PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *SALMONELLA* ISOLATED FROM NILE TILAPIA (*Oreochromis nilotics, L.*) ALONG LAKE VICTORIA BEACHES IN WESTERN KENYA

BY:

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ABSTRACT

Enteritis induced by nontyphoidal *Salmonella* represents major economic and social problems. Chemotherapeutic selection may have additional consequences for virulence evolution through acquisition of linked virulence genes. Various foods have been implicated as sources of Salmonellosis to humans. These include poultry, eggs, pork, and fish. Although prevalence of Salmonellosis is high in Western Kenya around Lake Victoria region, no particular food source has been identified to be associated with its transmission. Fish is the main source of protein for people living around the lake. Poor sanitary facilities at fishing beaches are likely to contaminate the lake waters. In Kenya, established quality control measures exist for export oriented but not locally consumed fish. The main objective of this study was to identify *Samonella* species and to perform their phenotypic and genotypic characterization in Winam gulf of L. Victoria. A total of 120 fish specimens were collected of which 63 were positive for various bacteria isolates as determined by standard culture techniques and serotyping. Twenty five (39.7%) were *Shigella sp*., 9 (14.3%) *Salmonella enterica* serotype *typhimurium*, 4 (6.3%) *S. enterica* serotype *enteritidis*, 7 (11.1%) *S. typhi*, 16 (25.4%) *Escherichia coli*, 1 (1.6%) *Proteus sp.,* and *E. aerogenes* respectively. Molecular typing of *Salmonella* involved the amplification of the house keeping gene for *malic acid dehydogenase* (*mdh*) with specific primers. Twenty *Salmonella* isolates had a gel ladder head resolved at 261 bp, which confirmed presence of *malic acid dehydrogenase* gene. Bands of 429 bp amplified regions using random sequence primers ST11 – ST15 further confirmed presence of *Salmonella* species in the isolates. Presence of *Salmonella* in *Oreochromis niloticus* was as a result of contamination of the fish during offloading but not during loading offshore.This study has provided vital data that is critical in assessing and controlling the risk associated with the presence of *Salmonella* in O. *niloticus* in the study area. For absolute safety, elimination of initial contamination of fish by enteric pathogens from the source should be ensured. Hence landing and marketing of fish from beaches with essential sanitary facilities can reduce the risk of cross contamination. Additional safety measures should include training in personal hygiene, sanitation and ensuring water quality.

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CHAPTER ONE

1.0 INTRODUCTION

Salmonella are microorganisms that belong to the family *Enterobacteriaceae* (Collier, 1998), and consist of Gram negative, non-spore forming bacilli. Most strains are motile. They produce acid and gas from glucose, mannitol and sorbitol except for a few that form acid only. They rarely ferment sucrose, adonitol or indole. They form Hydrogen Sulphide (H_2S) gas on triple sugar iron agar and use citrate as sole carbon source. They form lysine and ornithine decarboxylates (Collier, 1998). *Salmonella* causes a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia. Gastroenteritis associated with food borne outbreak is the most common clinical manifestation of the infection (Rodrigue *et al.*, 1990). Following oral ingestion, *Salmonella* colonizes the intestines and invades the intestinal mucosa. Invasion of enterocytes and mast cells results in the extrusion of infected epithelial cells into intestinal lumen followed by villus blunting and loss of absorptive surfaces (Wallis and Edward ,2000). *Salmonella* also elicits a polymorphonuclear leukocyte (PMN) influx into infected mucosa and induce watery diarrhoea, which may contain blood (Wallis and Edward ,2000).

Gastroenteritis being an important problem in developing countries, is normally related to unhealthy sanitary conditions. The development of antibiotic resistance in enteropathogens including *Salmonella* species has increased the problem (Ruiz *et al.*, 1999). Approximately 13 million cases of Salmonellosis occur worldwide annually with 70% of the reported cases occurring in developing countries (Murugkar *et al.,* 2005). According to World Health Organization (WHO) web based surveillance and global *Salmonella* distribution from 2000 to 2002, *Salmonella enterica* serotype *typhimurium*

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was the most commonly reported serotype in all the three years (2000 to 2002), accounting for 17% of non human isolates in 2000 globally (Galanis *et al*., 2006). About 600 deaths occur in Kenya annually from *Salmonella* infections, the most susceptible groups being the elderly, infants and Acquired Immune Deficiency Syndrome (AIDS) patients (Cathrine, 2004). Contamination of food; poultry, pork, beef, eggs, milk,cheese, shell fish, and fish can occur at multiple steps along food chain (Gomez *et al*., 1997; Esaki *et a*l., 2004). Earlier studies on contamination of fish and fisheries products with *Salmonella* have indicated that unless fish is in polluted waters, they would not be infected or they do not ordinarily harbour enteropathogenic bacteria (Janssen *et al.,* 1968). There is possibility that fish exposed to *Salmonella* through contaminated water or feed may become infected with pathogenic bacteria and may finally shed the organisms posing serious health hazard to fish farmers, workers, consumers, and hobbyists (Janssen *et al*., 1968). Human infections caused by pathogens transmitted from fish or the aquatic environment are quite common depending on the season, patients' contact with fish and related environment, dietary habits and the immune system status of the exposed individual. There are often bacterial species that are facultative pathogenic for both fish and man and may be isolated from fish without apparent symptoms of disease. The infection source may be fish kept either for food or as a hobby (Acha *et al*., 2003). However, quantification of the occurrence of these diseases is difficult because of many causes, hence many of such illnesses go unreported. The symptoms usually do not last long and are self-limiting in healthy people. Fish pathogen transmissions have been recorded following injury by cleaning aquarium with bare hands, after exposure to fish tank water, by handling tropical fish ponds, by contact with rare tropical fish (Kern *et al*., 1989; Guarda *et al*., 1992; Alinovi *et al.,* 1993 ; Bhahy *et al*., 2000). Transmissions

have also been found to occur after injuries from fish thorns, after fish bite, through contact with fish living in the wild, by contact with fresh or salt water environment, through processing fish in the food industry and preparation of dishes, or oral consumption of infected fish or related products (Hayman., 1991; O'Shea *et al.*, 1991; Darie *et al.*, 1993 ; Bleiker *et al.*, 1996; Said *et al.*, 1998 Notermans *et al.*, 2000 ; Seiberras *et al.*, 2000). Poor sanitary facilities at the fishing beaches in which feaces and untreated water are discharged into the lake are likely to contaminate lake waters and its resources leading to contamination of Nile tilapia fish.

