OCCURRENCE AND ANTIMICROBIAL PROFILES OF *SALMONELLA* SP IN FISH (*OREOCHROMIS NILOTICUS*) AND SELECTED FISH PONDS IN WESTERN KENYA

BY

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ABSTRACT

Salmonella, a gram-negative facultative rod shaped bacterium in the family *Enterebacteriaceae*, is found in the intestinal tracts of humans and animals. *Salmonella* causes salmonellosis, which manifests as two diseases in humans: enteric fever (typhoid) and acute gastroenteritis. Salmonellae are disseminated to the external environment, such as water, soil and plants, through human or animal excretion. Fish farming has become an important practice in Western Kenya where two species, namely Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) are reared for subsistence and for commercial sales. It has however been established that fish can serve as carriers or infectious source of pathogens such as Mycobacteria, *Streptococcus iniae, Vibrio* spp., aeromonads and *Salmonella* spp. Currently, there is a rise in the incidence of antimicrobial resistant bacteria including *Salmonella.* This has become of critical concern in the world, with about 1.3 billion cases and 3 million deaths reported annually due to multidrug resistant (MDR) *Salmonella.* In Kenya, the prevalence of MDR *Salmonella* has been rising steadily since it was first reported between 1997 and 1999. The aim of this study was to determine the incidence of *Salmonella* and analyze their antimicrobial profile in farmed fish and fish ponds in the community around Maseno University in Maseno division in Western Kenya. Nineteen *Salmonella* isolates were obtained from the flesh and intestines of fish (n=55) and water collected from eleven fish farms. Two isolates were *Salmonella enterica* serovar Typhi and 4 were *Salmonella enterica* serovar Typhimurium due to the presence of malate dehydrogenase (*mdh*) gene (261bp). Ninety five percent of the *Salmonella* isolates were resistant to Ampicillin, 89% to kanamycin, 84% to chloramphenical, 63% to streptomycin, 31% to tetracycline, 16% to gentamicin, and 11% to cotrimoxazole. Bla_{TEM}, a gene that confers resistance to β-lactams and cephalosporins was amplified in 61% *Salmonella* isolates. The incidence of *Salmonella* spp was 45% in the fish ponds and 15.7% fish. These findings indicate that fish from fish farms around Maseno University contain MDR *Salmonella* which may infect humans. It is therefore important that the farmers improve the hygiene of their fish farms and that consumers prepare their fish properly to cut the cycle of infection from man to fish and back to man.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Salmonella is a Gram-negative facultative rod-shaped bacterium in the family *Enterobacteriaceae*. *Salmonella* is well-studied from a structural, biochemical and molecular point of view, though its ecology is not known. Salmonellae live in the intestinal tracts of warm and cold blooded animals with some species being ubiquitous. Other species are specifically adapted to a particular host. In humans, *Salmonella* are the cause of two types of salmonellosis: enteric fever (typhoid) and acute gastroenteritis, resulting from bacterial invasion of the bloodstream, and food-borne infection and intoxication respectively (Todar, 2005).

The principal habitat of the salmonellae is the intestinal tract of humans and animals. However, *Salmonella* serovars can be found predominantly in one particular host, can be ubiquitous, or can have an unknown habitat. *S. typhi* and *S. paratyphi* A are mostly human and may cause grave diseases often associated with invasion of the bloodstream. Salmonellae are disseminated in the external environment (water, soil, sometimes plants used as food) through human or animal excretion. Humans and domestic and wild animals can excrete *Salmonella* either during or after clinical salmonellosis. The bacteria do not seem to multiply significantly out of digestive tracts, but can survive for several weeks in water and several years in soil if conditions of temperature, humidity, and pH are favorable (Todar, 2005).

Fish culture systems found in Kenya include semi-intensive culture of Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*), practiced by small-scale fish farmers in earthen ponds. The species used at any given site are mainly endemic to the region and more or less appropriate to the agro-climatic zone. Tilapia is normally a warm water fish and is mainly cultured in freshwater environment. Catfish are grown in the same agro-climatic region as tilapia (Ngugi *et al.*, 2007).

Generally, an ideal fish culture system needs to have the dissolved oxygen (DO) level maintained at 3mg/L or higher for tilapia and African catfish. This will enhance the growth of phytoplanktons. In terms of productivity, the plankton density (algal bloom) should allow one to see about 30-45 cm into the water. The pH of pond waters should be maintained between the optimum limits for fish, that is, between 6.5 and 9.0. Effort to check clay turbidity in pond water should be enhanced as muddy water can have a negative impact on fish and pond productivity (Ngugi *et al.*, 2007).

Nile tilapia grows best in waters with a temperature range of 20-35°C. At higher temperatures their metabolic rate rises, leading, in extreme cases, to death. They are able to survive levels of dissolved oxygen (DO) below 2.3 mg/L as long as temperature and pH remain favourable. They can grow up to 500grams in eight months if breeding is controlled and food supply is adequate. Juvenile tilapia feed on phytoplankton, zooplankton, and detritus, but adults feed almost exclusively on phytoplankton. Tilapia can reach sexual maturity at two months of age or at 10 cm or less in length. Hence, the major drawback with tilapia culture is their tendency to over breed, which can result in a large population of stunted (undersized) fish (Ngugi *et al.*, 2007).

Catfish generally reach maturity at two years of age at a weight of 200-500 grams. Females can produce between 10,000 and 150,000 eggs, depending on the size and age of the female. The optimal temperature for growth is 30°C; however, tropical temperatures in the range of 26-33°C are known to yield acceptable growth performance (Ngugi *et al.*, 2007). At temperatures below this range, growth rates decrease but survival is still good. However, it is documented by Ngugi *et al.*, (2007) that 28°C is the optimal temperature for both yolk sac adsorption and maximum growth rate. Catfish can withstand very low dissolved oxygen levels. A salinity range of 0-2.5 parts per thousand (ppt) is optimal for young catfish. Larval growth is acceptable in up to 5 ppt salinity, and survival is good up to 7.5 ppt. Catfish are omnivorous or predatory, feeding mainly on aquatic insects, fish, crustaceans, worms, molluscs, aquatic plants, and algae. They find food by probing through the mud on the bottom of the ponds. Their nutritional requirements in fish ponds (particularly for protein and lipids) are highly variable, and are influenced by factors such

as management practices, stocking densities, availability of natural foods, temperature, fish size, daily feed ration, and feeding frequency (Ngugi and Manyala, 2007).

In East Africa, fish diseases are not so common on fish farms due to low rates at which fish ponds are stocked and the relative hardiness of the fish that are usually farmed (Ngugi and Manyala, 2007). Diseases in fish may be evidenced by changes in their appearance and/or behavior and death. Fish diseases may result from exposure to excessive stress in the environment, which lowers their resistance to disease causing organisms. Common sources of stress for fish include: poor nutrition, poor sanitation, overcrowding in ponds, rough handling of fish by farm workers, presence of disease vectors and intermediate hosts (Ngugi *et al.*, 2007). Environmental conditions such as water temperature, salinity, oxygen levels, phytoplankton concentration, pH, light and nutrient concentration can modify the occurrence and concentration of indigenous aquatic pathogenic bacteria in water, and therefore their occurrence in farmed fish (Tamplin, 2001; Martinez-Urtaza *et al.*, 2008).

Transmission of disease/infections from fish to man are common depending on the season, contact with fish and related environment, dietary habits and the immune status of the exposed individual (Bhaftopadhyay, 2000). Some bacterial species are facultative pathogens of both fish and man and may not display any symptoms of disease in fish. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include mycobacteria, *Streptococcus iniae, Vibrio vulnificus, Vibrio* spp., aeromonads and *Salmonella* spp. (Lipp and Jose, 1997; Zlotkin *et al.*, 1998; Bhaftopadhyay, 2000).

A high incidence of bacteria resistant to antimicrobials used in aquaculture, including multiple resistant bacteria, have been found in fish farms and the surrounding aquatic environments (DePaola *et al.*, 1995). Microbial degradation, diffusion (Samuelsen *et al.*, 1992), light and temperature conditions (Lunestad, 1992) have been shown to be some of the factors that influence the turnover of antimicrobials in sediment. Accumulation of surplus antimicrobials and antimicrobial residues may occur in integrated fish farms when the ponds are only rarely emptied at the time of fish harvest. Such a buildup could establish selective pressure favoring selection and growth of

antimicrobial-resistant bacteria. Although increased levels of antimicrobial resistance in and around fish farms may only occur transiently, there is a potential risk that antimicrobial resistance genes could be disseminated into a wide range of bacteria in the aquatic environment (Barger *et al.,* 1997; Wegener, 1999).

Antimicrobials approved for use as animal growth promoters are not associated with antimicrobial therapy in humans to avoid selection of bacteria resistant to important drugs. Nevertheless, resistance to one antimicrobial within a class of antimicrobials often confers resistance to other members of the same group (cross-resistance) (Barger *et al.,* 1997; Wegener, 1999). The use of antimicrobials as growth promoters in animal husbandry has been linked to certain antimicrobial resistance patterns among human bacterial pathogens, suggesting that there is a possible flow of antimicrobial resistance strains between animal and human pathogens (Wegener, 1999). Antibiotic resistance is of critical concern in African countries. *Salmonella* is one of the antimicrobial resistant bacteria present in fish, which could be transmitted to man. Nontyphoidal salmonellosis (NTS) causes high rates of infections worldwide due to food poisoning in humans which is associated with contaminated food products of animal origin (Thorns, 2000). In many countries, it is the leading cause of food-borne infections and outbreaks (Tirado and Schmidt, 2001). Multidrug-resistant nontyphoidal salmonellosis is one of the most common causes of bacteremia in children (Graham, 2002). Currently, there are global pandemics of *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium DT104 (Saeed *et al.,* 1999).

Studies by Kariuki *et al.,* (2004) indicated that multidrug resistant (MDR) *Salmonella* is becoming a major problem in Kenya and that these resistant strains are quickly replacing the sensitive strains. Onyango *et al.*, (2007) demonstrated that the prevalence of MDR *Salmonella enterica* serovar Typhimurium was high in clinical samples from Maseno and Mukumu Hospitals in Western Kenya. Many people in Western Kenya depend on fish for diet and as a source of livelihood through sales. It is documented that fish can carry *Salmonella* for a long period without symptoms of infection (Fell *et al.*, 2000). Many community-based self-help groups and individual families have set up several fish farms in Western Kenya. Potential transfer of resistant

bacteria, including *Salmonella* spp from aquaculture to humans may occur through direct consumption of antimicrobial-resistant bacteria present in fish and associated products (Petersen *et al.,* 2002). The role of aquaculture in the transmission of *Salmonella* to humans in Western Kenya has not been studied. Resistance of these strains to ampicillin also needs to be studied since the studies in clinical samples at Maseno and Mukumu Hospital indicated a very high prevalence of bla_{TEM} gene, which confers resistance to penicillins and cephalosporins including ampicillin.

Epidemiological and molecular studies suggest that antimicrobial use in animal agriculture and antimicrobial resistant bacteria from animals can lead to antimicrobial resistant *Salmonella, Campylobacter,* and *Enterococcus* infections in humans (Aarestrup *et al.,* 2001; Hein *et al.,* 2003). Nucleic acid-based detection systems offer rapid and sensitive methods to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms (Fluit *et al.,* 2001). These are used for the detection of properties of microorganisms, such as virulence factors and antimicrobial resistance (Fluit *et al.,* 2001).

1.2 Problem statement

Many people in Western Kenya depend on fish for food because it is easily available and affordable. It is apparent that the prevalence of MDR *Salmonella* is on the rise and in turn there is an increase in morbidity and mortality, which according to Tassios *et al.,* (1997) is estimated to be 1.3 billion cases and 3 million deaths world wide annually. A study conducted by Onyango, (2003) revealed that in Western Kenya the prevalence of *S. typhimurium* was 23.3% and 60% among immunocompetent and immunocompromised children respectively. Recently, MDR serovar Typhi showing resistance to nearly all of the commonly available first line antimicrobial agents used for the treatment of these and other infections have been isolated in Kenya (Kariuki *et al.,* 2000). According to Kariuki *et al.,* (2004), the prevalence of MDR phenotype is increasing rapidly, it is speculated that MDR serovar Typhi strains have been spreading to other parts of Kenya and are gradually replacing the fully sensitive strain type, probably due to their survival advantage over sensitive strains.

Fish and shellfish are said to be passive carriers of *Salmonella* that demonstrates no clinical disease, and can excrete *Salmonella* spp without apparent symptoms (Fell *et al.*, 2000). It is documented that human contact with *Salmonella* infected fish could serve as a potential source of the pathogen to humans (Newaj-Fyuzul *et al.*, 2006) posing a serious health hazard to fish farmers and hobbyists (Janssen and Meyers, 1968). The occurrence of *Salmonella* in fish and fish farms in community around Maseno University has not been studied. Also, a link needs to be established between human infection and the presence of *Salmonella* in fish.

Onyango *et al.*, (2007) documented the occurrence of clinical Salmonellosis in Maseno and Mukumu Hospitals in Western Kenya. MDR *Salmonella enterica* serovar Typhimurium was isolated from those samples. However, data on the environmental sources of *Salmonella* is not available. Fish is thought to be one of the many reservoirs of *Salmonella* that infects humans living in around Maseno University. It was therefore important that a survey be conducted in community fish farms around Maseno University to establish the distribution of *Salmonella*, their antimicrobial resistance patterns.

1.3 Justification of the Study

Farmers in many areas across the country are turning to fish farming as a way of producing high quality food, either for family consumption or commercial purposes (Ngugi *et al.*, 2007). The need to produce more fish as a source of animal protein is even more pressing in Western Kenya where 40 per cent of the country's total population live, although the region consists of only 8 per cent of the total area of the country. Since the catches from major lakes are declining, increases in production through fish culture offer a promising alternative.

An earlier study by Onyango *et al.*, (2008), showed that Nile tilapia from Lake Victoria were contaminated by *Enterobacteriaceae*, including *Salmonella*. As stated earlier, fish can harbor *Salmonella* for a long period without any symptoms, and contact with infected fish can lead to human infection. It is however acknowledged that the sources of salmonellae are poorly understood, and this study provides vital data that is

critical in assessing and controlling the risk associated with the presence of salmonellae in the acquatic environment.

In many sub-Saharan African countries community acquired bacteremia is a major cause of high morbidity and death among children especially from poor settings (Kariuki *et al.,* 2006). According to a study conducted by Berkley *et al.*, (2005), the majority (68%) of the children admitted to hospital with severe bacteremia were below 3 years of age, and nearly half of these were in the below 1 year of age (Berkley *et al.*, 2005). Kariuki *et al.,* (2004) reported that *S. typhimurium* was the highest recorded enteric isolate from stool and cerebral spinal fluid obtained from patients at an out break of *S. typhimurium* in Kenyatta National Hospital in Kenya. Since the first report of multidrug resistant (MDR) serovar Typhi outbreaks, which occurred in Kenya between 1997 and 1999 in which the prevalence of MDR phenotype was 50 to 65% (Kariuki *et al.,* 2000), continuous surveillance has to date shown that the prevalence of MDR serovar Typhi has been rising steadily and that, at present, 70 to 78% of all serovar Typhi isolates from blood cultures from the main referral hospital in Nairobi are MDR (Kariuki *et al.,* 2004).

