	4.165186	0.023	3.238872
Within Groups	0.871281	16	0.054455			
Total	1.551727	19				

Aerobic plate count (APC) values for respective fish landing sites at 3 hour.

Anova: Single Factor α 0.05

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
DUNGA 3HRS	5	30.13366	6.026731	0.043884
USENGE 3HRS	5	29.98471	5.996942	0.019873
UHANYA 3HRS	5	30.36382	6.072765	0.001473
PORT VICTORIA 3HRS	5	29.63087	5.926175	0.10535

LSD	0.276873
HSD	0.373658
Scheffe	0.470101

Post Hoc	DUNGA 3H	USENGE 3H	UHANYA 3HRS
USENGE 3H	0.029789		
UHANYA 3	0.046033	0.075823	
PORT VICT	0.100557	0.070767	0.14659

Colored cells have signficant mean differences

ANOVA Cannot Reject Null Hypothesis because p > 0.05 (Means are the same)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between Groups	0.056705	3	0.018902	0.443231	0.725	3.238872
Within Groups	0.68232	16	0.042645			
Total	0.739024	19				

Aerobic plate count (APC) values for respective fish landing sites at 8 hour.

Anova: Single Factor α 0.05

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
DUNGA 8HRS	5	30.52887	6.105773	0.02392
USENGE 8HRS	5	29.36899	5.873798	0.030458
UHANYA 8HRS	5	30.13908	6.027815	0.004307
PORT VICTORIA 8HRS	5	29.10715	5.821429	0.014805

LSD	0.181732
HSD	0.245259
Scheffe	0.308561

Post Hoc	DUNGA 8H	USENGE 8H	UHANYA 8HRS
USENGE 8H	0.231976		
UHANYA 8H	0.077958	0.154018	
PORT VICT	0.284344	0.052369	0.206386

Colored cells have signficant mean differences

ANOVA Reject Null Hypothesis because p < 0.05 (Means are Different)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between Groups	0.262251	3	0.087417	4.758045	0.015	3.238872
Within Groups	0.29396	16	0.018372			
Total	0.556211	19				