The use of antibiotics in livestock, fish and poultry has accelerated the development of antibiotic resistant bacteria complicating treatment for both animals and humans. Chemotherapeutic selection may have additional consequences for virulence evolution through the acquisition of linked virulence genes. These routine practices are important factors in the emergence of antibiotic resistant bacteria that are subsequently transferred from animals to humans through food chain (Kariuki *et al.*, 2004). Extensive use of antimicrobial chemotherapeutic agents to common bacterial pathogens cannot be over emphasized (Kakai and Wamola, 2002). Serological methods play important role in identification and characterization of many bacteria species including those of *Salmonella* serotypes necessary for epidemiological evaluation. However, alternative techniques have been developed which allow more rapid and simple identification of isolates (Baudart *e t al.,* 2000)

Multidrug resistant strains of *Salmonella enterica* serotype *typhimurium* phage type (DT) 104 implicated in epidemics in Europe, the United States, and Canada has

reservoirs in cattle and is transmitted mainly through consumption of contaminated meat, milk and milk products (Threfall, 2000). However, there are no data from developing countries including Kenya on the likely sources of non-typhoidal *Salmonella* that causes human infections (Kariuki *et al.,* 2002).

Prevalence of *Salmonella enterica* serotype *typhimurium* was found to be 23.3% and 60% among immunocompromised and immunocompetent children in Western Kenya (Onyango *et al*., 2005). A clinic based surveillance for diarrhoeal diseases conducted between May 1997 and April 1998 in Asembo, a rural area along Winam gulf indicated that out of 729 specimens collected, 33 (14%) were *Salmonella* isolates (Roger *et al*., 2001). In a five month study of *Salmonella* septicemia in Kenyan children in malaria endemic areas carried out during the annual peak infection period (January to March, May, September to October 1989) indicated that *Salmonella enterica* serotype *typhimurium* septicemia occurred seven times more frequently than typhoid or other non typhoid infections (Nesbitt *et al*., 1989). Typical clinical features of fever, diarrhoea, and severe anaemia resembled *Plasmodium falciparum* malaria which they often co-exist with (Nesbitt *et al*., 1989). Mortality was found to be 18% and isolates exhibited a wide range of multidrug resistance (Nesbitt *et al*., 1989). However no particular food source has been linked to the above mentioned cases. The aforementioned data, Nesbitt *et al*., (1989), Rogers *et al*., (2001) and Onyango *et al*., (2005 and 2008) indicated that Salmonellosis is prevalent within Winam gulf of Western Kenya, therefore the main objective of this study was to perform phenotypic and genotypic characterization of *Salmonella* in randomly selected *O. niloticus* (Nile tilapia) fish in Lake Victoria beaches in Western Kenya.

1.1 Statement of the problem

Septicemic Salmonellosis due to *Salmonella enterica* serotype *typhimurium* is documented to occur seven times more frequently than typhoid infections, with mortality rate of 18% in malaria endemic areas (Nesbitt *et al.*, 1989). Isolates exhibited a wide range of multidrug resistance. Clinic based surveillance by CDC/KEMRI for diarrhoeal diseases (May 1997 – April 1998) in Asembo along Winam gulf found 14% of total isolates to be *Salmonella* (Rodger *et al.*, 2001). Brooks *et al.*, (2003 ;2006) also found 12% of nontyphoidal diarrhoea in Asembo .

Fish is the main source of protein for the people living around L.Victoria, and given the prevalence of water and foodborne disease; Salmonellosis, it was important that all possible infection routes of the pathogens be investigated and possible prevention measures recommended. It was with these in mind that the research study was developed.

1.2 Justification and Significance of the study

Fish industry plays an important role in East African economies. It supports 3-4 million people. It contributes about 3% of Uganda's and Tanzania's Gross Domestic product (G.D.P) and 0.4% of Kenya's G.D.P. It earns Kenya US$ 50 million, Uganda US$ 80 million and Tanzania US$ 100 million as foreign exchange (Gitonga , 2006).

L. Victoria is a source of most fresh water fish both for local and export markets in Kenya. Fish from Lake Victoria represents 85% of Kenya's fish supply and constitutes 25% of total catch from Africa's inland fisheries (Gitonga, 2006).

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Fish has been found to be a source of *Salmonella* infection (Massete, 1999). Fish landing environment has been identified as a major source of fish quality problems in Kenya (Nanyaro and Makene ,1998). While established quality control measures exist for export oriented fish, none exist for fish consumed locally, posing health hazards to local consumers (Ogwang *et al*., 2005). Isolation of *Salmonella* species from fish samples will provide information on the possible role of tilapia as a source of infection in the study areas. This would sensitize department of fisheries on the need to inspect and improve fish handling before it reaches local consumers. Information gathered from this study will enable LVEMP, VICRES, fisheries department and fisheries management to evaluate the success of the experiment on multi- hauling practices and procedures introduced after European Union ban on fish from Kenya in 1997 (Makene, 2005), that were meant to eliminate contamination of fish. The results from this study could provide valuable information to agencies and legislators, (FAO, WHO, and Ministry of health) involved in making policy decisions on food safety in Kenya.

1.3 Main objective:

To perform phenotypic and genotypic characterization of *Salmonella* species in randomly selected Nile tilapia (*Oreochromis niloticus*) in Winam gulf of Lake Victoria Western Kenya.

1.3.1 Specific objectives

(1) To identify *Salmonella* species in *O. niloticus* collected from the fishing sites within Winam Gulf; Luanda Kotieno, Uhanya, Dunga and Homa Bay along the shores of L. Victoria in Western Kenya.

(2) To perform phenotypic characterization of bacteria isolated from *O. niloticus* within the study areas mentioned in (1) above.

(3) To perform genotypic characterization of the isolated *Salmonella* species identified in (1) above.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Gastroenteritis

Gastroenteritis is a major cause of morbidity in the developing world (Bern *et al.*, 1992), and is normally related to unhealthy sanitary conditions. Gastroenteritis has the greatest adverse effect on children's growth and development (Black *et al.*, 1984). Approximately 5 episodes of diarrhoea per child per year occur among children below 5 years old and approximately 0.2% of the cases are fatal (Roger *et al.*, 2001). Sub-Saharan Africa is among the regions with the highest morbidity and mortality from diarrhoeal diseases (Bern *et al.*, 1992). Mortality is attributed to diarrhoea that leads to loss of electrolytes and fluids in the body, making the body weak. In normal host, bacteremia accompanies gastroenteritis about 5% of the time, whereas in Acquired Immune Deficiency Syndrome (AIDS), the incidence of bacteremia is much higher (Gooze, 1998). Gastroenteritis associated with foodborne outbreaks is probably the most common clinical manifestation of the infection (Rodrigue *et al.*, 1990).