According to Onyango *et al.,* (2007), about 80% of human population in Western Kenya is unemployed and thus depends heavily on agriculture for livelihood. Small scale business is also highly observed within the study population. Food is acquired either from the farms, open water bodies, or market place. However, poor sanitary conditions exist within the population accompanied by few or no health facilities available. Thus most of the infections are combated by use of over- the counter administration of drug. These together with the HIV/AIDs co-infection, has led to the increase of antimicrobial drug resistance within the population due to inappropriate use of antimicrobials, (Onyango *et al.*, 2007).

Whereas many stakeholders, such as Lake Basin Development Authority (LVDA); Kenya Marine and Fisheries Research Institute (KMFRI); and the Fisheries Department (FD), are working hard to develop the fish culture industry, very little record is available on the incidences of fish diseases, pathogens and particularly those that can

be passed on to man. A study by Onyango *et al.*, (2007) established a high rate of MDR *Salmonella enterica* serovar Typhimurium at Maseno and Mukumu Hospitals. Maseno Hospital lies on the borderline between the two constituencies. Ampicilin resistance gene (bla_{TEM}) was amplified in 90% of the isolates. The environmental sources of the *Salmonella* had not been studied in this area. This study compared the serotypes and antimicrobial resistance profiles of *Salmonella* isolates obtained from fish to those documented to affect humans at Maseno Hospital.

1.4 Significance of the Study

This study sought to determine the role of aquaculture in the dissemination of *Salmonella* to humans living in communities around Maseno University. 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 this pathogens to man. Whereas clinical strains of *Salmonella* have been studied at Maseno hospital, this study explains one of the possible environmental reservoir and the data obtained can be shared with stakeholders in the fish farming industry in Western Kenya, so that they can advise fish farmers and consumers on ways to break the cycle of infection from man to fish and back to human.

1.5 Main Objective

To determine the occurrence of *Salmonella* and analyze their antimicrobial profile in fish and water collected from selected fish farms in the community living around Maseno University in Maseno Division, Western Kenya.

1.5.1 Specific Objectives

1. To isolate *Salmonella* spp from selected fish ponds and fish (*Oreochromis niloticus*).

2. To determine the antimicrobial susceptibility of each of the isolated *Salmonella* spp.

3. To study the distribution of ampicillin resistance genes in the *Salmonella* isolates.

1.6 Hypotheses

2. The incidence of *Salmonella* spp is not high in fish and fish ponds in the community around Maseno University in Western Kenya

3. Antimicrobial resistance is not high among *Salmonella* isolated from fish and fish ponds

4. Ampicillin resistance in *Salmonella* spp conferred by the beta-lactamase (bla_{TEM}) gene is not widely distributed in fish farms

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Salmonella:* species and subspecies

The genus *Salmonella* is a member of the family *Enterobacteriaceae,* composed of bacteria related to each other both phenotypically and genotypically. *Salmonella* DNA base composition is 50-52 mol% Guanine + Cytosine (Todar, 2005), similar to that of *Escherichia, Shigella,* and *Citrobacter.* Different bacteria within this genus are also related to each other by DNA sequence (Fluit *et al.,* 2001).

Salmonella species are Gram-negative, flagellated facultatively anaerobic bacilli characterized by O, H, and Vi antigens (Baron, 1996). They are divided taxonomically into two species, *Salmonella enterica* and *Salmonella bongori* (V). *Salmonella enterica* comprises 6 subspecies: *S. enterica* subspecies *enterica* (I), *S. enterica* subspecies *salamae* (II), *S. enterica* subspecies *arizonae* (IIIa), *S. enterica* subspecies *diarizonae* (IIIb), *S. enterica* subspecies *houtenae* (IV), and *S. enterica* subspecies *indica* (VI) (Popoff, 2003). *Salmonella* is currently classified into more than 2,500 serovars using the Kauffmann-White scheme (Popoff, 2003). *Salmonella enterica* subspecies I consists of almost 1,500 serovars (Popoff, 2001), The different host ranges, diseases, and virulence potentials demonstrated by the various serovars belonging to *S. enterica* subspecies I (Baumler, 1998; Edwards, 2002) are thought to be caused by genetic variation (Kim, 2006).

2.1.1 Morbidity and Mortality of Salmonellosis

Diseases caused by *S. enterica* serovars are especially prevalent in developing countries, such as Southeast Asia, Africa, and South America (See figure 1 below) (Boyle *et al.,* 2007) Typhoidal *Salmonella* serovars, such as *Salmonella enterica* serovars Typhi and Paratyphi, cause systemic illness that leads to an estimated 20 million cases and 200,000 deaths worldwide each year (Crump *et al.,* 2004). The worldwide incidence of nontyphoidal salmonellosis is estimated at 1.3 billion cases and 3 million deaths annually (Tassios *et al.,* 1997), 95% of which are thought to be foodborne (Mead *et al.,* 1999). In

Kenya, invasive nontyphoid salmonellosis infections in children less than 5 years of age are an important cause of morbidity and high mortality; having an incidence of 505 out of every 100,000 and 28% mortality despite good healthcare. They are ranked second only to pneumococcal pneumonia in importance as the leading bacterial cause of child mortality (Mwangi *et al.*, 2002; Berkeley *et al.*, 2005).

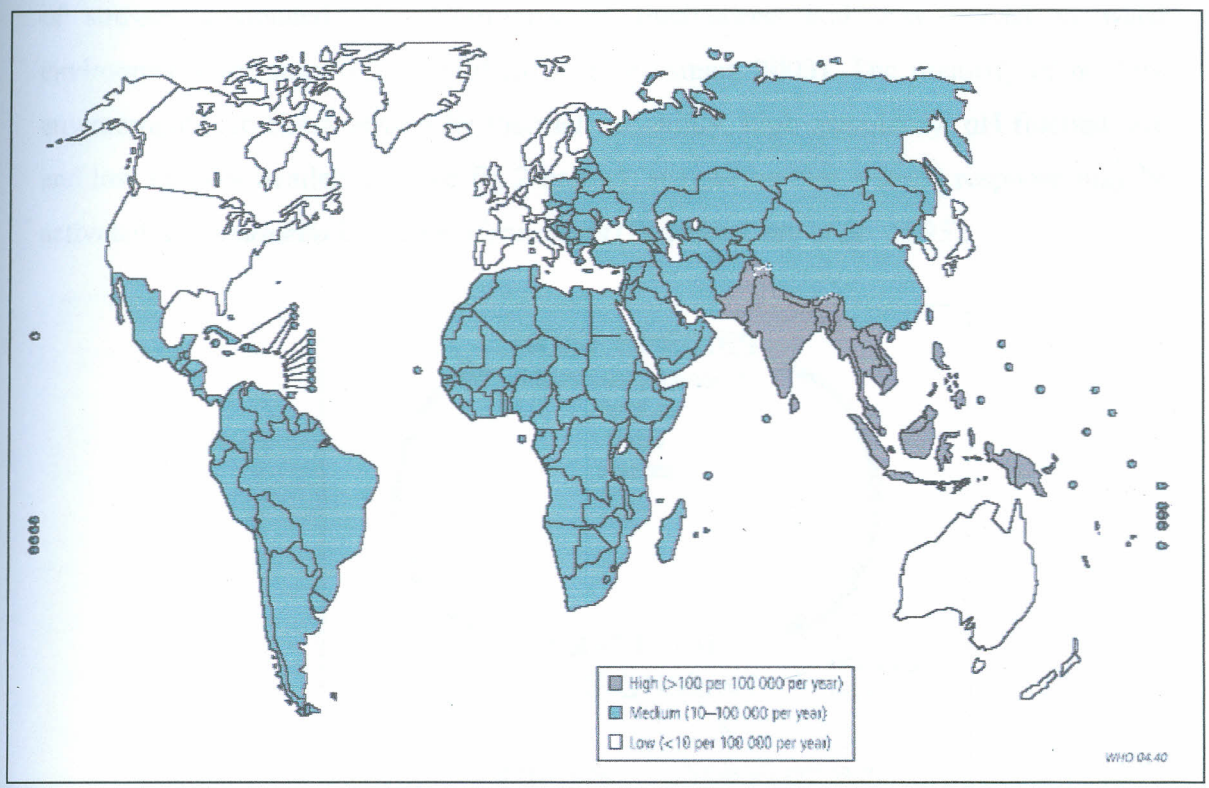

Figure 1: Global distribution of typhoid: Areas marked gray have the highest incidence (>100 cases per 100,000 people) per year; areas marked green have medium incidence (10-100 cases per 100,000 people) per year; and white areas have the lowest incidence (<10 cases per 100,000 people) per year (Crump *et al.*, 2004).

Antimicrobial resistant *Salmonella* can cause even greater morbidity than their susceptible counterparts due to treatment failure, increased infection severity, and increased rates of disease in people taking antimicrobials for other reasons (Varma *et al.*, 2005a; Varma *et al.*, 2005b). This has negatively affected the population by increasing morbidity and mortality rates, reducing desired treatment outcomes, and increasing the need for hospital admission as well as increasing the cost of hospital care (Onyango *et al.*, 2008).

2.1.2 *Salmonella* in external environment

Salmonella is frequently isolated from water sources, which serve as bacterial reservoirs and may aid transmission between hosts (Foltz, 1969; Cherry *et al.,* 1972). *Salmonella* is also constantly released into the environment from infected humans, farm animals, pets, and wildlife (Baudart *et al.,* 2000). The bacteria withstands a wider variety of stresses associated with environmental fluctuations and may persist in water environments for some time (Winfield and Groisman, 2003). The majority of nonhost environments are characterized by thermal variability, high osmolarity, pH fluctuations, and low nutrient availability (see fig 2 below), suggesting that a stress response may be activated in *Salmonella* in such environments (Fedorka-Cray *et al.,* 1995).

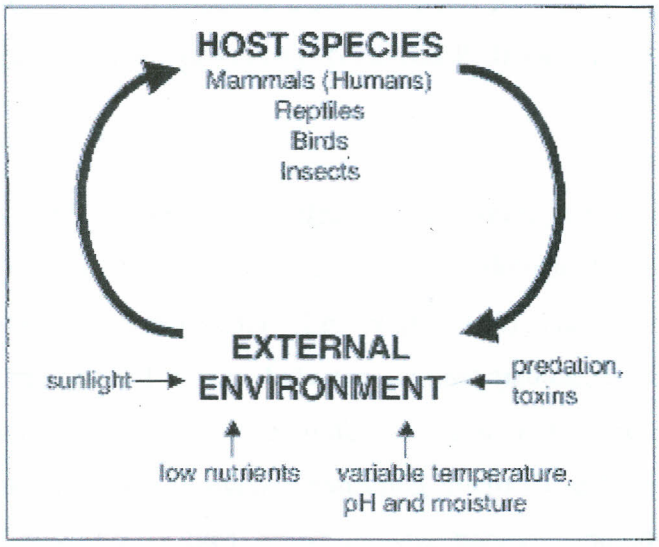

Figure 2: Lifestyle of *Salmonella. Salmonella* cycles actively between host (mammals, including humans, reptiles, birds and insects) and external environment where it withstands hostile conditions such as predation and toxins, variable temperature, pH and moisture, low nutrients and sunlight to infect new host (Figure courtesy of Fedorka-Cray *et al.,* 1995).

The ubiquitous nature of *Salmonella* may facilitate a cyclic lifestyle consisting of passage through a host into the environment and back into a new host (Thomason *et al.,* 1977). Compared to other bacteria, *Salmonella* has high survival rates in aquatic environments (Chao *et al.,* 1987). For example, a study of an antibiotic-resistant strain of *Salmonella enterica* serovar Typhimurium DT-104 indicated that it can survive for

several months in aquatic environments, with enhanced survival in sediments relative to overlying water (Moore *et al.*, 2003). Winfield and Groisman (2003) suggested that the long-term survival of *Salmonella* in the external environment which includes soil, water, and a variety of surfaces ensures its successful passage to the next host.

Numerous studies have investigated the survival of enteric bacteria in aquatic ecosystems (Burton *et al.*, 1987). *Salmonella* species are usually found in higher concentrations in sediments than in overlying water (Morigo *et al.*, 1986). Soil and sediment particles are believed to function as microecological niches in which bacterial species can survive and perhaps replicate (Brettar and Hofle, 1992). Association with soil particles can provide the bacteria with both high concentrations of nutrients, due to the release of organic molecules from attached algal cells, and protection against predation, by providing shelter against grazing protozoans (Fish and Pettibone, 1995).

2.1.3 *Salmonella* infection in man

Colonization is the first step of any infection. For enteropathogenic bacteria, this poses a formidable task as the target host organ is already colonized by a dense microbial community, the microflora, or "microbiota" (Ley *et al.*, 2006). The intestinal epithelium constitutes the major mechanical barrier between luminal pathogens and the mucosal immune system. An intact epithelial barrier function is thus critical to protect the host organism from invading luminal pathogens, restricting microbial entry and propagation (Lalmanach and Lantier, 1999). In turn, enteroinvasive bacteria, including *Salmonella*, are capable of traversing this barrier by infecting epithelial cells and ultimately translocating to the mucosal and submucosal layers. Eventually, enteroinvasive pathogens will enter the intestinal microcirculation, resulting in systemic infection and causing septic illness (Lalmanach and Lantier, 1999). The innate immune system plays an essential role in the early responses to *Salmonella* infections and may be enough to control progression to disease (Lalmanach and Lantier, 1999).

Following ingestion, the pathogen is able to colonize the intestinal tract and penetrate the intestinal epithelium to ultimately gain access to systemic sites, such as the liver and spleen, through lymphatic and blood circulation. Bile affects the expression of

serovar *Typhimurium* genes that are important for virulence, such as *Salmonella* plasmid virulence factor (*spv*) and invasive gene (*inv*), and this has been proposed to enhance colonization and persistence within the gallbladder (Prouty and Gunn, 2003). *S. typhimurium* invasion genes (*inv*) are necessary for bacterial invasion of intestinal epithelial cells and are thought to allow *Salmonella* to enter and cross the intestinal epithelium during infection by manipulation of the host cytoskeleton (Gruenheid and Finlay, 2003). The invasion gene operon, *invA*, is reported by Lance *et al.,* (1999) to be essential in *Salmonella* for full virulence where it is thought to trigger internalization required for invasion of deeper tissues (Lance *et al.,* 1999). The initial events following Salmonella infection include adherence to and invasion of the enterocytes (Gianella *et al.,* 1973) and the M cells of the gut-associated lymphoid tissue (GALT) (Cater and Collins, 1974; Jones *et al.,* 1994).

In vitro, *Salmonella* replicates to high numbers within epithelial cells and macrophages, yet *in vivo*, infected cells usually contain only one or two bacteria (Mastroeni 2004; Brown *et al.,* 2006). Martinez-Moya *et al.,* (1998), noticed that *Salmonella* do not replicate or cause cytotoxicity in fibroblasts but simply remain in a persistent state. Boyle *et al.,* (2007) provided evidence that persistence in fibroblasts occurs *in vivo*. This may be key to understanding why chronic *Salmonella* infections are not cleared by the immune system, since the host has no means of detecting bacteria hiding within these cells.

2.1.4 Clinical manifestation of typhoidal and non-typhoidal salmonellosis

Typhoidal *Salmonella* serovars, such as *Salmonella enterica* serovars Typhi and Paratyphi, cause systemic illness that leads to an estimated 20 million cases and 200,000 deaths worldwide each year (Crump *et al.,* 2004). In Southeast Asian countries, such as Vietnam, the prevalence of typhoidal salmonellosis is high, and patients often suffer from recurrent or relapsed infections (Crump *et al.,* 2004; Parry, 2004). Why the immune system is unable to mount a lasting protective response against typhoidal *Salmonella* species remains unknown.

Nontyphoidal salmonellosis is caused by *Salmonella enterica* serovars Typhimurium, Enteritidis, Newport, and Heidelberg and typically presents as self-limiting gastroenteritis, although in immunocompromised individuals, serious complications can ensue (Hohmann, 2001; Rabsch, 2001).

2.2 Fish Farming in Western Kenya

The Fisheries Department in Kenya was started in 1954 with a program of dam and pond stocking in Western Kenya (*December 3, 2008;* www.fao.org/docrep /008/v4050b/ v4050B00.HTM). Zonneveld (1983) implied that some estimates for the Western and Nyanza Provinces alone indicated over 30,000 ponds. However, he termed these statistics as confused as no accurate census had been undertaken. Available estimates ranged from 18,000 to 32,000. Most of these ponds were said to be in Nyanza and Western Provinces. In 1983, a World Bank funded research study found out that there were only 4,842 fish ponds in the whole of Western Kenya. Since then, LBDA has continually monitored newly constructed and abandoned fish ponds and currently the number of functional fish ponds in the LBDA region is 6,738 (*December 3, 2008;* www.fao.org/docrep/008/v4050b/ v4050B00.HTM).

The average pond size in western Kenya was found to correlate with the size of the farm. Overall water availability does not appear to be a major constraint. Over 90 percent of the ponds are perennial seasonal ponds and represent about 2 percent while the remaining occasionally dries up during severe drought. The initial stocking of ponds has been done mainly on the basis of supplies from other farms 50%, 30% from government fish farms or demonstration ponds, 10% from the wild (lakes and reservoirs) and the remaining from farmers' own ponds. Tilapia monoculture represents over 75% of fish species culture with polyculture of tilapia and catfish currently standing at 15%, trout takes about 5% and the remaining 5% consist of catfish culture and other species. Mixed farming of two indigenous species, the Nile tilapia and catfish, is practised in Western Kenya since many years but the average production rate has remained low at about 50 kilograms per hectare per year (Ngugi and Manyala, 2007).

The market demand and existing marketing channels for tilapia have been studied in the LBDA region, Western Kenya. Tilapias are among the highest valued fish in Kenya (Zonneveld, 1985) and fetch the highest price of all species on the market. In areas around the lake, fresh fish is preferred, but in distant areas where fresh fish is scarce due to the distribution and preservation problems, processed fish (sun-dried, smoked and fried) is readily acceptable. (*December 3, 2008;* www.fao.org/docrep/008/v4050b /v4050B00.HTM).

2.2.1 *Salmonella* in Fish and water

Main factors influencing the risk associated with the presence bacterial pathogens in aquaculture include the farm location, the species being farmed, husbandry practices and systems (including feeding), post-harvest processing, and eating habits in food preparation and consumption (*December 16, 2009;* www.efsa.europa.eu/efsa_locale-1178620753812_ 1211902132 140.htm).

Occurrence of pathogens such as *Salmonella* in water and fish is generally due to external contamination sources, such as farms located in polluted areas, use of excreta as fertilizers and faecal effluents from human sewage, farms or wild animals (*December 16, 2009;* www.efsa.europa.eu/efsa_locale-1178620753812_1211902132 140.htm). *Salmonella* has been isolated in tropical aquaculture systems where fecal wastes were not used as fertilizers (Dalsgaard, 1998; WHO, 1999). But when contamination occurs some *Salmonella* serotypes can persist in the marine environment for months, even years (Gaulin *et al.*, 2002). Apparently *Salmonella* is unable to multiply within the aquatic environment in temperate waters and fish intestine is not its normal habitat (Dalsgaard, 1998). However, experimental evidence has shown that fish infected with *Salmonella* maintained the infection for up to 39 weeks (Brunner, 1974).

Salmonella spp has previously been isolated in Nile tilapia by (Onyango *et al.*, 2008) in the Winam Gulf of Lake Victoria and by Sifuna *et al.*, (2008) in fish (*Rastrineobola argentea*) sold in six markets in Kisumu city. 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).

2.2.2 Genetic identification of *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar typhimurium is an invasive bacterial pathogen that can cause severe, fatal invasive disease in young infants, the elderly and immunocompromised hosts (Tennat *et al.*, 2010).

Malate dehydrogenase gene (mdh) 261bp is a housekeeping gene for *Salmonella enterica* serovar *typhimurium* whose product, malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate in the tricarboxylic acid cycle (Entrez gene, 2010). Berg and Martelius (1995) identified this gene by polymerase chain reaction in all *Salmonella enterica* serovar Typhimiurium isolates and established that it was missing in other *Salmonella* serovars. This 261bp product is exclusively an *S. typhimurium* gene and can be used to identify this strain.

2.3 Acquisition of antimicrobial resistance

When dividing bacteria are exposed to bactericidal concentration of antibiotics, the density of viable cells does not decline exponentially. During exposure to antibiotics, the rate of mortality of bacteria decreases, and a substantial fraction of bacteria may survive and even start to grow again (Dalacher *et al.*, 2000). These organisms survive because they have acquired some resistance to the antibiotic and will give rise to a clone of cells that are resistant to that antibiotic.

Antimicrobial resistance in bacteria can be caused by several mechanisms: (i) the presence of an enzyme in the bacteria that inactivates the antimicrobial agent; (ii) the presence of an alternative enzyme that could be used in place of the enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent; (iv) posttranscriptional or post-translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of the antimicrobial agent; and (vii) overproduction of the target of the antimicrobial

agent. In addition, resistance may be caused by other previously unrecognized mechanism (Fluit *et al.,* 2001). The coexistence of several of these mentioned mechanisms in the same host can lead to multidrug resistance (MDR) (Depardieu *et al.,* 2007).

Among the members of the family *Enterobacteriaceae*, transfer of antibiotic resistance genes is due largely to broad-host-range plasmids that carry transposons (Davis, 1997). Many of the resistance genes in the transposons have been found to be mobile gene cassettes carried as part of integrons (Fluit and Schmitz, 1999). Four classes of integrons have been described based on the similarities between their integrases, but the majority described belong to class 1. This type has been found in multiple *Enterobacteriaceae*, including *Escherichia coli*, *Citrobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., and *Salmonella* spp. (Davis, 1997; Guerra *et al.,* 2000; Nastasi and Mammina, 2001).

2.3.1 *Salmonella* Antimicrobial resistance

For more than 40 years since its discovery, chloramphenicol was the drug of choice for the treatment of typhoid (Shanahan *et al.,* 1998; Mirza *et al.,* 2000; Rahman *et al.,* 2002). However, the emergence in the late 1980s of multidrug-resistant (MDR) serovar Typhi (Rahman *et al.,* 2002) isolates resistant to ampicillin, chloramphenicol, and cotrimoxazole in outbreaks reported in the Indian subcontinent (Arabian Gulf, the Philippines (Rowe *et al.,* 1997), and South Africa (Coovadia *et al.,* 1992) has led to the use of the fluoroquinolones as alternative drugs (Chinh *et al.,* 2000). Among the first reports of clinical treatment failure due to serovar Typhi resistant to nalidixic acid and showing then an increased ciprofloxacin MIC (0.125 µg/ml) was in 1991 in a patient who had recently returned to the United Kingdom from India (Wain *et al.,* 1997).

Molecular characterization of the serovar Typhi outbreak strains revealed that resistance to commonly used antimicrobial agents, including chloramphenicol, ampicillin, and trimethoprim, was encoded by plasmids of the HI incompatibility group (Rowe *et al.,* 1997; Mizra *et al.,* 2000). In most strains of serovar Typhi, resistance to the quinolones has been attributed to point mutations in the genes encoding DNA gyrase

(*gyrA* and *gyrB*) or DNA topoimerase IV (*parC* and *parE*) enzymes, which are located within the quinolone resistance-determining region (QRDR) of the chromosomes of bacteria (Giraud *et al.,* 1999; Hirose *et al.,* 2002; Phung le, 2002; Rahman *et al.,* 2002).

2.3.2 *Salmonella* genomic island 1

The MDR profile is conferred by an MDR gene cluster included in a chromosomal genomic island called *Salmonella* genomic island 1 (SGI1) (Boyd *et al.,* 2000; Boyd *et al.,* 2001). The SGI1, was cloned by Boyd *et al.,* (2000) from the genome of a Canadian isolate and comprises a 43-kb region between *thdF* and a novel retron sequence. A year later, the entire SGI1 element was sequenced by Boyd *et al.,* (2001) and, in addition to the MDR region, was found to contain at least 25 or more open reading frames (ORFs), including an integrase gene and an excisionase gene, some of which showed similarity to genes commonly found on conjugative plasmids. In *S. enterica* serovar Typhimurium DT104, the SGI1 is located between the *thdF* and *int2* genes of the chromosome. (Boyd *et al.,* 2000; Boyd *et al.,* 2001). In other *S. enterica* serovars SGI1 is located between *thdF* and the *yidY* gene (Boyd *et al.,* 2002; Boyd *et al.,* 2001; Doublet *et al.,* 2003). All of the antibiotic resistance genes are located near the 3′ end of SGI1 and are part of a complex class 1 integron that belongs to the In4 group (Boyd *et al.,* 2002).

The MDR gene cluster of SGI1 is bound by inverted repeats (IR) namely, IRi at the intergrase end and IRt at the transposition module (*tni* region) and thus can be considered a complex In4-type integron (Boyd *et al.,* 2002). Further, the MDR region is surrounded by 5-bp direct repeats, strongly suggesting it was integrated by a transposition event (Boyd *et al.,* 2002; Patridge *et al.,* 2001a; Patridge *et al.,* 2001b). There is a duplication of the 5′-CS in SGI1, each one followed by a gene cassette. The first cassette carries the *aadA2* gene, which confers resistance to streptomycin and spectinomycin, and a 3′-CS with a truncated *sul1* (*sul1*Δ) gene. The second cassette contains the β-lactamase gene *pse-1*, which confers resistance to ampicillin and a 3′-CS with a complete *sul1* gene conferring resistance to sulfonamides. Flanked by the two cassettes are the *floR* gene, which confers cross-resistance to chloramphenicol and florfenicol, and the tetracycline resistance genes *tetR* and *tet*(G) (Arcangioli *et al.,* 1999; Bolton *et al.,* 1999).

2.3.3 Integron Mediated Antimicrobial Resistance in *Salmonella*

Integrons contain 1 or more resistance genes present as mobile gene cassettes and inserted into various arrangements between 2 conserved DNA regions, creating arrays of different antimicrobial resistance genes (Levesque, 1995). Over 60 gene cassettes and 4 distinct classes of integrons have been identified to date (Levesque, 1995; Hall, 1997). Cassette-associated genes conferring resistance to beta-lactams, aminoglycosides, trimethoprim, chloramphenicol, and quaternary ammonium compounds used as antiseptics and disinfectants have been found. Also, class I integrons include a sulfonamide resistance gene (*sul1*) in the backbone structure (Hall, 1997).

Class 1 integrons contain a 5′ conserved segment (5′-CS) which consists of the *intI1* gene, encoding the site-specific integrase, and the associated *attI1* site, the primary site of recombination, and the 3′ conserved segment (3′-CS) of variable length but generally consisting of *qacEΔ1*, encoding low-level resistance to some antiseptics; the *sul1* gene, encoding sulfonamide resistance; and *orf5*, a gene of unknown function (Fluit and Schmitz, 1999). The gene cassettes, of which over 60 have been described (Fluit and Schmitz, 1999; Peters *et al.,* 2001), consist of the coding region and the downstream 59-base element (59-be), which is responsible for recognition and mobilization of cassettes. The IntI1-catalyzed recombination between the *attI1* and 59-be sites is the main reaction responsible for inserting gene cassettes into the integron (Patridge *et al.,* 2000).

2.3.4 Selected techniques used to detect antimicrobial resistance in *Salmonella*

2.3.4.1 Antimicrobial disk diffusion method

This is a qualitative test, which uses paper discs impregnated with the antimicrobial of choice at varied concentrations appropriate for either elimination or limitation of the activity of bacteria *in vitro* or *in vivo.* The discs are placed on agar plate and the drug diffuses into the agar thus extending the bactericidal or bacteristatic effect. In this method, activity of the drug against the bacteria correlates with the zone of growth inhibition around the disc. The method is used routinely for testing common, rapidly growing, and certain fastidious bacterial pathogens. Performance depends on reagents

(Mueller Hinton agar and Diagnostic sensitivity test agar are media specially designed for this purpose) preparation, pH, moisture, and effects of Thymidine/Thymine, storage of discs and inoculation of preparation (Lorian, 1996). The size of the zone of inhibition is inversely proportional to the MIC of the organism. This method is an indirect measure of the susceptibility based on MIC zone size correlation. The activity of the drug against the bacteria correlates with the zone of bacterial inhibition around the disk (Lorian, 1996; Mendoza, 1998). Based on the zones of inhibition, a qualitative report of "susceptible," "intermediate" or "resistant" can be determined for rapidly growing non-fastidious aerobic bacteria (Lorian, 1996; Mendoza, 1998).

2.3.4.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was first described by Mullis and Faloona, (1987), and the first diagnostic application of PCR was published by Saiki *et al.*, (1988a). The technique became broadly used after the introduction of a thermostable DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase) (Saiki *et al.,* 1988b) and the development of automated oligonucleotide synthesis and thermocyclers. PCR involves cycles of heating the sample for denaturing, annealing of the primers, and elongation of the primers by a thermostable DNA polymerase. In theory, each round of amplification gives a doubling of the number of DNA target molecules, but the process is seldom 100% efficient because of the presence of inhibitors, and in later rounds of amplification DNA polymerase may become limited.

2.4 *Salmonella* resistance to β-lactams and cephalosporins (Penicillins and Ampicillin)

The β-lactamases are among the best-studied antimicrobial resistance enzymes because they confer resistance to β-lactam antibiotics (Livermore, 1996). The occurrence of bla_{TEM-1} was first reported in isolates of *Escherichia coli* and *Salmonella enterica* serovar Paratyphi in 1965 shortly after ampicillin was introduced into clinical use (Datta and Kontomichalou, 1965). In early 1980s, it was the most prevalent resistance gene in clinical microbial populations throughout the world (Madeiros, 1997). The TEM-1 β-lactamase primarily confers resistance to penicillins, including ampicillin. However, in

the 1980s, novel TEM β-lactamases emerged that were capable of hydrolyzing both penicillins and extended-spectrum cephalosporins. This was caused by point mutations due to extended usage of extended spectrum cephalosporins. The first extended-spectrum β-lactamase (ESBL) *bla*$_{TEM}$ allele was isolated in 1983 (Sirot *et al.*, 1987), and todate, about 160 variants of *bla*$_{TEM-1}$ that differ in amino acid sequence have been identified.