Salmonella is a genus of Gram negative rod shaped bacteria in the family *Enterobacteriaceae*, that causes a wide range of human diseases such as enteric fever, gastroenteritis and bacteremia. *Salmonella* is mainly an intestinal parasite of vertebrates and are pathogenic for many species of animals. Following oral ingestion, *Salmonella* colonizes the intestines and invades the intestinal mucosa. Invasion of enterocytes and mast cells result in the extrusion of infected epithelial cells into intestinal lumen with consequent villus blunting and loss of absorptive surfaces (Wallis and Edward, 2000). *Salmonella* also elicit a polymorphonuclear leukocyte (PMN) influx into infected mucosa

and induce watery diarrhoea which may contain blood (Wallis and Edward ,2000). Salmonellosis in humans caused by non typhoidal *Salmonella* strains usually results in self limiting diarrhoea that does not warrant antimicrobial therapy. However, there are occasions when infections can lead to life threatening systemic infections that require effective chemotherapy (Helms, 2002). In many developing countries, bacteremia due to invasive non typhoidal *Salmonella* has been associated with an increased risk of death, particularly in children with severe malaria (Oundo *et al*., 2002; Walsh, 2002) and among immunocompromised adults, particularly with HIV and AIDS (Arthur, 2001). Non-typhoidal *Salmonella* bacteremia has also been reported as a complication of the management of severe malarial anaemia causing clinical deterioration after blood transfusion. It is estimated that approximately 13 million cases of Salmonellosis occur worldwide annually, out of which about 70% of the reported cases come from China, India, and Pakistan (Murugkar *et al*., 2005). The development of antibiotic resistance in enteropathogens including *Salmonella* species has increased the problem. Resistance to some β – lactam antibiotics, tetracycline, chloromphenicol or trimethoprim is reported with increasing frequency (Ruiz *et al*., 1999). Among the resistant *Salmonella* serotypes is *Salmonella enterica* serotype *typhimurium* (White *et al*., 2003). The resistant strains have caused severe infections and death in animals and humans worldwide (White *et al*., 2003; Kariuki, 2004).

Bacteria constituting the genus *Salmonella* posses 3 different types of antigens. The agglutinating properties of the somatic 'O', flagella 'H' and capsular 'Vi' antigens are used to differentiate among more than 2,500 serologically distinct types of *Salmonella* (Popoff *et al*., 2003). Following the scheme of Le Minor (Le Minor, 1984),

enterobacteria of the genus *Salmonella* belong to a single species *Salmonella enterica* and seven subspecies have been identified by applying biochemical tests. Within *S. enterica* subspecies *S. entericae*, the most common 'O'-antigen serogroups are A, B, C_1, C_2, D and E. Group D, *Salmonella enterica* serotype *typhimurium* is more virulent than group B, *Salmonella* (Fierer, 2001).

It is estimated that 2 to 4 million cases of Salmonellosis and more than 500 deaths occur in United States annually (FDA, 2006). In 1985, 16,000 confirmed cases of Salmonellosis in Chicago dairy farm in United States were reported . Approximately 95% of the human *Salmonella* infections are foodborne corresponding to approximately 30% of the deaths caused by food borne infections in United States. In most developed countries, outbreaks of non-typhoidal *Salmonella* infection have been caused mainly by *Salmonella enterica* serotype *typhimurium* and *S. enterica* serotype *enteritidis* (Ridley *et al*., 1998; Threfall *et al*., 2003).

Salmonella infection is even more detrimental in the developing world. In developing countries, non typhoidal *Salmonella* (NTS) accounts for an increasing proportion of human infections and represent about 20 to 30% of *Salmonella* serotypes, in particular multi drug resistant *Salmonella enterica* serotype *typhimurium* which causes serious outbreaks (Kariuki *et al*., 2004). In Africa alone, in the year 2002, nine hundred and six five *Salmonella* species were isolated (Cameroon, 247; Mali, 334; Morocco, 76; Senegal, 220; Tunisia, 388) Galanis *et al.,* (2006). In Zaire and Rwanda, multidrug resistant *S. enterica* serotype *typhimurium* was the main cause of bacteremic illness in children (Lepage *et al*., 1990; Green *et al*., 1993).

In Kenya *S. enterica* serotype *typhimurium* was the main isolate in adults with *Salmonella* bacteremia (Kariuki *et al.*, 2000; Arthur, 2001). A previous study by Onyango *et al.,* (2005) in Western Kenya also indicated that prevalence of *S. enterica* serotype *typhimurium* was 23.3% and 60% among immunocompromised and immunocompetent children respectively. Approximately 95% of these cases were foodborne and of animal origin.

2.2 Aetiology and sources of infection

Outbreaks of gastroenteritis infections are often a result of exposure to a common source of etiological agent (Michael and Bean ,1999). Infection is usually through direct or indirect contact with animal and human faeces or in the environment or contact with contaminated food, water or objects such as feeding bowls (Daniel *et al.*, 2003). The majority of the 1.3 billion annual cases of *Salmonella* – caused human gastroenteritis result from ingestion of contaminated food products, such as undercooked beef, pork, eggs, milk, Cheese, Shellfish and fish (Pang, *et al.*, 1995; Gomez *et al.,* 1997; Esaki *et al.,* 2004). *Salmonella* infections are also contracted following consumption of fresh fruits or vegetables contaminated by infected fertilizer (Tauxe, 1997). Reptilian pets have also served as reservoirs and hence sources of *Salmonella* for infected owners (Kourany *et al.*, 1970; Sanyal *et al.,* 1997). This indicates that *Salmonella* species lack special host adaptations and are capable of colonizing a wide variety of macro - organisms (Foltz, 1969). Their ubiquitous nature facilitates a cyclic lifestyle consisting of passage through a host into the environment and back into a new host (Thomason *et al.,* 1977). Birds and flies are important vectors for rapid widespread dissemination of *Salmonella* in the environment (Davies and Wray, 1996). Flies that come in contact with contaminated

materials (manure, food, water) are capable of transmitting *Salmonella* (Mian *et al.*, 2002). Rapid dissemination of *Salmonella* between hosts also occurs by passage of bacteria from infected farm animals to vegetables as a result of field fertilization with raw contaminated manure. Compared to *E. coli, Salmonella* withstands a wider variety of stresses associated with environmental fluctuations and may persist in water environment for sometime. *Salmonella* can be disseminated in soil and sediment even in the absence of active fertilization, as a result of water currents, underground springs, and rain run off carrying contaminated material (Chao *et al.,* 1987; Abdel-Monem *et al.,* 1990). Percolation of waste water through soil filters out bacteria that become trapped in this environment (Chao *et al.,* 1987). Animals are important sources of *Salmonella* for food borne human disease. Animals consume *Salmonella* from soil or contaminated processed feed. The bacteria are then shed alive in the infected animals' feaces. During slaughtering and processing, *Salmonella* may contaminate animal carcasses. Contact of *Salmonella* with flowers, stems or fruits of tomato plants leads to infiltration and colonization of plant tissues (Guo *et al.,* 2001; 2002). Presence of bacteria on household surfaces has important health consequences. Survival of *Salmonella* in bathrooms and toilets following bacterium – induced illness is known to demonstrate high contamination levels for up to several weeks (Barker *et al*., 2000).

Salmonella is frequently isolated from water sources (Cherry *et al*., 1972) which serve as bacteria reservoirs and may aid transmission between hosts (Foltz, 1969). Like *E. coli*, *Salmonella* is constantly released into the environment from infected humans, farm animals, pets and wildlife (Baudart *et al.,* 2000). Despite efforts to contain and sanitize human waste, *Salmonella* can survive for 10 to 15 days in a septic system

(Parker *et al.,* 1982). Seepage from septic tanks and sewage injection well fields as well as sewage and storm runoff facilitate bacterial passage into surface waters (Paul *et al*., 1995; Baudart *et al*., 2000).