While ESBL *bla*$_{TEM}$ alleles have the ability to confer resistance to extended-spectrum cephalosporins, they have also retained their earlier ability to confer resistance to penicillins (Queenan *et al.*, 2004). The ability of some *bla*$_{TEM}$ alleles to confer resistance to both cephalosporins and penicillins suggests that the frequency of those alleles should increase because they confer novel advantageous phenotypes. ESBL *bla*$_{TEM}$ may co-occur with *bla*$_{TEM-1}$ or replace it as the most frequently encountered allele in environments where cephalosporins are heavily used. However, *bla*$_{TEM-1}$ is still the most commonly occurring allele in many microbial populations where cephalosporin resistance has been selected for (Queenan *et al.*, 2004, Kruger *et al.*, 2004). The fact that *bla*$_{TEM-1}$ is the most common gene in numerous microbial populations indicates that there may be a selective advantage for bacteria that express *bla*$_{TEM-1}$ rather than other *bla*$_{TEM}$ alleles (Mroczkowska and Barlow, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in the community living around Maseno University. Maseno is situated in Nyanza province, Western Kenya, its geographical coordinates are 0° 0' 0" South, 34° 36' 0" East (*October 16, 2008;* www.maplandia.com/kenya/ western/maseno). It has an average altitude of 4,934 feet above sea level (*October 16, 2008*; http://www.fallingrain.com/world/KE/7/Maseno.html). Maseno town is the headquarters of Maseno Division, one of four administrative divisions of Kisumu District. According to the 1999 census, Maseno division has a population of 65,304, of whom 2,199 are classified as urban (*October 16, 2008;* www.answers.com/topic/maseno-1). The township lies next to administrative boundary of Western and Nyanza Provinces.

The climate condition of this area is equatorial sub-humid savannah type which is influenced by continental rainfall regime of Lake Victoria basin. Rainfall is bimodal, long rains experienced during March to July. Short rains experienced during August to October. Mean annual precipitation 1250mm. Temperatures are highest during dry season with peak in February. Annual mean daily minimum and maximum temperature ranges as follows respectively 10-40 C and 26-36 C (*February 11, 2011*; http://www. chulaimbohospital.or.ke /index.php?option).

Approximately 45% of the population in the division is poor. This is due to underutilization of the available arable land despite the fact that the area has two rainfall seasons. Maseno division is second to Winam in terms of high population with a density of 387 persons per sq. km (*February 11, 2011;* http://www.chulaimbohospital. or.ke/index.php?option)

The fish farms used for this study are located within community around Maseno University. Some are in Nyanza province while others in Western province. Most of these fish farms are located near homesteads and are owned and managed by individual families. Some are owned and managed by community self help groups.

Most of the ponds in the study area are small to medium in size where for the purpose of this research, a pond is said to be small if it has less than 5,000 fish. A medium pond has between 5,000 and 10,000 fish while a large pond has more than 10,000 fish. These ponds are earthen and most receive water from the underground or diverted streams. Fish species commonly stocked are the Nile tilapia though a few farms stock the African catfish in addition to Nile tilapia.

3.2 Sampling technique, and sample collection

Eleven fish farms were selected from the villages surrounding Maseno University. Since there are not many fish farms in these villages, knowledge of the farm location and consent of the fish farm owner were the two main selection criteria for inclusion to this study.

From the selected fish farms, three fish ponds were randomly selected from each farm if there were more than three fish ponds. If there were three or fewer ponds on the farm, all the ponds were used for the study. A questionnaire (appendix 1) was administered to the fish farmer to asses the conditions of the fish farm.

Five Nile tilapia (*Oreochromis niloticus*) were collected randomly from the selected ponds in each farm using a fishing net and were packed individually in sterile plastic bags and transported in a cool box to the laboratory at Maseno University for analysis within two hours of capture. Three UV sterilized bottles were used to collect water from different points of each of the selected ponds in the farm. Water samples were analyzed within six hours of collection.

3.3. Measurement of pond physico-chemical parameters

Physico-chemical parameters of sampled pond water were measured, such that the dissolved oxygen level was measured using a dissolved oxygen meter (Hanna inc. Germany), whereas salinity and temperature were measured using a salinity meter (Hanna inc. Germany), and the pH was measured using a portable pH meter (Hanna inc. Germany).

3.4 Isolation of *Salmonella* species

In the laboratory at Maseno University, each fish surface was disinfected by dipping in 70% ethyl alcohol for 2 minutes then rinsed 3 times with sterile distilled water following the method of Newaj-Fyuzul *et al.,* (2006). The flesh and the intestines were separately obtained from the fish using a sterile surgical blade and macerated using a sterile mortar and pestle, and then mixed with sterile PBS to a concentration of 10% w/v. Five millimeters of the slurry was used to inoculate tubes of double-strength selenite fecal (SF) broth (HIMedia Laboratories Pvt. Ltd Mumbai, India) which was incubated for 18-24 hours at 37°C. This was streaked on salmonella shigella agar (SSA) (HIMedia Laboratories Pvt. Ltd Mumbai, India) and incubated at 37°C for 18-24 hours. Colonies suspected to be *Salmonella* and other lactose and non-lactose fermenters were selected and inoculated in tubes of brain heart infusion (BHI) broth (HIMedia Laboratories Pvt. Ltd Mumbai, India), incubated at 37°C for 18 hours then stored at 4°C for further tests.

Water samples collected from the same ponds where fish was collected, centrifuged at 2,400 rpm for 20 minutes and the pellet resuspended in 5ml of the supernatant and used to inoculate tubes of double-strength SF broth. These were incubated for 18-24 hours at 37°C then streaked onto plates of SSA which were incubated at 37°C. The suspected *Salmonella* colonies and other lactose and non-lactose fermenters were stocked in BHI broth (HIMedia Laboratories Pvt. Ltd Mumbai, India) at 4°C for further analysis.

Salmonella confirmatory test was done by streaking the suspected colonies on plates of brilliant green agar (BGA) (Fluka, Sigma-Aldrich Chemie GmbH, Switzerland) and Xylose lysine desoxycholate (XLD) agar (HIMedia Laboratories Pvt. Ltd Mumbai, India).

All the isolates were subjected to methyl red Voges Proskauer test where indole production was checked by inoculating the isolates into tubes of peptone water (HIMedia Laboratories Pvt. Ltd Mumbai, India) then incubated at 37°C for 24 hours. Five drops of Kovac's reagent (HIMedia Laboratories Pvt. Ltd Mumbai, India) were added to each tube. The isolates were inoculated into methyl red Voges Proskauer (MRVP) broth

(HIMedia Laboratories Pvt. Ltd Mumbai, India) and incubated at 37°C for 24 hours. The MRVP broth was divided into two tubes. Five drops of methyl red (MR) indicator were added to the first tube to test for mixed acid metabolic pathway. Five drops of α-naphthol solution were added to the second tube, shaken for one minute then 5 drops of 40% potassium hydroxide solution were added to the same tube to test for the Voges Proskauer (VP) metabolic pathway. For a positive VP test, the media in the tube turned red after one minute. The isolates' citrate utilization was established by inoculating them onto Simmon's citrate (SC) agar (HIMedia Laboratories Pvt. Ltd Mumbai India) and incubated at 37°C for 48 hours then observations made. The carbohydrate fermentation pattern, hydrogen sulphide and gas production was established by inoculation into tubes of Triple sugar iron (TSI) agar (Fluka, Sigma-Aldrich Chemie GmbH, Switzerland) then incubated at 37°C for 24 hours. The isolates were stocked in Tryptic soy broth (TSB) at 4°C for later reference.

3.4.1 *Salmonella* DNA extraction

Salmonella DNA was extracted following the method of Levings *et* al, (2005) by resuspending a single colony in 1 ml of sterile distilled water. The mixture was then heated at 100°C for 5 min to rupture the cells, then centrifuged at 13,000rpms for 10 minutes to pellet cell debris. The supernatant containing DNA was carefully pipetted out into sterile eppendorf tubes and stored frozen at -20°C.

3.4.2 Test for Malic Dehydrogenase gene (*mdh*) for *Salmonella enterica* serovar Typhimurium

The following primer sequence was used in a conventional PCR to identify the malic dehydrogenase gene in the *Salmonella* isolates:

Gene	Oligonucleotide sequence	Product size
mdh	F: 5'- TCG CAA CGG AAG TTG AAG TG 3'	261 BP
	R: 5'- CGC ATT CCA CCA CGC CCT TC -3'	

Table 1: PCR master mix composition for identification of *mdh* gene (Amavitsi *et al.*, 2005)

	x1	x19
H_2O	36µl	684µl
10x PCR buffer	5 µl	95µl
$MgCl_2$	1µl	19µl
Primer 1 *mdh* F	0.5µl	9.5µl
Primer 2 *mdh* R	0.5µl	9.5µl
dNTPs	2µl	38µl
Taq Polymerase	0.25µl	4.75µl

Note: Primer 1F, 2R refers to the resistance gene primer sequences for *mdh* gene

Forty five microliters of PCR master mix + 5 µl chromosomal DNA template were aliquot into 0.2 ml PCR tubes. The mixture was thoroughly vortexed and then centrifuged at 10,000 g for 6 seconds (eppendorf centrifuge 5415D, Germany).

The prepared mixture was then amplified using MJ Gradient Thermocycler (PTC -225, Peltier Thermocycler, BioEnzymes, Germany) PCR with the following conditions: at 94°C for 5 minutes for denaturization, 94°C for 25 seconds, annealing temperature of 54 °C for 45 seconds, at 35 cycles and extension temperature of 72 °C for 45seconds, cooling at 72° C for 7 minutes then 4° C until removed. The amplified amplicons were then loaded on to a casted 1.5% agarose gel (1.5 grams agarose powder + 100ml of 1 × TBE buffer) with a gene marker of 100bp, a negative control and positive control. This was then let to run for 25 minutes at 135V after which the UV pictures were taken using the UV photo transilluminator (Gel Logic 100 Imaging System, Kodak).

3.5 Antimicrobial susceptibility testing

The isolates were cultivated on nutrient agar plates for 16 hours to establish a culture in the logarithmic growth phase then a small colony of each isolate was suspended in sterile normal saline (0.9% sodium chloride) solution to make a turbidity of 0.5 McFarland. This was then applied evenly onto the surface Muller Hinton agar in a petri-plate using a sterile cotton tipped swab to make a uniform lawn. Combi disk 34 2/4

octodiscs (HIMedia Laboratories Pvt. Ltd Mumbai India) were then carefully applied onto the agar surface. The plate was inverted and incubated for 18 hours (Soussy *et al.*, 2000). Susceptibilities of *Salmonella* isolates to ampicillin, tetracycline, cotrimoxazole, chloramphenicol, gentamicin, kanamycin, streptomycin, and sulfamethoxazole was determined by measuring the zones of inhibition using a millimeter ruler.

3.6 Test for ampicillin resistance gene (bla_{TEM})

The following primer was used for identification of ampicillin resistance gene (bla_{TEM})

Gene	Primer	Product size
bla_{TEM}	5′ GCA CGA GTG GGT TAC ATC GA 5′ GGT CCT CCG ATC GTT GTC AG	310bp

Table 2: PCR master mix composition for identification of ampicillin resistance genes (Gebreyes and Altier, 2002)

	×1	×20
H_2O	26.5µl	530µl
10 x Qiagen Buffer	5µl	100µl
Q-Buffer (Qiagen)	10 µl	200µl
Primer 1F	0.5 µl	10µl
Primer 2R	0.5 µl	10µl
dNTPs	2 µl	40µl
Hot start Taq poly	0.5 µl	10µl

Note: Primer 1F, 2R refers to the resistance gene primer sequences for bla_{TEM} gene on study.

Forty five of PCR master mix + 5 µl bacterial DNA were aliquot into 0.2 ml eppendorf tubes.

The prepared mixture was then amplified using MJ Gradient Thermocycler (PTC–225, Peltier Thermocycler BioEnzymes, Germany) PCR with the following conditions: building up of Hot start temperature at 95°C for 15 min, denaturization temperature at

94°C for 1 minute, for 40 cycles, annealing temperature of 55 °C for 45 seconds, (for gentamicin resistance gene the annealing temperature was 53°C for 30 seconds and 35 cycles) 72°C for 30 seconds, extension temperature at 72° C for 7 minutes then 4°C until removed.

The amplified amplicons were then loaded on to a casted 1.5% agarose gel (2.25 grams agarose powder + 150ml of 1 × TBE buffer) stained with ethidium bromide (5 µg/ml), with a DNA ladder of 100bp and a negative control. This was then let to resolve for 45 minutes at 135V after which the UV pictures were taken using the UV photo transmitter (Gel Logic 100 Imaging System, Kodak).

3.7 DATA ANALYSIS

Records of the fish farms, date of collection and all salient information (pH, temperature, salinity and dissolved oxygen) of the fish farms was entered accurately in Microsoft excel worksheet (MS office 2007). All the fish and water samples were inoculated in selenite fecal broth for 24 hours to enrich the enteric pathogens. Samples of bacteria growing in selenite fecal broth was streaked on salmonella shigella agar plates where all samples with black-centered colonies after 24 hour incubation were recorded in a note-book and also entered in the excel worksheet under the farm of collection. These black centered colonies were then streaked on xylose lysine deoxycholate agar plates and incubated for another 24 hours. Samples with alkaline colonies with black centers were noted and recorded in a different column in the note-book. These colonies were further streaked on brilliant green agar where discreetly selected pink *Salmonella* colonies were selected, recorded and stocked for further studies.

The biochemical test done included the indole production test, mixed acid fermentation or methyl red test, Voges proskauer or butane diol pathway, citrate utilization and sugar fermentation tests using the triple sugar iron agar slants. All the data was recorded either as positive or negative for each of the test done in the note book and in the excel work sheet. The results were interpreted using the interpretative chart by Wu (1995) and indicated in the inference column (see appendix 3).

The results for the antimicrobial susceptibility testing done using the Kirby-Bauer disk diffusion method were observed after between 16 and 19 hours by measuring the

diameter of the zones of inhibitions if there were any. The distances measured in millimeters were interpreted using the zone size interpretative chart (HiMedia Laboratories Pvt Ltd). The data for each salmonella sample was recorded in the note book and in an excel work sheet (see appendix 4). The percentages of isolates resistant to a particular antimicrobial drug was computed and presented as a bar graph using Microsoft excel (MS office 2007). The formula used was:

Number of isolates resistant/total number of isolates x 100

The DNA of the isolates was extracted and used for the genotypic identification of *Salmonella enterica* serovar Typhimurium and the identification of bla_{TEM} gene. The electrophoretic gels were viewed over a UV transilluminator and photographed using a digital camera.

All the data was coded and enterered into the statistical package for social sciences (SPSS version 17) and correlated. The student T test was used at 95% confidence interval to determine if there was any significant difference between the fish farms in relation to the presence of Salmonella.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Survey of fish ponds for *Salmonella* spp in western Kenya

4.1.1 *Salmonella* isolation

A total of 120 non-lactose fermenting (NLF) black-centered colonies were isolated on SS agar. From these, twenty-one pink colonies were selected on Brilliant Green agar (BGA) and ultimately 19 alkaline black-centered colonies were isolated on xylose lysine deoxycholate (XLD) agar.

The 19 isolates were confirmed to be *Salmonella* by a series of biochemical tests such that they were negative for indole production when Kovac's reagent was added to 24 hour-cultures in peptone water (figure 3). These isolates were positive for the mixed acid pathway tested by addition of methyl red indicator to organisms cultured in methyl red Voges Proskauer (MRVP) media (figure 4), and negative for acetoin, an intermediate product tested in the Voges Proskauer pathway by addition of α-naphthol and 40% potassium hydroxide to cultures in MRVP media. Seventeen isolates tested positive for citrate utilization on Simmon's citrate agar (Figure 5).