Detection of *Salmonella* correlates with proximity to sewage discharge area (Alonso *et al.,* 1992, Baudart *et al.,* 2000). For example *Salmonella* was isolated from 65% of the water samples collected along Peavine Creek in Decatur Ga, which flows through urban industrial and residential areas (Cherry *et al.,* 1972). In addition , *Salmonella* was detected within 350 feet of the subsurface origin of a small stream in the same area suggesting that contamination occurs rapidly once water emerges from the earth (Cherry *et al.,* 1972). Compared to other bacteria, *Salmonella* has high survival rates in aquatic environments (Chao *et al*., 1987) .It outlives both *Staphylococcus aureus* and the waterborne *Vibrio cholerae* in ground water and in heavily eutrophied river water (DiRita, 2001).

Industrialization, mass food production, decreasing trade barriers and human migration have disseminated and increased the incidence and severity of food borne diseases world wide (Gomez *et al*., 1997; Todd, 1997). The most common causes of illness is eating improperly prepared or stored foods. When food is improperly handled, *Salmonella* bacteria are able to proliferate and produce toxins that cause illness. Anything contaminated with *Salmonella* that is not heated to destroy the bacteria before being eaten may cause illness. After ingestion, symptoms begin within 1 to 3 days and may include abdominal pain, diarrhoea, fever, and sometimes vomiting. The illness usually lasts for 4 to 7 days and most persons recover without treatment. However in some persons, the

diarrhoea may be severe. In these patients, the *Salmonella* infection may spread from the intestines to the blood stream and other body parts. At this point it can cause death unless the patient is treated with antibiotics (Velge *et al*., 2004)

In recent years, concerns have been raised about particular strains of bacteria that have become resistant to traditional antibiotics in both animals and humans (Velge *et al.*, 2004). The use of antibiotics in livestock, fish and poultry has accelerated the development of antibiotic resistant bacteria, complicating treatment for both animals and humans. Chemotherapeutic selection may have additional consequences for virulence evolution through the acquisition of the linked virulent genes (Velge *et al*., 2004). A number of studies have indicated the development of resistance strains from the use of antimicrobials in human medicine, veterinary medicine, animal husbandry as well as agriculture practices (Velge *et al*., 2004). In many developed countries, most antimicrobial resistant *Salmonella* infections are acquired from eating contaminated foods of animal origin (Angulo *et al*., 2000; Gorman, 2004). Resistance to combinations of several classes of antimicrobials has led to the emergence of multidrug resistant (MDR) strains that may be transmitted through food to humans (Threfall *et al*., 1997). One notable MDR strain is *Salmonella enterica* subspecies *enterica* serovar *typhimurium* definitive type 104 (DT 104). It was first recognized in the United Kingdom (Threfall *et al*., 1994) and since then has been reported in many parts of the world (Kariuki *et al*., 1992) and from various host species including pets (Threfall *et al*., 2002). Another important phage type of serovar *typhimurium* often exhibiting MDR is DT 193, a MDR strain responsible for outbreaks in humans in the late 1980s and early 1990s, mainly in Europe (Threfall, 2000). Two of the major outbreaks due to DT 193 in Italy and United

Kingdom were traced to contaminated pork products. Multidrug resistant strain of *S. enterica* serotype *typhimurium* phage type (DT) 104 implicated in epidemics in Europe, the United States and Canada has reservoirs in cattle and is transmitted mainly through consumption of contaminated meat, milk and milk products (Threfall, 2000). However, there is no data from developing countries including Kenya on the likely sources of nontyphoidal *Salmonella* that cause human infections (Kariuki *et al*., 2002).

Previous study by Kariuki *et al.,* (2000) working on cases in Kenyatta National Hospital Nairobi Kenya isolated 52.3% of *S. enterica* serotype *typhimurium* and 38.4% of *S. enterica* serotype *enteritidis* of total *Salmonella* serovars. It was however difficult to incriminate any particular common food source. Clinic based surveillance for diarrhoeal diseases conducted in Asembo, a rural area within Winam gulf in Western Kenya, between May 1997 – April, 1998, showed that 33 (14%) out of 729 diarrhoeal specimens collected had *Salmonella* isolates, but the main source of infection was unknown. In relation to that, studies by Onyango *et al*., (2005) in Western Kenya found prevalence of *S. enterica* serotype *typhimurium* to be 23.3% and 60% among immunocompromised and immunocompetent children respectively without knowledge of the main source of infection. Thus there was need to discern the source of infection within the selected beaches.

2.3 Role of fish in transmission of enteric pathogens

Human infections caused by pathogens transmitted from fish or the aquatic environment are quite common depending on the season, patients' contact with fish and related environment, dietary habits and the immune system status of the exposed individual.

There are often bacterial species that are facultatively pathogenic for both fish and man and may be isolated from fish without apparent symptoms of disease. The infections source could be fish kept either for food or as a hobby (Acha *et al*., 2003). However, quantification of the occurrence of these diseases is difficult because of many causes, typically gastrointestinal illness go unreported. The symptoms usually do not last long and are self-limiting in healthy individuals. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include *Mycobacteria*, *Streptococcus iniae*, *Vibrio vulnificus, Vibrio* spp., *Aeromonads*, *Salmonella* spp. and others (Lipp *et al*., 1997; Zlotkin *et al.,* 1998; Bhaftopadhyay, 2000). Human infections by this fish pathogen are usually through contact with infected fish while handling them, water or other constituents of fish life environment (Acha *et al.,* 2003).

Fish pathogen transmissions have been recorded following injury by cleaning aquarium with bare hands, after exposure to fish tank water, by handling tropical fish ponds, and by contact with rare tropical fish (Alinovi *et al.,* 1993; Kern *et al*., 1989; Guarda *et al*., 1992; Bhahy *et al*., 2000). Transmissions have also been found to occur after injuries from fish thorns, after fish bite, through contact with fish living in the wild, by contact with fresh or salt water environment, through processing fish in the food industry and preparation of dishes, or oral consumption of infected fish or related products (Hayman., 1991; O'Shea *et al*., 1991; Darie *et al*., 1993 ;Bleiker *et al*., 1996; Said *et al*., 1998; Notermans *et al*., 2000; Seiberras *et al*., 2000).

Apart from factors relating to the living environment (exposure), the development of an infectious disease is markedly affected by internal factors such as the physiological status

of consumer, particularly by immunosuppression and stress as in the case of HIV/AIDS (Von-Reyn *et al.*, 1996). Fish and shellfish appear to be passive carriers of *Salmonella*, they demonstrate no clinical disease and can excrete *Salmonella* spp. without apparent trouble.

The contamination of this organism is derived from terrestrial sources and fish may serve as a source for *Salmonella* spp. infection (Metz, 1980; Minete, 1986; Fell *et al.*, 2000). An outbreak of *S. blockley* infections following smoked eel consumption occurred in Germany (Fell *et al.*, 2000). The consumed eel came from four different local smokehouses, but could be traced back to fish farms in Italy .

This outbreak indicated that eel may be a source of *Salmonella* spp. infection and that the smoking process may not eliminate bacterial contamination from raw fish. *S. enterica* serotype *Paratyphi B* var. Java phage type Dundee was isolated from the stool of a 14-month old boy who suffered from diarrhoea, vomiting, and fever for two days. The same isolate was identified from the water of home fish tank (Senanayake *et al.*, 2004). Fish was the source of *Salmonella* spp. in this case. Most outbreaks of food poisoning associated with fish is derived from the consumption of raw or insufficiently heat treated fish, which may be contaminated with bacteria from water environment (*Vibrio* spp., *C. botulinum*) or terrestrial sources (*C. perfringens*, *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *V. cholerae*), or fish products recontaminated after heat processing (Senanayake *et al.*, 2004).

The spread of drug resistance among bacteria species that have emerged as human pathogens causing diarrhoea diseases has raised concern (Singh and Sanyal, 1997). This is an indication of prevalence of contamination of fish by bacteria leading to constant use

of antimicrobials. Studies on antibiotic multiresistant bacteria confirms the prevalence of bacterial infection leading to continuous use of antimicrobials in the production of ornamental fish (Del Rio-Rodriguez *et al.*, 2002).

Many farmers use antibiotics either to treat fish or and as growth promoters. A survey of fish farms in Trinidad indicated that 67% of the farmers use antimicrobial agents in one way or the other (Newaj-Fyzul *et al.*, 2005). Ornamental fish are extensively produced in fish farms or large aquaria systems where diseases are common and antimicrobials are routinely used for prophylaxis and treatment including in the shipment water (Meier and Schmitt, 1992). A study carried out to investigate prevalence of *Salmonella* in ornamental fish in Trinidad indicated that 2.3% of fish slurry was contaminated with *Salmonella* and 7.1% shipment water was also contaminated with *Salmonella.* This indicates that fish slurry and pond water infested by *Salmonella* could transmit *Salmonella* to their owners and other handlers. The high resistance to commonly used antibiotics may have therapeutic problems in management of fish in the country (Newaj - Fyzul *et al.*, 2005).

In Singapore, a large number of antibiotics commonly employed in humans and veterinary medicine have been used for the treatment of diseases in the ornamental fish industry. Singapore and South America are the two main shippers of ornamental tropical fish to the United Kingdom (Cheong, 1996; Davenport, 1996). Despite the risks that ornamental fish represent as a source of potentially harmful bacteria for fish and man (Del Rio-Rodriguez *et al.*, 2002), but research on this risk is poor. One of the consequences of the increased use of antimicrobials in fish therapy has been emergence of resistant bacterial fish pathogens, which in turn have limited options of antimicrobial treatment available (Lewin, 1992). Shotts *et al.*, (1997) found antibiotic multidrug

resistant isolates of motile bacteria in water and ornamental tropical fish imported from South East Asia. Isolation of antibiotic multidrug resistant bacteria species and its plasmids isolated from food fish production site in South East Asia represented a major economic problem for the fish producers (Saitanu *et al.*, 1994). Ansary *et al.,* (1992) defined the antibiotic resistance profile and frequency of plasmids carriage of 34 strains of bacteria isolated from several species of cultured food fish in Malaysia, an indication of infestation of food fish with bacteria.

Antibiotic resistant strains of bacteria have also been isolated in cultured food fish in Thailand (Saitanu *et al.*, 1994) and in Asia (Inglis *et al.*, 1997). These multidrug resistant capabilities and homogeneity may in turn account for their survival and prevalence in fish systems where antibiotics are applied (Sugita *et al.*, 1989). Researchers have raised concern regarding the possibility of transfer of multidrug resistant bacteria from tropical aquarium fish to cold water species or transfer of plasmids conferring antibiotic resistance from tropical aquarium fish bacteria to bacteria that cause disease in cold water species. Hence the multidrug resistant strain of bacteria harboured in ornamental tropical fish poses a threat to native fauna, aquaculture and humans. Experimental evidence indicate that when fish is infected with *Salmonella* it can maintain the infection for 39 weeks (Brunners, 1974) during which it transmits the pathogen to the other animals in contact.

The biochemical composition of fish or intrinsic factors and their interrelationships with post-mortem extrinsic factors contribute substantially to the perishability of fish as a food commodity because they determine the initial contamination (Huss *et al.*, 1997). The

physical, chemical and bacteriological characteristics of fish tend to vary with species, feeding habits, seasonality, spawning cycles, methods of catching, fishing ground, environment, initial microbiological load and possibly geographical location. Microbiological form of spoilage is more prevalent than either enzymatic or oxidative rancidity (Liston, 1980) accounting for a higher percentage of spoilage in the fish industry (Shewan, 1961). This prevalence has been attributed to a number of interactive spoilage both intrinsic and extrinsic factors and the wide range of growth tolerance limits of spoilage bacteria (Huss *et al.*, 1997).

Bacteria are characterized according to their optimal growth requirements which include among others water, oxygen, pH, temperature and redox potential (Eh). Bacteria living on the surface of marine animals are phenotypically capable of utilizing amino acids, peptides and other non-carbohydrate sources (Liston, 1980). Utilization of these substrates leads to production of slightly alkaline conditions especially in the stored fish products . *Enterobacteriaceae* including coliforms are not isolated from fish captured away from the coastline. Its presence on fish implies that the catch was mishandled at landing beaches (FOSRI, 1997). Bacteria from skin and gills are predominantly aerobic although facultative bacteria particularly *Vibrio* may occur in high numbers on pelagic fish. Obligatory anaerobic bacteria are common on the surface of fish but occur in significant numbers in the intestines (Bramsnaes, 1965). Bacteria on fish from temperate waters are mostly Gram negative, while those on fish from tropical warm waters are Gram positive (Liston, 1980; Huss, 1994). Common bacteria found on living fish in warm waters include *Bacillus, Micrococcus* and *Corynebacterium* which constitute 50-60% of total microflora. Microflora found on fish from cold waters are *Psychrobacter*

(*Moraxella*), *Pseudomonas*, *Actinobacter, Shewanella, Flavobacterium, Cytophaga* and *Vibrio.* Microflora on fresh water fish exhibit similar pattern of preference except that *Aeromonas* replaces *Vibrio*. The pathogenic bacteria namely: *Campylobacter jejuni, Yersinia entercolitica, E. coli, Shigella spp*, and *Salmonella spp,* have all been isolated from fish taken from waters subjected to human sewage pollution or terrestrial run-off (Njiru, 2005). *Shigella* species is specific and adapted to humans and primates, and its presence in environment is associated with feacal contamination (Rodger *et al.*, 2001). *Shigella* strains are the cause of shigellosis (bacillary dysentery), an infection of the gut. Food borne outbreaks have been linked to contaminated water. Seafood, including shrimp cocktail and tuna salads has been implicated in a number of outbreaks of shigellosis.