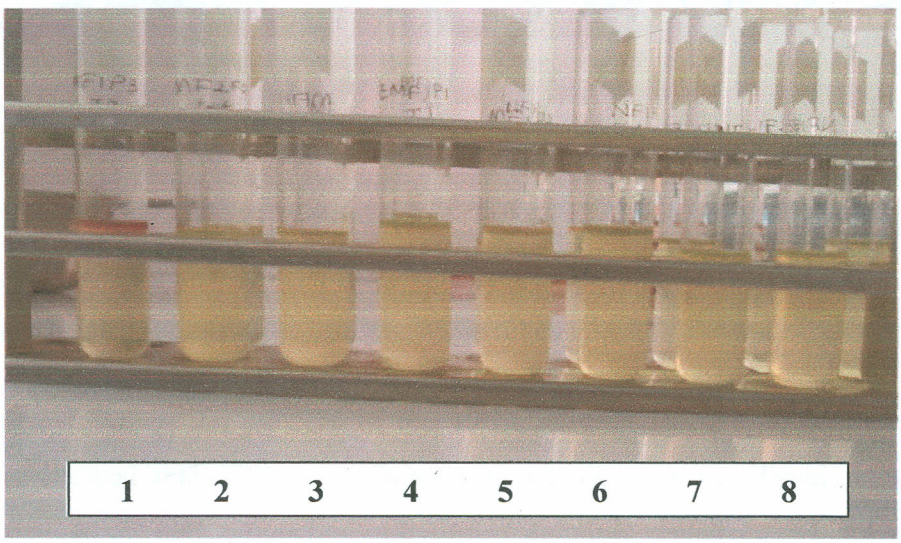

Figure 3: Indole test: peptone water after addition of Kovac's reagent. Tube 1 shows a positive test while tubes 2 to 8 show negative tests.

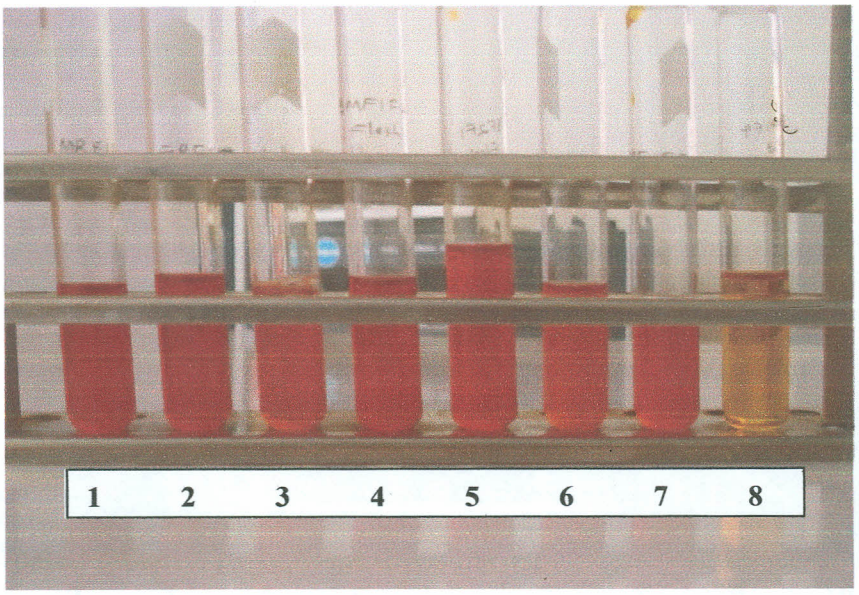

Figure 4: Methyl red test in MRVP media. Tubes 1 to 7 present positive reaction for the mixed acid pathway and tube 9 present a negative test.

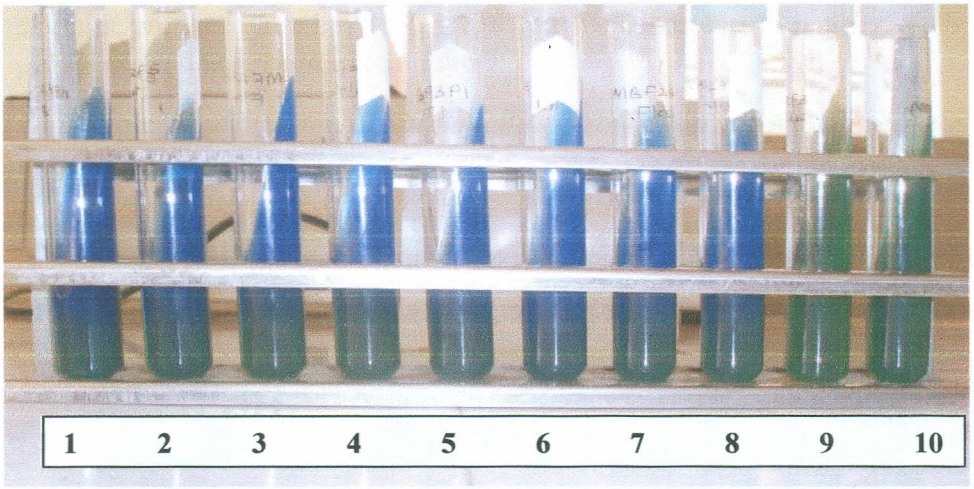

Figure 5: Citrate utilization test on Simmon's citrate agar slant. Tubes 1 to 8 present positive test for citrate utilization, while tubes 9 and 10 show a negative test

Sugar fermentation patterns of the 19 *Salmonella* isolates on tryptic sugar ion (TSI) agar showed that 11% were *Salmonella enterica* serovar Typhi presenting an alkaline slant, acidic butt, production of hydrogen sulphide visualized as black precipitate in the media and absence of gas production. Eighty nine percent gave an acid butt, acid slant, produced hydrogen sulphide and produced gas. These *Salmonella* were classified as other genera (Appendix 2).

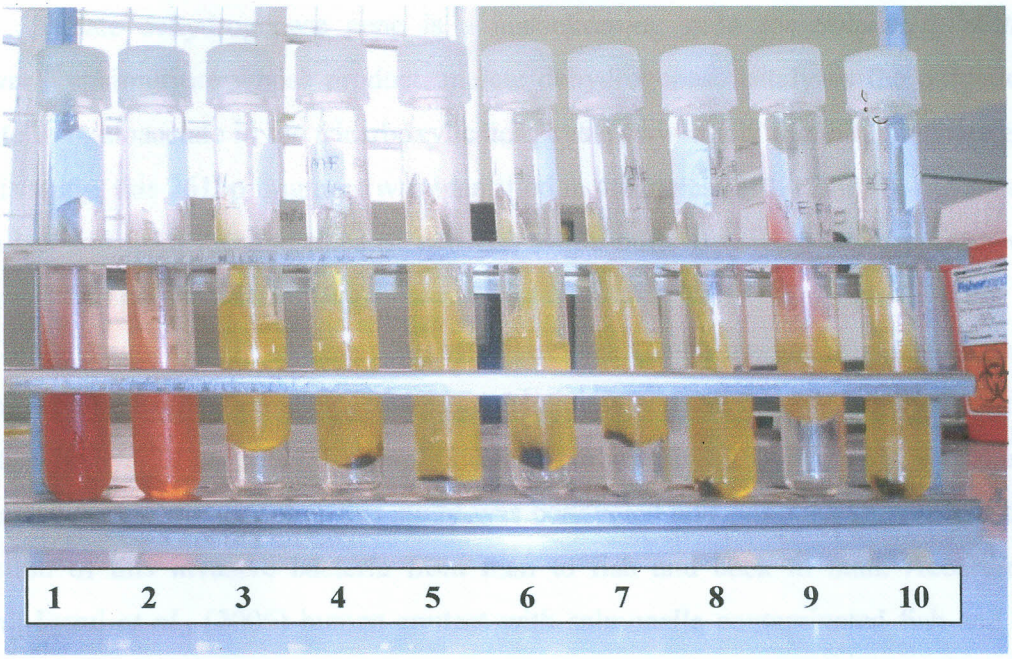

Figure 6: Triple sugar iron (TSI) slants showing negative tests in tube 1 and 2; hydrogen sulphide produced in tubes 4, 5, 6, 7, 8 and 10. Tubes 3 to 10 have acidic butt, gas produced, and acid slant except tube 9.

4.1.2 Genotypic identification of *Salmonella typhimurium* according to Amavitsi *et al.*, (2005)

A band of 261bp representative of Malic Dehydrogenase (*mdh*) gene was amplified in the DNA of four isolates: S5, S7, S8 and S14 (Figure 7).

Figure 7: PCR gel showing distinctive bands size of *mdh* 261bp gene products. Note: From top left to right: lane 1 is DNA ladder, lane 2 through 19 are experimental samples while lane 20 is NC (negative control)

Malate dehydrogenase gene is a housekeeping gene for *Salmonella enterica* serovar Typhimurium whose product, malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate in the tricarboxylic acid cycle (www.ncbi.nlm.nih.gov/sites/gene). In this study, this 261bp fragment was identified in 4 isolates (21%). Two *S. typhimurium* were isolated from the intestines of two fish while other two were isolated from water from two fish ponds.

Onyango (2007) isolated 20 antibiotic resistant *Salmonella enterica* serovar Typhimurium from clinical specimens at Mukumu and Maseno Mission Hospital in Western Kenya. In his study, he amplified the *mdh* gene in all isolates. Maseno Hospital is located within the area that this study was conducted and possibly, there's a cycle of infection of this invasive bacteria from man to fish and back to man. According to Newaj-Fyzul *et al.*, (2006) human contact with salmonella contaminated fish and pond water could pass the pathogen to humans posing a health hazard.

Salmonella enterica serovar Typhimurium is an invasive bacterial pathogen that can cause severe, fatal invasive disease in young infants, the elderly and immunocompromised hosts (Tennant *et al.*, 2010). The invasive nature of *Salmonella enterica* serovar Typhimurium was demonstrated in this study where it was recovered from flesh of two fish. One was recovered in the intestine of one fish and the other from water.

Table 3: Distribution of *Salmonella* isolates in sampled fish and fish ponds

SN	Isolate	Pond water n = 22	Flesh n = 57	Intestines n = 57
1	*Salmonella enterica* serovar Typhimurium	9%	4%	0
2	*Salmonella enterica* serovar Typhii	9%	0	0
3	*Salmonella* (others)	27%	0	12%
4	None	55%	96%	88%
	Total	**100%**	**100%**	**100%**

Percentage (%) based on the isolates and total sample size. SN 4, none refers to the number of samples that did not contain any *Salmonella* spp.

From this study, 45% of the 22 ponds sampled were contaminated by *Salmonella* (table 3 above). This prevalence was very high compared to that obtained by Newaj-Fyuzul *et al.*, (2006) of 5.9% in pond water in Trinidad. In this case, this high prevalence can be attributed to the fact that these were open ponds without any perimeter fence and that most of the fish farmers kept other animals such as cattle, goats, sheep, chicken, dogs and cats whose excreta could easily be washed into the ponds. *Salmonella* is an enteric bacteria and its presence in water is attributed to contamination sources (*December 16, 2009;* www.efsa.europa.eu/efsa_locale-1178620753812_1211902132 140.htm) possibly by the excreta of the animals reared by the farmers. All the ponds were slightly acidic with pH ranging from 4.7 to 6.20 (5.27, Table 5). This mean was below the expected range of 6.5-9.0. Student's T-test analysis (table 4) indicated these physico-chemical parameters of sampled ponds were not significantly different from one another at $P < 0.05$ and thus may not be responsible for the distribution of the microorganisms observed. Therefore, external factors, such as pollution are responsible for contamination of the ponds with both *Salmonella* and other *Enterobacteriaceae.*

Table 4: Mean observed physico-chemical parameters of the ponds, expected values and Student T-test analysis of the observed physico-chemical parameters

	Test Value = 0					
					95% C.I of the Difference	
	n	Expected	Mean Observed	Std Dev.	Lower	Upper
pH	11	6.5 – 9.0	5.27	0.42	4.99	5.56
Salinity (ppt)	11	0 – 2.5	0.30	0.14	0.20	0.39
DO	11	≥ 2.3	3.41	2.06	2.02	4.79
Temperature(° C)	11	20 – 35	24.66	1.73	23.49	25.82

pH = preferential Hydrogen, ppt = parts per thousand, ° C = Degree Celsius. Expected values adapted from Ngugi *et al.*, (2007).

Table 5: Summary of fish farms information, mean physico-chemical parameters and *Salmonella* spp isolated

						Mean Physico-chemical parameters				*Salmonella* species Isolated		
Fish Farm S/N	**Size of pond**	**Water source**	**Fish feeds**	**Target Market**	**Use of antimicrobials**	**Salinity (ppt)**	**pH**	**DO**	**Temp (°C)**	***Salmonella enterica* serovar Typhii**	***Salmonella enterica* serovar Typhimurium**	***Salmonella* (Others)**
1	Small	Ground water	Local	Local	No	0.3	5.12	2.4	27.47	-	1	3
2	Small	Ground water	Local	Local	No	0.3	4.70	2.2	25.67	-	-	-
3	Medium	Diverted river/stream	Commercial	Local	yes	0.2	5.66	2.93	26.63	1	2	1
4	Small	Ground water	Local	Local	No	0.4	4.88	3.07	23.15	-	-	1
5	Large	Diverted river/stream	Local	Local	No	0.2	5.26	2.36	27.03	-	-	2
6	Small	Ground water	Local	Local	No	0.3	5.19	2.8	23.3	-	-	1
7	Small	Ground water	Local	Local	No	0.2	4.92	1.5	27.3	-	-	1
8	Small	Diverted river/stream	Commercial	Local	No	0.2	5.11	2.5	24.17	-	-	1
9	Medium	Diverted river/stream	Local	Local	No	0.1	6.20	2.82	23.8	1	-	1
10	Medium	Ground water	Local	Local	No	0.3	5.59	2.99	25.3	-	1	1
11	Small	Ground water	Commercial	Local	No	0.3	5.38	3.7	21.9	-	-	1
Mean Observed						**0.30**	**5.27**	**3.41**	**24.7**			

Ponds classified as small if it stocks less than 5000 fish, large if it stocks between 5000 -10000 fish and large if it stocks more than 10000 fish. Fish feeds are either locally collected or commercially acquired. Target market for all farms is local.

Two farms, farm 1 and farm 3 (table 5), produced the highest number of *Salmonella*, each having 4 isolates. In farm 3, two isolates (*S. typhimurium* and unidentified *Salmonella* species) were recovered from fish intestines, one from fish flesh (*S.enterica* serovar Typhimurium) and one (*Salmonella enterica* serovar Typhi) from pond water. This farm has 28 medium-sized ponds, each having between 5,000 and 10,000 fish. It is watered by a diverted river, which possibly explains the high prevalence of *Salmonella* to be from surface run-offs, since all other physiological parameters are within the range of all the other ponds. This is the only farm that has experienced cases of fish disease characterized by swollen intestines in dead fish. No diagnostic records were available for this study. In the second farm (farm 1), three *Salmonella* isolates (one *S. typhimurium* and two unidentified *Salmonella* spp) were recovered from pond water while one unidentified *Salmonella* species was recovered from fish flesh. This farm was located near a major highway in a valley. Therefore surface run-offs from the two facing slopes easily collected in the fish farms and into the ponds.