E. coli resides in the lower intestines of warm blood animals and man, an environment that provides a lot of nutrients for bacterial growth. Strains that colonize intestinal tract are non pathogenic and maintain intestinal physiology (Huss, 1994). Four pathogenic strains have been isolated in environments polluted with feacal material or sewage namely; Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC) (Verocytoxin producing *E. coli* (VETC) or *E. coli* 015: 47, Enteroinvasive *E. coli* (EIEC). Most infections with *E. coli* are related to contamination of water with faecal materials or sewage or handling of food under unhygienic conditions (Huss, 1994). Spoilage bacteria are characterized both by their dominance in the microflora of spoiling fish and their ability to produce spoilage compounds. Accumulated metabolic products of bacteria are the primary causes of organoleptic spoilage of raw fish. The microbial activity on reduced compounds produce

the characteristic fishy, ammonia and sulphide odours and change the texture to slimy and pulpy constitution associated with spoiled fish (Howgate, 1982).

The method of capture has profound effect on keeping quality of fish. Fish caught by hook and line die or are killed faster when brought into air and the method minimizes stress and its associated deterioration attribute. Fish caught by seine net has a better quality index than fish caught in a trawl net, which tends to compact fish and in so doing presses out guts with their high bacterial contents. The spilled bacteria utilize the available substrate and their metabolic products constitute deterioration. Gill netting entails fish struggling, which in turn quickens the onset of rigor and subsequent deterioration (Mayer and Ward, 1991; Sorensen and Mjelde, 1992). The bacteria may gain access through puncture wounds and bruises during death struggle and may multiply rapidly in these localized areas. The degree of struggling before death reduces the levels of glycogen in the fish muscles, which has a negative impact on quality as far as texture is concerned. A method that inflicts stress or struggle hastens the onset of spoilage. The population of microbes associated with live fish reflects microflora of the environment or fishing ground at the time of capture and this constitutes to initial microbial load. The initial microflora on the surface of fish is directly related to the water environment while the flora in the gastrointestinal tract corresponds to the type of food and condition of the fish (Liston, 1980).

According to Huss *et al.*, (1997), intrinsic and extrinsic factors determine the initial bacterial contamination. This is further enhanced by the poikilothermic nature of fish, the presence of trimethylamine oxide (TMAO) which increases the redox potential, the presence of large amounts of non protein nitrogen (NPN) and low carbohydrate content

which increases the pH in the flesh post-mortem. Bacterial numbers range from 10^2 to 10^6 CFU/cm^2 on the skin, 10^3 to 10^5 on the gills and in the intestines, from very few in non-feeding fish to 10^7 or greater in feeding fish (Liston, 1980; Mayer and Ward, 1991). During storage, the counts on a given morphological entity may increase by a minimum of one logarithm (Liston, 1980).

The intrinsic and extrinsic factors are interrelated during post mortem fish spoilage and their effects are only enhanced by inappropriate handling practices and facilities along the handling chain. Thrower (1987), Ward and Baj (1998) documented various post-harvest practices and storage procedures which enhance microbiological contamination and subsequently influence spoilage. During capture operations, fish come in contact with nets, ropes, deck boards, human hands and clothing. This contact continues during packing and storage operations below deck. Extensively handled fish may carry significant numbers of gram-positive bacteria, some of which may be spoilage or pathogenic bacteria. Gutting of fish is one of the practices where fish is exposed to agents of spoilage (Huis In't Veld, 1996). Offloading operations provide opportunities for bacterial contamination through offloading equipments, pumps, conveyers, baskets and boxes that redistribute surface contamination (Huis In't Veld, 1996). Insufficient cleaning may lead to bacterial build up which in turn will act as a source of subsequent contamination. Public auction markets where fish may be displayed on or in wooden, metal or plastic containers in the open are potential bacteriological sources. Delays in chilling when ambient temperatures are high can shorten shelf life during subsequent storage (Liston, 1992). The type of production material and design of the containers may not allow them to be cleaned and disinfected effectively. The presence of Vermin e.g.

rodents, birds, domestic animals and people (handlers and auctioneers) are additional sources of contamination (Thrower, 1987). At the fish processing plant, further handling occurs during wet processing operations like sorting, filleting and trimming. These operations transfer Gram-positive bacteria usually associated with humans directly from fish and gut to filleted flesh surfaces. It has been estimated that fillets and other products from fresh fish processors usually carry counts of 10^3 to 10^5/g or more (Liston, 1980). If the product is refrigerated later on, the dominant bacteria on the product after a day or two of storage will be Gram negative due to inability of Gram positive bacteria to grow competitively under refrigeration. The bacteria may also be transferred from the processing environment such as contaminated surfaces, knives, and machines.

Most of the animal protein consumed by the local population in Western Kenya comes from Lake Victoria (Abila *et al.*, 1997). However preliminary observations on landing beaches indicates that a much lower quantity of fish, composed mainly of immature fish and fish rejected by factory agents for being of poor quality remain behind for local consumption (Abila *et al.*,1997). Mitigation of environmental problems in Lake Victoria indicates four immediate microbiological pollution sources, namely municipal untreated sewage, runoff, and storm water and animal waste , maritime transport wastes ,of which municipal untreated sewage, runoff, and storm water are the most important immediate microbiological pollutants (Kayambo and Sven, 2006). Direct discharge of municipal untreated effluent into rivers and the lake directly contribute to degradation of rivers and lake-water quality for habitats and domestic use. The low standard of health in the Winam Gulf region are caused by a general lack of awareness of good hygiene practices, direct contamination of beach waters through bathing and washing and uncontrolled

waste disposal around the shore line (Kayambo and Sven, 2006). The effluents have significantly increased biological oxygen demand (BOD) load thus significantly increasing the occurrence of water borne diseases such as typhoid and cholera which are common in the region (Kayambo and Sven, 2006). The effect of runoff and storm water that collect a lot of animal, plant and human wastes from point and nonpoint sources and channel these to the rivers and the lake, create an environment that supports microbiological pathogens. The number of people without sewers in urban populations is high, and with 10% per year in most of the larger towns (against 2-4% in most parts of the lake basin), the situation is likely to get worse. In rural areas there are no sewers at all. Other sectors like wildlife, agriculture, urban, forestry and rural settlement have been implicated to contribute to microbiological pollution of the lakes (Kayambo and Sven, 2006). These activities increase eutrophication process thus creating a vast conducive environment for the survival of microbes which eventually infect the fish. *Salmonella spp.* have in recent years been isolated from various fresh and marine waters contaminated by urban sewage (Ogindo, 2005). Veterinary authorities from Spain and Italy detected an unacceptable level of bacteriological contamination in fish from all the three East African countries (Abila *et al.*, 1997). As a result, in March 1997, the European commission directed that all fish imports from the region be subjected to bacteriological examination for *Salmonella* and other pathogens on entry to the European Union (Abila *et al.*, 1997). An inspection mission was therefore sent to the three countries in May 1997 to assess the situation in detail. In 1998, European Union (EU) banned fish exports from East Africa as a result of cholera outbreak in E. Africa and Mozambique, while in 1999 the ban was due to suspicion of presence of pesticides in Lake Victoria.

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The last ban which was due to claims of bacteriological contamination was lifted in April 2000 for Tanzania, August 2000 for Uganda and November 2000 for Kenya (Gitonga, 2006). While established quality control measures exist for the export oriented fish according to the European Union Directive on Hygiene (91/493/EEC), that deals with handling and processing of fish from the point of capture to its eventual arrival on the market, none exists for the fish consumed locally and this poses a great danger to the health of local consumers (Ogwang *et al.*, 2005).

Regardless of earlier ban of fish imports from Kenya in 1996 by European Union citing poor sanitary conditions at landing beaches and lack of refrigeration facilities, studies carried out along L.Victoria indicated poor sanitation conditions with 83% of the population using pit latrines and 14% not using toilets at all (Rwabigene, 2005). During heavy rains, the pit latrines overflow flushing the faecal waste directly into the lake. The condition has been worsened by discharge of untreated effluents directly into the lake. The impact of run off waters has been high pollution rates in the L. Victoria and its resources with pathogens from faecal waste and untreated effluents contaminating the water and infecting tilapia fish. Constant discharge of untreated effluents erode rocky shores which are breeding sites for fish causing disturbances of breeding ecosystem, leading to reduction of fish population and diversity. (Njiru, 2005). Degradation of wetlands which are potential filters trapping sediments, nutrients and pathogenic bacteria has exposed fish to contamination. Over enrichment of the lake have resulted in eutrophication creating high biological oxygen demand at lake shores where waste water is being discharged, leading to water borne diseases and loss of fish species (Okungu *et al.*, 2005). Recommendations by Lake Victoria Environmental Management Project

(LVEMP) to introduce multihauling practices and procedures to improve fish quality during landing and to eliminate fish contamination by inshore waters has not been adhered to due to lack of follow up, hence fish within these beaches are likely to be contaminated (Ogwang *et al*., 2005).

2.4 Phenotypic Characterization of *Salmonella* species

Pathogenic bacteria posses a variety of virulence associated surface structures. These are: lipopolysaccharide, capsules, surface layers , flagella and fimbriae that are used for phenotypic identification. These structures react variedly to different test chemicals both on suspension and on solid media plates.

Phenotypic identification of *Salmonella* involves culturing the specimen in various differential and selective media. Prior incubation of the specimen on Selenite F broth (enrichment medium) is done for maximum recovery of the microorganisms. The isolates are then subcultured on differential (MacConkey) and selective (DCA, KIA, SIM, TSI,) media. The Kauffmann –White Scheme is a serological method used for phenotypic classification of *Enterobacteriaceae* that test for cell wall lipopolysaccharide protein 'O' antigen and 'H' flagella protein agglutination. This method has been used widely by microbiologists in identification of unknown bacteria isolates. The method was developed by WHO testable antigens and is used to associate the serovar to a particular identification group (A,B,C. or D). According to Kauffmann –White Scheme method of classification, *Salmonella* were classified broadly into four subgenera based on biochemical reactions (Brenner, 1998).

Serological classification of *Salmonella* is by Kauffmann White Scheme which forms the basis of serotyping of *Salmonella* and depends on the identification by agglutination of the structural formula of 'O' and 'H' antigens of the strains (Brenner, 1998). Most strains of *Salmonella* closely resemble each other biochemically, although there are a number of exceptions. No organism should be excluded on the basis of a single test. The usual reactions include (1) fermentation of glucose, maltose, mannitol and sorbital with production of acid and gas; 2) absence of fermentation of salicin, sucrose and adonitol; (3) failure to produce indole, to hydrolyse urea, or to deaminate phenylalamine and (4) a positive methyl red reaction and a negative Voges -proskauer reaction (Brenner, 1998).

Antimicrobial resistant bacterial diarrhea is a significant public health problem throughout the developing world. Laboratory services are an essential component of curative and preventive health care activities worldwide. Laboratory investigations are a vital part of the clinical assessment and the results determine selection of drugs for patient management. In addition to confirmatory diagnosis, laboratory records can be used for disease and drug surveillance. The importance of surveillance of the antimicrobial susceptibility pattern cannot be over emphasized as a guide to the use of antimicrobial agents.

Unfortunately, these laboratories are far from offering antibiotic sensitivity testing services. Frequently, antibiotics are prescribed and administered to patients without laboratory diagnosis and determination of antibiotic sensitivity patterns. Worse still, the administration of antibiotic is often given to patients with viral or fungal infection. Broad spectrum antibiotics are frequently given in place of specific ones to substitute for

bacterial identification and sensitivity testing. Intensive use of antimicrobial agents for prophylaxis and treatment of patients in hospitals make them a prime site for emergence, maintenance and spread of resistant pathogens. This may lead to drug wastage and emergence of more resistant bacterial strains (Kakai and Wamola, 2002).

Established conventional methods to detect and identify *Salmonella* require selective enrichment and plating followed by biochemical tests. The diagnostic value of biochemical traits is generally combined with serological characterization, resistance to antibiotics, plasmid profiling, and phage- typing assays. (Bennasar *et al.,* 2000). The whole process requires several days. It also requires more than 150 specific serum (Klinger, 1998). Some strains cannot be identified due to the untypeability of isolates.

The shortcomings of phenotypically based typing methods have led to the development of typing methods based on the, microbial genotype which minimize problems with typeability and reproductibility and in some cases enable the establishment of large databases of characterized organisms (Michael and Bean, 1999). Genotypic methods also provide a higher power of discrimination and allow a more rapid identification attainable by non specialized laboratories (Bennasar *et al* 2000).

Problems affecting the operations of laboratories at the peripheral level are widespread. These include lack of properly designed laboratory rooms, unreliable access to clean water or electrical power, shortage of equipment and supplies, lack of effective equipment, maintenance services and little regular support supervision from higher units. Consequently, the laboratories are functioning below capacity (Waiyaki , 1993).

2.5 Genetic characterization of *Salmonella*

All strains of *Salmonella* appear to be at least 70% related to each other by DNA hybridization (Crosa, 1973) and are considered members of a single genospecies (Lindberg, 1984). A part from the use of intergenic spacer regions that show extensive sequence and length variations which can be used to characterize bacteria at genus and species level (Jensen, 1993; Dolzani., 1995) and subspecies (Dolzani, 1995; Beltran, 1998), it is also documented that the house keeping genes e.g. *malic acid dehydrogenase (mdh)* can be used for *Salmonella* species identification (Dolzani, 1995). Random sequence primers ST11-ST15 are also used for *Salmonella* species identification. ST11-ST15 are DNA fragments corresponding to regions of the genome which are highly conserved in *Salmonella* species. They enable target nucleic acid sequence from *Salmonella* to be selectively amplified and detected. Two structural genes (*fliC* and *fliB*) coding for flagellins are used for phenotypic identification in most *Salmonella* strains. *fliC* alleles have conserved sequence on the distal parts of the gene, therefore making the gene of any serotype easy to amplify and hence for species identification (Macnah, 1987). During long standing association with its host, *Salmonella* has evolved a sophisticated mechanism to modulate host cell functions. Many virulence associated genes are acquired during evolution of pathogenic bacteria via horizontal gene transfer. These genes are often clustered on the plasmids or on chromosomal 'Pathogenicity Island' and 'Pathogenicity Islets'. There are various bacterial host cell attachment mechanisms, one such strategy found within certain bacteria that infect epithelial cell surfaces is mediated by fimbriae (Korhomen, 1990).