No *Salmonella* was isolated from farm 2. This farm recorded physico-chemical properties within the range of all the other ponds. This was a small commercial farm (fish stock <5,000 fish) having African catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*). All other conditions were same as for many other farms, such as, earthened pond receiving ground water and fish fed on locally collected feeds such as table left overs, leaves and vegetables as well as chicken droppings. According to Winfield and Groisman (2003), *Salmonella* may persist in water environments for sometime withstanding various stresses that are associated with environmental fluctuations. These environment fluctuations may include temperature variability, high osmolarity, pH fluctuations and low nutrient availability (Fedorka-Cray *et al.,* 1995). Therefore variation in these physico-chemical properties of the ponds may not be considered to be important in this distribution pattern.

Four percent fish contained *Salmonella* in their flesh while 12% contained *Salmonella* in their intestines. The total number of contaminated fish was 15%. This is prevalence is slightly lower than the 23.5% obtained by Newaj-Fyuzul *et al.*, (2006), in

apparently healthy ornamental fish in Trinidad. In Kenya, a higher frequency of *Salmonella* in fish was recorded by Onyango *et al.*, (2008) at Winam gulf of Lake Victoria in Western Kenya. He reported an incidence of 31.7% in 120 fish from several beaches of the Winam gulf. The lower incidence obtained in this study is because the level of contamination unlike the open beaches of Lake Victoria that are subjected to several microbiological pollutions including runoff and storm-water that contain deposits from wildlife, agriculture, urban, forestry and rural settlements.

4.2 *Salmonella* Antimicrobial Profile

4.2.1 *Salmonella* antimicrobial susceptibility testing

The zones of inhibition of bacterial growth on a plate of Muller Hinton agar were measured and interpreted using the zone size interpretative chart (HiMedia Laboratories Pvt Ltd). Ninety five percent isolates were resistant to Ampicillin, 89% to kanamycine, 84% to chloramphenical, 63% to streptomycin, 31% were resistant to tetracycline, 16% to gentamycin and 11% to cotrimoxazole (Figure 9).

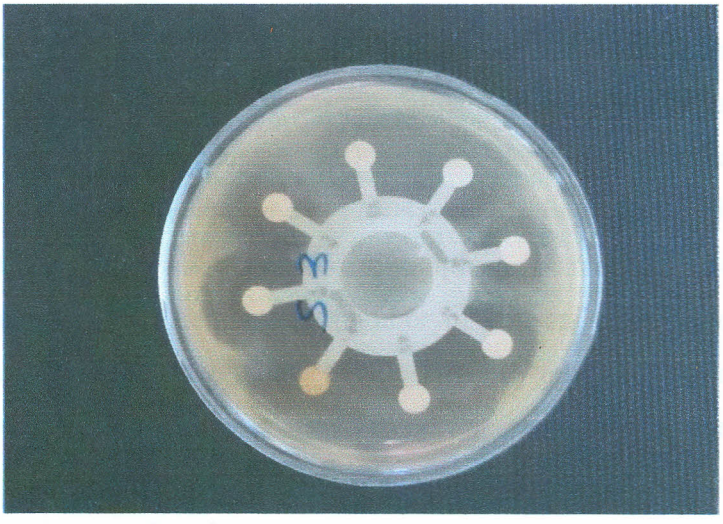

Figure 8: A Kirby-Bauer test: An antimicrobial impregnated octodisc on Muller Hinton agar plate showing different zones of inhibition to the different antimicrobials.

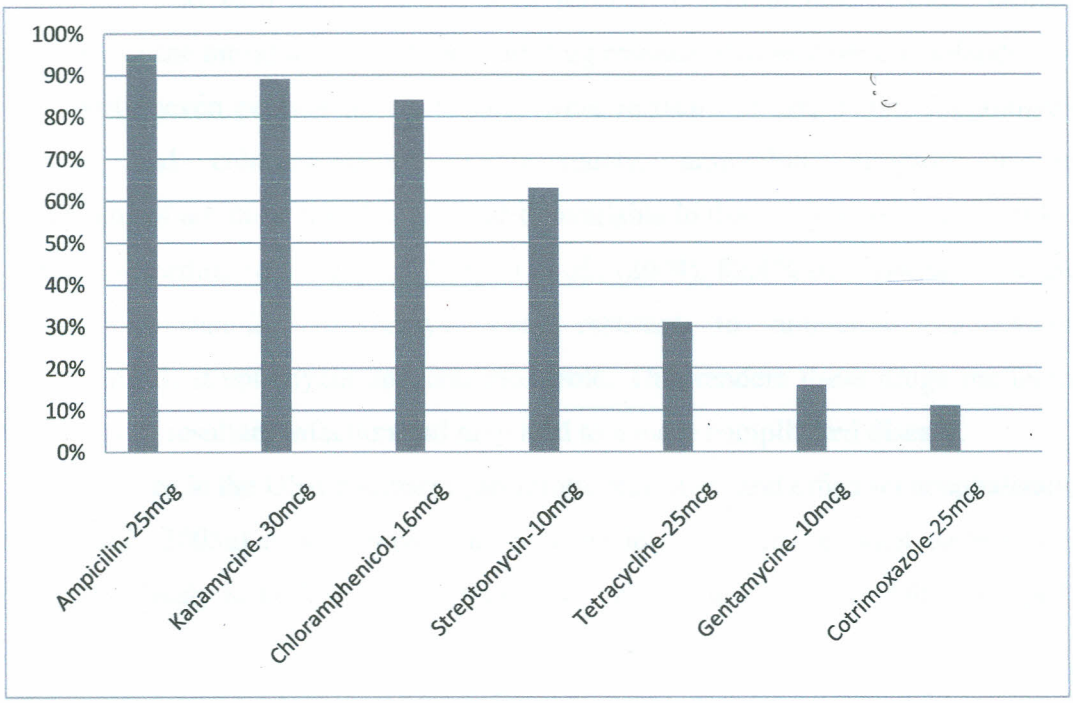

Figure 9: Antimicrobial profile of *Salmonella* isolates as given by the Kirby Bauer disk diffusion method.

The results obtained in this study are in tandem with findings from other studies that antimicrobial resistance is high among *Salmonella* isolates (Chen *et al.*, 2004). 89.4% exhibited multidrug resistance (MDR), since they were resistant to 3 or more antimicrobials. Studies by Kariuki *et al.,* (2004) and Kariuki *et al.*, (2006) showed the prevalence of MDR *Salmonella* spp in clinical samples to be more than 80%.

The two farms with the highest number of isolates (farm 1 and farm 3) also produced isolates that were resistant to the highest number of antimicrobials. A *Salmonella typhii* from farm 3 was resistant to seven antimicrobials, namely, ampicillin, tetracycline, cotrimoxazole, streptomycin, kanamycin, gentamicin and chloramphenicol. In this farm, an unknown fish disease had been previously reported and the owner had used some antimicrobials in the farm. The previous use of antimicrobials could have contributed to this increased resistance in the bacterial species, including *Salmonella* that survived. The second most resistant pattern (resistant to six antimicrobials, namely: ampicillin, tetracycline, streptomycin, kanamycin, gentamycin and chloramphenicol) was obtained in an isolate from farm 1, a small commercial farm situated in a valley between

two hills and next to a highway. The high level of contamination in these farms is responsible for the introduction of these multidrug resistant strains from the uplands.

Fourty seven percent of the isolates were resistant to ampicillin, streptomycin, kanamycin and chloramphenical. Unfortunately, ampicillin, streptomycin and chloramphenicol are the drugs that are readily available in the Kenyan market (Kariuki *et al.*, 2004). According to a study by Kariuki *et al.*, (2004), 82.4% of *Salmonella* isolated from clinical samples in Kenya were resistant to ampicillin, tetracycline, chloramphenical, streptomycin and cotrimoxazole. This renders these drugs ineffective for treatment of resultant infection and may lead to a more complicated disease.

In studies in the USA comparing antibiotic resistance and effect on hospitalization (Varma *et al.*, 2005a) it was shown that patients infected with resistant isolates were slightly more likely to be hospitalized than were patients infected with fully susceptible isolates.

4.3 *Salmonella* resistance to ampicillin

4.3.1 Genotypic analysis of ampicillin resistance of the isolated *Salmonella*

The bla_{TEM} gene was amplified in 58% of the *Salmonella* isolates as shown in the figure 10 below:

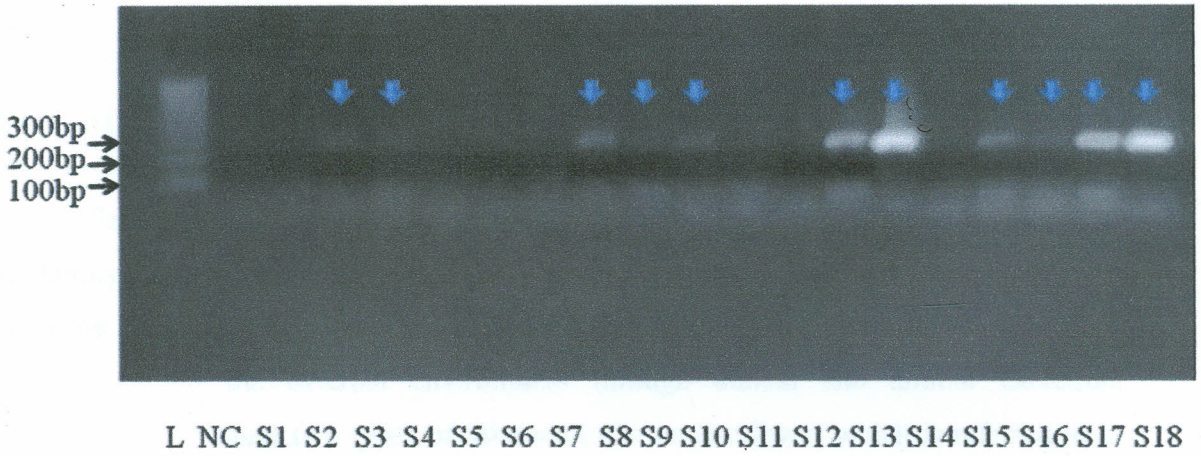

Figure 10: A PCR gel showing distinctive bands size of *bla*$_{TEM}$ 310bp gene products. Note: From top left to right: lane 1 is DNA ladder, lane 2 is NC (negative control) and lanes 3 to 20 are the experimental samples.

The molecular mechanism of ampicillin resistance in the *Salmonella* isolates was studied. DNA from 18 isolates that were resistant to ampicillin were amplified for the β-lactamase gene. Of these, 61.1% expressed the β-lactamase gene (*bla*$_{TEM}$). *Bla*$_{TEM}$ is one of the two β-lactamase genes (the other is *bla*$_{PSE1}$), which confer resistance to ampicillin (Frech *et al.*, 2003). The genes are usually located on transposons; such as *Tn*3 (Frech *et al.*, 2003) and in usual cases, isolates negative of *bla* $_{PSE1}$ are positive of *bla*$_{TEM}$ (Onyango *et al.*, 2007). The prevalence of *bla*$_{TEM}$ gene in *Salmonella enterica* serovar Typhimurium was even higher (90%) in the study conducted by Onyango *et al.*, (2007), in clinical samples collected from Mukumu and Maseno hospitals in Western Kenya. With this high prevalence of the β-lactamase gene, the therapeutic use of ampicillin Salmonellosis is not recommended for patients in this area.

Other molecular mechanisms of resistance that may have conferred resistance to β-lactams for the other isolates were not studied. However, it is documented by Poole, (2004) that resistance to β-lactams can also result from mutations that reduce levels of outer membrane proteins involved in uptake, altered target proteins (penicillin-binding proteins) to reduce β-lactam binding, or increased expression of efflux pumps that export the antibiotics.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

Salmonella is a gram negative facultative rod-shaped bacterium that belongs to the family *enterobacteriaceae*. The principal habitat of *Salmonella* is the intestinal tracts of warm and cold blooded animals, though some species are ubiquitous. It is disseminated to the external environment through human and animal excretion. Salmonellae can cause two types of salmonellosis: enteric fever, which is referred to as typhoid, resulting from bacterial invasion of bloodstream; and acute gastroenteritis, a food-borne infection and intoxication.

Strains of *Salmonella* that are resistant to several antimicrobial drugs (multidrug resistant strains) have been isolated in many areas around the world and are quickly replacing the fully susceptible strains. This has become a problem since treatment and management of the diseases they cause has become not only difficult, but also expensive. Multidrug resistant strains of *Salmonella enterica* serovar Typhimurium have previously been isolated in clinical samples at Maseno hospital.

Fish farming has become an important practice in Western Kenya as many individual families and self-help groups keep fish in earthen ponds for commercial sales and also for a source of proteins. The community living in Maseno around Maseno University rears Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) in small to medium sized ponds that do not exceed a population of 10,000 fish.

It has been demonstrated that human contact with *Salmonella* infected fish or pond water could lead to infection. This poses a great risk to fish farmers and hobbyists, as well as fish consumers if the fish is not well prepared. This study, which sought to establish the role of fish farming in the dissemination of *Salmonella* to humans in Maseno, established that 45% of the fish ponds and 9% of fish collected from the community living around Maseno University were contaminated by *Salmonella* within the study period, Oct- Dec, 2008. Eighty nine percent of these were multidrug resistant, since they were resistant to at least three antimicrobial drugs (ampicillin, streptomycin, gentamicin, kanamycin, chloramphenicol, tetracycline and cotrimoxazole). Ninety five

percent were resistant to ampicillin, while 61.1% of these contained the beta lactamase (bla_{TEM}) gene product after PCR amplification.

The findings of this study indicate that *Salmonella* is present in fish and fish farms in the community around Maseno University in Western Kenya. *Salmonella* is an enteric bacteria and is a contaminant in the fish ponds; it is hypothesized that the microbe may have found its way into the pond through excretion from humans living near the farms or from animals, such as sheep, goats, cows, dogs and cats kept by farmers. The contamination found its way into the ponds easily since all were open and non of them had a perimeter wall around.

Salmonella enterica serovar Typhimurium and *Salmonella enterica* serovar Typhi recovered in some ponds are human pathogens that can cause invasive non-typhoidal salmonellosis and systemic typhoid respectively. Human contact with pond water or poorly prepared infected fish could be a source of infection. The disease complication could be worse and difficult to treat owing to the fact that most of the isolates (89.4%) were multidrug resistant especially to conventional drugs such as, tetracycline, chloramphenical and ampicillin.

The presence of ampicillin resistant gene, bla_{TEM}, accounts for resistance to penicillins and cephalosphorins, including ampicillin. Its distribution in different farms is quiet high and an issue of concern. Though some of the *Salmonella* strains were not identified in this study and may not be human pathogens, the presence of this gene is still an issue of concern since it can be transferred from these resistant bacteria to the susceptible stains through horizontal gene transfer between bacteria inhabiting in the same environment making it difficult to treat the resultant infection.

This study draws the conclusion that the occurrence of *Salmonella* spp in fish and fish farms is high and that most of the *Salmonella* isolated are multidrug resistant.

The following recommendations are made against the findings of this study:

- Following the isolation of *Salmonella* in fish and fish ponds, it is important that fish farmers improve the hygiene standards of their fish ponds by fencing off their farms to keep away animals, both domestic and wild, and also to improve

drainage around the ponds to avoid to avoid direct flow of surface run-offs from getting into the ponds.

- Following the indication that the prevalence of *Salmonalla* spp in fish farms is high. There is need that more study be conducted to establish the actual source of the bacteria to the farms and the reason for the increased resistance as observed. This will help in surveillance of Salmonellosis in humans in relation to external reservoirs and possibly help in establishing ways to control further spread.
- Since this research was only restricted within the parameters of fish farms, there is need that the situation of the fish at the markets also be studied to establish the effect of post fishing contamination by *Salmonella* spp.
- This study established a high prevalence of multidrug resistance in *Salmonella* isolates from fish and fish farms, but only looked at the ampicillin resistance gene. Therefore, the presence and distribution of other antimicrobial resistance genes need to be studied.

REFERENCE

Aarestrup F. M., Seyfarth A. M., Emborg H. D., Pedersen K., Hendriksen R. S., and **Bager F. (2001).** Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother*; 45:2054–2059.

Amavisti, P., Boonyawiwat, W., and Bangtrakulnont, A. (2005). Characterization of *S. enterica* serovar *typhimurium* and Monophasic *Salmonella* Serovar 1, 4, [5], and 12: i: - Isolates in Thailand. *J.Clin.Microbiol.* 2736–2740.

Arcangioli M. A., Leroy-Sétrin S., Martel J. L., and Chaslus-Dancla E. (1999). A new chloramphenicol and florfenicol resistance gene flanked by two integron structures in *Salmonella typhimurium* DT104. *FEMS Microbiol. Lett.* 174:327-332.

Bager F., Madsen M., Christensen J., and Aarestrup F. M. (1997). Avoparcin used as a growth promoter is associated with the occurrence of vancomycinresistant *Enterococcus faecium* in Danish poultry and pig farms. *Prev. Vet. Med.* 31:95–112.

Baron S., editor. (1996). *Salmonella, Medical Microbiology*. Galveston (TX): University of Texas Medical Branch . USA

Baudart J., Lemarchand K., Brisabois A., and Lebaron P. (2000). Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer regions. Appl. *Environ. Microbiol.* 66:1544–1552.

Baumler A. J., Tsolis R. M., Ficht T. A., and Adams L. G. (1998). Evolution of host adaptation in *Salmonella enterica. Infect. Immun.* 66:4579-4587.

Berg, O. G., and Martelius M. (1995). Synonymous substitution-rate constants in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium and their relationship to gene expression and selection pressure. J. Mol. Evol. 41449-456.

Berkley J. A., Lowe B. S., Mwangi I., Williams T., Bauni E., Mwarumba S., Ngetsa C., Slack M. P., Njenga S., Hart C. A., Maitland K., English M., Marsh K., and Scott J. A. (2005). Bacteremia among children admitted to a rural hospital in Kenya. *New Engl J Med*, 352:39-47.

Bhaftopadhyay P. (2000). Fish-catching and handling. In: Robinson R.K (ed.): *Encyclopedia of Food Microbiology.* Vol 2, Academic press, London. Pp 1547.

Blazquez, J., Negri M. C., Morosini M. I., Gomez-Gomez J. M., and Baquero F. (1998). A237T as a modulating mutation in naturally occurring extended-spectrum TEM-type beta-lactamases. Antimicrob. Agents Chemother. 42:1042-1044.

Bolton L. F., Kelley L. C., Lee M. D., Fedorka-Cray P. J., and Maurer J. J. (1999). Detection of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol. *J. Clin. Microbiol.* 37:1348-1351.

Boyd D. A., Peters G. A., Ng L. K., and Mulvey M. R. (2000). Partial characterization of a genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104. *FEMS Microbiol. Lett.* 189:285-291.

Boyd D. A., Peters G. A., Cloeckaert A., Boumedine K. S., Chaslus-Dancla E., Imberechts H., and Mulvey M. R. (2001). Complete nucleotide sequence of a 43-kb genomic island associated with the multidrug resistance region of *Salmonella enterica* Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J. Bacteriol.* 83:5725-5732.

Boyd D., Cloeckaert A., Chaslus-Dancla E., and Mulvey M. R. (2002). Characterization of variant *Salmonella* genomic island 1 multidrug resistance regions from serovars Typhimurium DT104 and Agona. *Antimicrob. Agents Chemother.* 46:1714-1722.

Boyle E. C., Bishop J. L., Grass G. A., and Finlay B. B. (2007). *Salmonella*: from Pathogenesis to Therapeutics. Meeting Review- Copyright © 2007, American Society for Microbiology. *J. of Bact.*, 189:5; 1489–1495

Brown S. P., Cornell S. J., Sheppard M., Grant A. J., Maskell D. J., Grenfell B. T., and Mastroeni P. (2006). Intracellular demography and the dynamics of *Salmonella enterica* infections. *PLoS Biol.* 4:e349.

Brettar I., and Hofle M. G. (1992). Influence of ecosystematic factors on survival of *Escherichia coli* after large-scale release into lake water mesocosms. *Appl. Environ. Microbiol.* 58:2201–2210.

Bruneau A., Rodrigue H., Ismael J., Dion R., and **Allard R. (2004).** Outbreak of *E. coli* O157:H7 associated with bathing at a public beach in the Montreal-Centre region. Can Commun Dis Rep.;30:133–136

Brunner G. H. (1974). Experiments on the possibilities and course of infections with *Salmonella enteritidis* and *Salmonella typhimurium* in fresh water fish. *Zentralbl. Bakteriol., Mikrobiol. and Hyg* (B) 158:412-431

Burton G. A., Gunnison D., and Lanza G. R. (1987). Survival of pathogenic bacteria in various freshwater sediments. *Appl. Environ. Microbiol.* 53:633-638.

Carter P. B., and Collins F. M. (1974). The route of enteric infection in normal mice normal mice. *J. Exp. Med.*, 139:1189–1203.

Chao W., Ding R., and Chen R. (1987). Survival of pathogenic bacteria in environmental microcosms. *Chinese J. Microbial Immunol.* (Taipei) 20: 339–348.

Chen S., Zhao S., White D.J., Schroeder C. M., Lu R., Yang H., McDermott P. F., Ayers S., and Meng J. (2004). Characterization of multiple-antimicrobial-resistant *Salmonella* serovars isolated from retail meats. *Appl Environ Microbiol.* 70:1–7.

Cherry W. B., Hanks J. B., Thomason B. M., Murlin A. M., Biddle J. W., and Croom J. M. (1972). Salmonellae as an index of pollution of surface waters. *Appl. Microbiol.* 24:334–340.

Chinh N. T., Parry C. M., Ly N. T., Ha H. D., Thong M. X., Diep T. S., Wain J., White N. J., and Farrar J. J. (2000). A randomized controlled comparison of azithromycin and ofloxacin for treatment of multidrug-resistant or nalidixic acid-resistant enteric fever. *Antimicrob. Agents Chemother.* 44:1855–1859.

Clinical and Laboratory Standards Institute, (2002). Performance standards for antimicrobial susceptibility testing; twelfth informational supplement. Approved standard M100-S12. *National Committee for Clinical Laboratory Standards, Wayne, Pa .*

Cohen S. N., Change A. C. Y., and Hsu L. (1972). Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of National Academy of Sciences USA.* 69:2110-2114.

Coovadia Y. M., Gathiram V., Bhamjee A., Mlisana K., Pillay N., Garratt R. M., Madlalose T., and Short M. (1992). The emergence of multi-antibiotic-resistant strains of *Salmonella typhi* in northern Natal-Kwa Zulu. *S. Afr. Med. J.* 81:280–281.

Crump J. A., Luby S. P., and Mintz E. D. (2004). The global burden of typhoid fever. *Bull.* W.H.O. 82:346–353.

Dalacher S., Derendorf H., Hollenstein U., Brunner M., Joukhadar S., Hofmann A., Georgopoulos H., Eichler H. G., and Muller M. (2000). A combined in vivo pharmacokinetic-*in vitro* pharmacodynamic approach to stimulate target site pharmacodynamics of antibiotics in humans. *J. Antimicrob. Chemother.* 46: 733-741.

Dalsgaard, A. 1998. The occurrence of human pathogenic *Vibrio* spp. and *Salmonella* in aquaculture. *International Journal of Food Science and Technology* 33: 127-138.

Datta N., and Kontomichalou P. (1965). Penicillinase synthesis controlled by infectious R factors in *Enterobacteriaceae*. Nature **208:**239-241.

Davies J. E. (1997). Origins, acquisition, and dissemination of antibiotic resistance determinants, p. 15-27. *In* D. J. Chadwick and J. Goode (ed.), Antibiotic resistance: origins, evolution, selection and spread. John Wiley & Sons, Inc., New York, N.Y.

DePaola A., Peeler J. T., and Rodrick G. E.. (1995). Effect of oxytetracycline medicated feed on antibiotic resistance of gram-negative bacteria in catfish ponds. *Appl. Environ. Microbiol.* 61:2335–2340.

Doublet B., Lailler R., Meunier D., Brisabois A., Boyd D., Mulvey M. R., Chaslus-Dancla E., and Cloeckaert A. (2003). Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster in *Salmonella enterica* serovar Albany. *Emerg. Infect. Dis.* 9:585-591.

Depardieu F., Podglajen I., Leclercq R., Collatz E., and Courvalin P. (2007). Modes and Modulations of Antibiotic Resistance Gene Expression. *Clinical Microbiol. Rev.,* 20:1;79–114

Edwards R. A., Olsen G. J., and Maloy S. R. (2002). Comparative genomics of closely related salmonellae. *Trends Microbiol.* 10:94-99.

Fedorka-Cray P. J., Kelley L. C., Stabel T. J., Gray J. T., and Laufer J. A. (1995). Alternate routes of invasion may affect pathogenesis of *Salmonella enterica* serovar Typhimurium in swine. *Infect. Immun.* 63:2658–2664.

Fidelma E. B., and Daniel L. H. (1998). *Salmonella* virulence plasmid: Modular Acquisition of the *spv* Virulence Region by an F – Plasmid in *S. enterica* subspecies I and Insertion into the Chromosome of subspecies II, IIIa, IV and VII isolates. *Genetics*, 149: 1183 – 1190.

Fierer J., and Guiney D. G. (2001). Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection. *J. Clin. Invest.*, 107:7; 775 – 780.

Fish J. T., and Pettibone G. W. (1995). Influence of freshwater sediment on the survival of *Escherichia coli* and *Salmonella* sp. as measured by three methods of enumeration. *Lett. Appl. Microbiol.* 20:277–281.

Fluit A. C., Visser M. R., and Schmitz F. (2001). Molecular Detection of Antimicrobial Resistance. *Clinical Microbiol. Rev.*, 14:4; 836–871

Fluit A. C., and Schmitz F. J. (1999). Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur. J. Microbiol. Infect.* Dis. 18:761-770.

Foltz V. D. (1969). *Salmonella* ecology. *J. Am. Oil Chem. Soc.* 46:222–224.

Gebreyes, W. A., and Altier, C., (2002). Molecular Characterization of Multidrug – Resistant *S. enterica subsp. enterica serovar typhimurium* isolates from swine. *J. Clin. Microbiol.*, 40 (8): 2813 – 2822.

Gianella R. A., Washington O., Gemski P., and Formal S. B. (1973). Invasion of HeLa cells by *S. typhimurium*: a model for study of invasiveness of *Salmonella. J. Infect. Dis.*, 128:69–75.

Giraud E., Brisabois A., Martel J. L., and Chaslus-Dancla E. (1999). Comparative studies of mutations in animal isolates and experimental in vitro and in vivo-selected mutants of *Salmonella* spp. suggest a counterselection of highly fluoroquinolone-resistant strains in the field. *Antimicrob. Agents Chemother.* 43:2131–2137.

Graham S. M. (2002). Salmonellosis in children in developing and developed countries and populations. *Curr. Opin. Infect. Dis.* 15:507–512.

Gruenheid S., and Finlay B. B. (2003) Microbial pathogenesis and cytoskeletal function. *Nature.* 422:775–781.

Guerra B., Soto S., Cal S., and Mendoza M. C. (2000). Antimicrobial resistance and spread of class 1 integrons among *Salmonella* serotypes. *Antimicrob. Agents Chemother.* 44:2166-2169.

Gaulin, C., Vincent, C., Alain, L., Ismaïl, J. (2002). Outbreak of *Salmonella* Paratyphi B linked to aquariums in the province of Quebec, 2000. *Canada Communicable Disease Report*, 28 (11):89-96.

Hein I., Schneck C., and Knogler M. (2003) *Campylobacter jejuni* isolated from poultry and humans in Styria, Austria: epidemiology and ciprofloxacin resistance. *Epidemiol Infect*;130:377–386.

Hirose K., Hashimoto A., Tamura K., Kawamura Y., Ezaki T., Sagara H., and Watanabe H. (2002). DNA sequence analysis of DNA gyrase and DNA topoisomerase IV quinolone resistance-determining regions of *Salmonella enterica* serovar Typhi and serovar Paratyphi A. *Antimicrob. Agents Chemother.* 46:3249–3252

Hall R. M. (1997). Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. *Ciba Found Symp*; 207:192–202.

Hohmann E. L. (2001). Nontyphoidal salmonellosis. *Clin. Infect. Dis.* 32:263–269.

Janssen A. W. and Meyers C. D. (1968). Fish: Serologic evidence of the infection with human pathogens. *Science,* 159:547-548

Jones B. D., Ghori N., and Falkow S. (1994). *S. typhimurium* initiates murine infection by penetrating and destroying the specialized M cells of the Peyer's patches. *J. Exp. Med.*, 180:15–23.

Kankwatira A. M., Mwafulirwa G. A., and Gordon M. A. (2004). Nontyphoidal salmonella bacteraemia—an under-recognized feature of AIDS in African adults. *Trop. Doct.* 34:198–200.

Kariuki S., Revathi G., Kariuki N., Kiiru J., Mwituria J., and Hart C. A. (2006). Characterisation of community acquired non-typhoidal Salmonella from bacteraemia and diarrhoeal infections in children admitted to hospital in Nairobi, Kenya. *BMC Microbiol.*, 6:**101**:1-10

Kariuki S., Revathi G., Muyodi J., Mwituria J., Munyalo A., Mirza S., and Hart C. A. (2004). Characterization of Multidrug-Resistant Typhoid Outbreaks in Kenya. *J. of Clin. Microbiology.* 42:**4**;1477–1482

Kariuki S., Gilks C., Revathi G., and Hart C. A. (2000). Genotypic analysis of multidrug-resistant *Salmonella enterica* Serovar Typhi, Kenya. *Emerg. Infect. Dis.* 6:649–651.

Karuse M., Fang F. C., and Guiney D. G. (1992). Regulation of plasmid virulence gene expression in *Salmonella Dublin* involves an unusual operon structure. *J. Bacteriol.*, 174: 4482 – 4489.

Kim H., and Park S. (2006). Comparison of *Salmonella enterica* Serovar Typhimurium LT2 and Non-LT2 *Salmonella* Genomic Sequences, and Genotyping of Salmonellae by Using PCR. *Appl Environ Microbiol.* 72:**9**; 6142–6151.

Kruger, T., Szabo D., Keddy K. H., Deeley K., Marsh J. W., Hujer A. M., Bonomo R. A., and Paterson D. L. (2004). Infections with nontyphoidal *Salmonella* species producing TEM-63 or a novel TEM enzyme, TEM-131, in South Africa. Antimicrob. Agents Chemother. **48:**4263-4270.

Lalmanach A. C, Lantier F. (1999). Host cytokine response and resistance to *Salmonella* infection. *Microbes Infect.*; 1:719–726.

Lance F. B., Kelley L. C., Lee M. D., Fedorka – Cray P. J., and Maurer J. J. (1999). Detection of Multidrug – Resistant *S. enterica serotype typhimurium* DT 104 based on a Gene which confers cross –resistance to Florfenicol and chloramphenical. *J.Clin. Microbiol.*, 37:**5**; 1348 – 1351.

Levesque C., Piche L., Larose C., and Roy P. H. (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother*; 39:185–191.

Levings R. S., Lightfoot D., Partridge S. R.,. Hall R. M., and Djordjevic S. P. (2005) The Genomic Island SGI1, Containing the Multiple Antibiotic Resistance Region of *Salmonella enterica* Serovar Typhimurium DT104 or Variants of It, Is Widely Distributed in Other *S. enterica* Serovars. J Bacteriol. 2005 July; 187(13): 4401–4409.

Ley R. E., Peterson D. A., and Gordon J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell.*;124:837–848.

Lipp E. K., and Rose J. B. (1997). The role of seafood in food borne diseases in the United States of America. Rev. *Sci. Tech. OIE*, 16:620-640

Livermore, D. M. (1996). Are all beta-lactams created equal? Scand. J. Infect. Dis. Suppl. **101**:33-43.

Lorian, V. (1996): Antibiotics in Laboratory Medicine 4th ed. Williams and Wilkins.

Lunestad B. T. (1992). Fate and effects of antibacterial agents in aquatic environments, p. 152–161. *In* C. Michel and D. J. Alderman (ed.), *Chemotherapy in aquaculture: from theory to reality.* Office international des epizooties, Paris, France.

Martinez-Moya M., de Pedro M. A., Schwarz H., and Garcia-del P. F. (1998). Inhibition of *Salmonella* intracellular proliferation by non-phagocytic eucaryotic cells. *Res. Microbiol.* 149:309–318.

Martinez-Urtaza, J., Lozano-Leon, A., Varala-Pet, J., Trinanes, J., Pazos, Y, Garcia-Martin, O. (2008). Environmental determinants of the occurrence and distribution of *Vibrio parahaemolyticus* in the rias of Galicia, Spain. *Applied and Environmental Microbioogyl.* 74 (1):265-274.

Mastroeni P. and Sheppard M. (2004). *Salmonella* infections in the mouse model: host resistance factors and in vivo dynamics of bacterial spread and distribution in the tissues. *Microbes Infect.* 6:398–405.

Mead P. S., Slutsker L. and Dietz V. (2003). Food related illness and death in the United states. *Emerg Infect Dis.*, 5:607-625, Available at www.cdc.gov/ncidod/EID/ Vol5no5/pdf/meadpdf

Medeiros, A. A. (1997). Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin. Infect. Dis. 24:S19-S45.

Mendoza, M. T. (1998). What's New in Antimicrobial Susceptibility Testing?. Phil *J. Microbiol. Infect. Dis.*, 27 (3):113-115.

Mirza S., Kariuki S., Mamun K. Z., Beeching N. J., and Hart C. A. (2000). Analysis of plasmid and chromosomal DNA of multidrug-resistant *Salmonella enterica* serovar Typhi from Asia. *J. Clin. Microbiol.* 38:1449–1452.

Moore B. C., Martinez E., Gay J. M., and Rice D. H. (2003). Survival of *Salmonella enterica* in Freshwater and Sediments and Transmission by the Aquatic Midge *Chironomus tentans* (Chironomidae: Diptera). *Appl Environ Microbiol.* 69:8; 4556–4560.

Morinigo M. A., Borrego J. J., and Romero P. (1986). Comparative study of different methods for detection and enumeration of *Salmonella* spp. in natural waters. *J. Appl. Bacteriol.* 61:169-176.

Mroczkowska J. E. and Barlow M. (2008). Fitness Trade-Offs in bla_{TEM} Evolution. Antimicrob Agents Chemother. 52(7): 2340–2345.

Mullis K. B., and Faloona F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.

Mwangi I., Berkley J., Lowe B., Peshu N., Marsh K., and Newton C. R. (2002) Acute bacterial meningitis in children admitted to a rural Kenyan hospital: increasing antibiotic resistance and outcome. *Pediatr Infect Dis J*, 21:1042-1048.

Nastasi A., and Mammina C. (2001). Presence of class 1 integrons in multidrug-resistant, low-prevalence *Salmonella* serotypes, Italy. *Emerg. Infect. Dis.* 7:455-458.

Newaj-Fyuzul A., Adesiyun A. A., and Mutani A. (2006). Prevalence of antimicrobial resistance of *Salmonella* spp. isolated from apparently healthy ornamental fish and pond water in Trinidad. *J of food, Agric. and Environm.* 4:1; 27-29

Ngugi C. C., Bowman J. R., and Omolo B. O. (2007). Species suitable for culture in Kenya. *A New Guide to Fish Farming In Kenya.* USA: Aquaculture CRSP. p29-37

Ngugi C. C., and Manyala (2007). Aquaculture extension service in Sub-Sahara Africa, Annex 3-Kenya. FAO corporate document repository. Accessed at www.fao.org/docrep/007/y5641e/y5641e00.HTM

Onyango D., Machoni F., Kakai R., and Waindi E. N. (2008). Multidrug resistance of *Salmonella enterica* serovars Typhi and Typhimurium isolated from clinical samples at two rural hospitals in Western Kenya. *J. Infect Developing Countries,* 2:2;106-111

Onyango M. D., Sarah W., Rose K. Eliud N. W. (2009). Isolation of Salmonella and Shigella from fish harvested from the Winam Gulf of Lake Victoria, Kenya. *J Infect Developing Countries*; 3(2):99-104.

Onyango D. O. (2007). Drug susceptibilities and mechanisms of drug resistance among *salmonella typhimurium* isolates from persons in western Kenya. *PhD thesis* pp 7-8. Maseno University.

Onyango D. O. (2003). Prevalence of enteric pahogens amongst HIV- positive children in Western Kenya with Diarrhoea as a presenting sign. *M.Sc, Thesis* pp 64 – 77. Maseno University.

Parry C. M. (2004). Typhoid fever. *Curr. Infect. Dis. Rep.* 6:27–33.

Partridge S. R., Recchia G. D., Scaramuzzi C., Collis C. M., Stokes H. W. and Hall R. M. (2000). Definition of the *attI1* site of class 1 integrons. *Microbiology* 146:2855-2864.

Partridge S. R., Brown H. J., Stockes H. W., and Hall R. M. (2001a). Transposons Tn*1696* and Tn*21* and their integrons In4 and In2 have independent origins. *Antimicrob. Agents Chemother.* 45:1263-1270.

Partridge S. R., Recchia G. D., Stokes H. W., and Hall R. M. (2001b). Family of class 1 integrons related to In4 from Tn*1696. Antimicrob. Agents Chemother.* 45:3014-3020.

Peters E. D. J., Leverstein-van H. M. A., Box A. T. A., Verhoef J., and Fluit A. C. (2001). Novel gene cassettes and integrons. *Antimicrob. Agents Chemother.* 45:2961-2964.

Petersen A., Andersen J. S., Kaewmak T., Somsiri T., and Dalsgaard A. (2002). Impact of Integrated Fish Farming on Antimicrobial Resistance in a Pond Environment. *Appl. Environ Microbiol.,* 68:**12**; 6036–6042

Phung le V., Ryo H., and Nomura T. (2002). Specific *gyrA* mutation at codon 83 in nalidixic acid-resistant *Salmonella enterica* serovar Typhi strains isolated from Vietnamese patients. *Antimicrob. Agents Chemother.* 46:2052–2053.

Poole, K., 2004. Resistance to beta-lactam antibiotics. *Cell Mol. Life Sci.* 61: 2200–2223.

Popoff M. Y., Bockemuhl J., and Gheesling L. L. (2003). Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Res. Microbiol.* 154:173-174.

Popoff M. Y. (2001). Antigenic formulas of the *Salmonella* serovars, 8th revision. W.H.O. Collaborating Centre for Reference and Research on *Salmonella.* Institute Pasteur, Paris, France.

Prouty A. M., and Gunn J. S. (2003). Comparative analysis of *Salmonella enterica* serovar Typhimurium biofilm formation on gallstones and on glass. *Infect. Immun.* 71:7154–7158.

Queenan, A. M., Foleno B., Gownley C., Wira E., and Bush K.. (2004). Effects of inoculum and beta-lactamase activity in AmpC- and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates tested by using NCCLS ESBL methodology. J. Clin. Microbiol. 42:269-275.

Rabsch W., Tschape H., and Baumler A. J. (2001). Non-typhoidal salmonellosis: emerging problems. *Microbes Infect.* 3:237–247.

Rahman M., Ahmad A., and Shoma S. (2002). Decline in epidemic of multidrug resistant *Salmonella typhi* is not associated with increased incidence of antibiotic-susceptible strain in Bangladesh. *Epidemiol. Infect.* 129:29–34.

Rowe B., Ward L. R., and Threlfall E. J. (1997). Multidrug-resistant *Salmonella typhi*: a worldwide epidemic. *Clin. Infect. Dis.* 24:1; S106–S109.

Saeed A. M., Gast R. K., Potter M. E., and Wall P. G. (1999). *Salmonella enterica* serovar *Enteritidis* in humans and animals — epidemiology, pathogenesis, and control. Iowa State University Press, Ames, Iowa.

Saiki R. K., Scharf S., Faloona F., Mullis K. B., Horn G. T., and Ehrlich H. A. (1988a). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for the diagnosis of sickle-cell anemia. *Science* 230: 1350–1354.

Saiki R. K., Gelfand D. H., Stoffel S., Scharf S. J., Higuchi R., Horn G. T., Mullis K. B., and Ehrlich H. A. (1988b). Primer-directed enzymatic amplification with a thermostable DNA polymerase. *Science*, 239:487–491.

Samuelsen O. B., Torsvik V., and Ervik A. (1992). Long-range changes in oxytetracycline concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after a medication. *Sci. Total Environ.* 114:25–36.

Schmitz F. J., Hafner D., Geisel R., Tollman O., Kirschke C., Verhoff J., Köhrer K., and Fluit A. C. (2001). Increased prevalence of class 1 integrons in *Escherichia coli. Klebsiella* species, and *Enterobacter* species isolated over a 7-year period in a German hospital. *J. Clin. Microbiol.* 39:3724-3726.

Shanahan P. M., Karamat K. A., Thomson C. J., and Amyes S. G. (1998). Molecular analysis of and identification of antibiotic resistance genes in clinical isolates of *Salmonella typhi* from India. *J. Clin. Microbiol.* 36:1595–1600.

Sifuna A. W., Njagi E. N., Okemo P, Munyalo A, Orinda G.O., Kariuki S. (2008). Microbiological quality and safety of Rastrineobola argentea retailed in Kisumu town markets, Kenya. East Afr Med J. 85(10):509-13.

Sirot, D., Sirot J., Labia R., Morand A., Courvalin P., Darfeuille-Michaud A., Perroux R., and Cluzel R. (1987). Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel beta-lactamase. J. Antimicrob. Chemother. 20:323-334

Soussy C. J., Carret G., Cavallo J. D., Chardon H., Chidiac C., Choutet P., Courvalin P., Dabernat H., Drugeon H., Dubreuil L., Goldstein F., Jarlier V., Leclercq R., Nicolas-Chanoine M. H., Philippon A., Quentin C., Rouveix B., and Sirot J. (2000). Comite´ de l'Antibiogramme de la Socie´te´ Franc¸aise de Microbiologie. Communique´ 2000–2001. *Pathol. Biol.* 48:832–871.

Tamplin, M. L. (2001). Coastal Vibrios: Identifying relationships between environmental condition and human disease. *Human and Ecological Risk Assessment* Volume 7 (5):1437- 1445.

Tassios P. T., Markogiannakis A., Vatopoulos A. C., Katsanikou E., Velonakis E. N., Kourea-Kremastinou J. and Legakis N. J. (1997). Molecular epidemiology of antibiotic resistance of *Salmonella enteritidis* during a 7-year period in Greece. *J. Clin. Microbiol.* 35:1316-1321.

Tennant S. M., Diallo S., Levy H., Livio S.,. Sow S. O, Tapia M., Fields P. I., Mikoleit M., Tamboura B., Kotloff K. L., Nataro P., Galen J. E., and Levine M. M. (2010). Identification by PCR of Non-typhoidal *Salmonella enterica* Serovars Associated with Invasive Infections among Febrile Patients in Mali. PLoS Negl Trop Dis. 4(3): e621.

Thomason B. M., Dodd D. J., and Cherry W. B. (1977). Increased recovery of salmonellae from environmental samples enriched with buffered peptone water. *Appl. Environ. Microbiol.* 34:270–273.

Thorns C. J. (2000). Bacterial food-borne zoonoses. Rev. Sci. Technol. 19:226-239.

Tirado, C., and Schmidt K. (2001). WHO Surveillance Programme for Control of Foodborne Infections and Intoxications: results and trends across greater Europe. *J. Infect.* 43:80-84.

Todar K. (2005). Todar's Online Textbook of Bacteriology. *Available at* http//www.textbookofbacteriology.net/

Varma J. K., Greene K. D., Ovitt J., Medalla F., and Angulo F. J. (2005a) Hospitalization and antimicrobial resistance in *Salmonella* outbreaks, 1984–2002. *Emerg Infect Dis,*11:943–946.

Varma J. K., Mølbak K., and Barrett T. J., (2005b). Antimicrobial resistant non-typhoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. *J Infect Dis.,* 191:554–561.

Wain J., Hoa N. T., Chinh N. T., Vinh H., Everett M. J., Diep T. S., Day N. P., Solomon T., White N. J., Piddock L. J., and Parry C. M. (1997). Quinolone-resistant *Salmonella typhi* in Viet Nam: molecular basis of resistance and clinical response to treatment. *Clin. Infect. Dis.* 25:1404–1410.

Wegener H. C., Aarestrup F. M., Jensen L. B., Hammerum A. M., and Bager F. (1999). Use of antimicrobial growth promoters in food animals and *Enterococcus faecium* resistance to therapeutic antimicrobial drugs in Europe. *Emerg. Infect. Dis.* 5:329–335.

WHO. (1999). Food safety issues associated with products from aquaculture: report of a joint FAO / NACA / WHO study group. www.who.int/foodsafety/publications/fs_management/aquaculture/en/index.html (accessed on February 24, 2011)

www.efsa.europa.eu/efsa_locale-1178620753812_1211902132 140.htm *Accessed on December 16, 2009;*

www.fao.org/docrep/008/v4050b/v4050B00.HTM Aquaculture development and research in sub-Saharan Africa, East region of Africa, Aquaculture development and research in Kenya (*accessed on December 3, 2008*).

www.maplandia.com/kenya/western/maseno. (*accessed on October 16, 2008*)

www.ncbi.nlm.nih.gov/sites/gene (*accessed on February 12, 2010*)

www.fallingrain.com/world/KE/7/Maseno.html. (*accessed on October 16, 2008*)

www.answers.com/topic/maseno-1. (*accessed on October 16, 2008*)

http://www.chulaimbohospital.or.ke/index.php?option (*Accessed on February 22, 2011*)

Zlotkin A., Eldar A., Ghifino C., and Bercovier H. (1998). Identification of *Lactococcus garviae* by PCR. *J. Clin. Microbiol.*, 36:983-985.