Each *Salmonella* genome has approximately 12 fimbrial operons, many of which play a role in virulence (Townsend *et al.*, 2001). Most of these fimbrial operons share fundamental similarities in genetic organization (Hultgren *et al.*, 1992). In addition to the fimbriae that are shared between serovars, *Salmonella enterica* serotype *enteritidis* contains SEF14 fimbriae that are missing in *S. enterica* serotype *typhimurium*. SEF14 fimbriae require 4 proteins for biosynthesis. The major subunit *(sef A*), Chaperon (*sef B*) and Usher (*sef C*) have previously been characterized (Thorns *et al.*, 1990; Clouthier *et al.*, 1993; Turcotte and Woodward, 1993). The minor subunits (*sef D*) was previously identified but not thought to be part of SEF14 fimbriae (Clouthier *et al.*, 1994). SEF14 fimbriae have a role in virulence in that they are essential for binding to microphages (Edwards *et al.*, 2000). DNA sequence from SEF14 operon of *S. enteritidis* have shown that *sef A* encodes the most abundant subunit of the fimbrial shaft. It is also documented that *sef ABC* genes are transcribed as part of a single mRNA transcript and that *sef B* and *sef C* are not expressed in the absence of *sef A* and no transcription start site or promoters are found immediately upstream of *sef B* or *C*, hence the transcription of *sef B* and *sef C* is initiated from *sefA* promoter region.

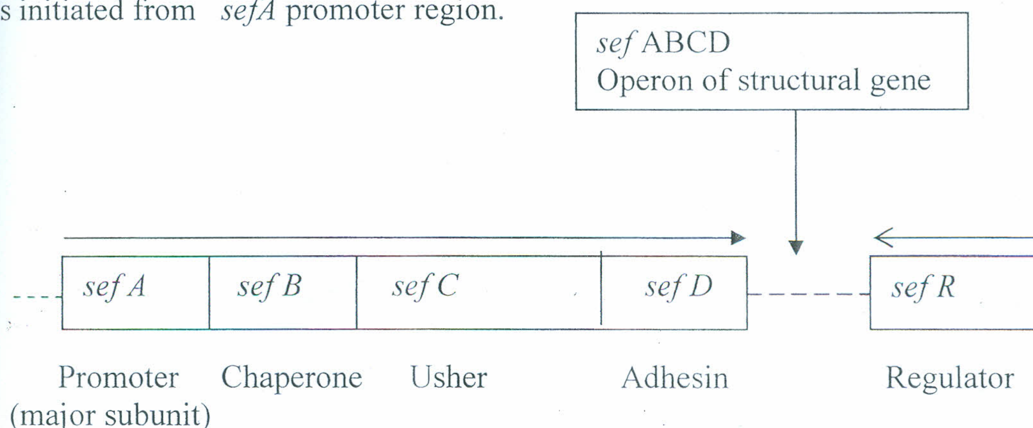


Fig. 1 *sef* pathogenicity island: Organization of the genes, direction of transcription (arrows), and predicted functions of the ORFs. (Adapted from Robert *et al.*, 2000).

Due to the importance of *Salmonella* in food borne diseases, many typing methods have been used to trace the outbreaks to the contaminated source and elucidate the epidemiology of its infections. The most commonly employed molecular typing methods based on characterization of plasmid DNA includes; plasmid profile typing, plasmid fingerprinting and identification of plasmid mediated virulence genes (Collier, 1998). Chromosomally based methods employed are ribotyping, random cloned chromosomal sequence (RCCS) typing, Insertion sequence (IS) 200 typing, pulse field gel electrophoresis (PFGE) and polymerase chain reaction (PCR). Some of the random amplified polymorphic DNA typing (RAPD) methods used include enterobacterial repetitive extragenic consensus typing (ERIC-PCR) and repetitive extragenic palindromic element typing (RER-PCR).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study sites

Sample collection was done in four fishing beaches: Uhanya in Bondo District representing the northern end of the lake, Luanda Kotieno also in Bondo District representing an open inshore beach, Homa Bay beach in Homa bay District representing Southern end of the lake, and Dunga beach in Kisumu city representing a closed shoreline in an industrialized area. These are the main sources of fish consumed in Western Kenya. The area along Winam gulf is highly populated. Fishing is the main occupation along the fishing beaches alongside subsistence farming.
(Appendix I).

3.2 Sample size determination

The sample size was determined according to formula $n = \frac{Z^2pq}{d^2}$

Fischer *et al.*, 1998 in which:

n= the desired sample size if the target population is infinite

Z= the standard normal deviate at the required confidence level

P= the proportion in the target population estimated to have characteristics being measured

q= 1-p

d= the level of statistical significance set

According to Fischer *et al., (*1998) if there is no estimate available of the proportion in the target population assumed to have characteristics of interest, 50% should be used. Therefore, if the proportion of target population is 0.5 or 50%, the Z – statistics was 1.095, at the level of significance, 0.05

Substituted for the above formulae as:

$$n = \frac{(1.095)^2 \times (0.5) \times (0.5)}{(0.05)^2}$$

$$n = \frac{(1.095)^2 \times (0.25)}{(0.05)^2} = \frac{1.1990 \times 0.25}{0.05 \times 0.05}$$

n= 119.9

$\simeq$ 120

Hence the total fish sample from the four beaches was 120

3.3 Study sample and transportation

The fish species used in this study was *Oreochromis niloticus* (Nile tilapia). Choice of fish species was based on the fact that it is the most sought after fish species locally, and it is the third commercially important fish in L. Victoria.

Sampling was done within a period of three months (February – April 2007). Ten (10) Nile tilapia were randomly sampled from each fishing beach at every sampling time. Three samples of fish were collected. Fishing was done by gill netting, beach seine, and trawling. The sampled fish were then placed in sterile plastic bags and transported in iced cool box at 8°C to Department of Zoology laboratory, Maseno University within 12 hours from collection time and subjected to conventional microbiological tests.

3.4 Phenotypic identification of *Salmonella*

Phenotypic identification was done according to Newaj Fyazul *et al.,* 2005, protocol with minor modifications. Working surface was cleaned using 10% Jik solution and 70% ethyl alcohol before and after inoculation work. Fish samples were externally disinfected by dipping them in 70% ethyl alcohol for 2 minutes, followed by three washings with sterile distilled water.

Whole fish excluding bones was macerated using a sterile mortar and pestle, immersed in phosphate buffered saline (PBS) of pH 7.5 to achieve 10% w/v suspension of fish. Five milliliters of each fish tissue slurry was used to inoculate selenite F broth medium (Himedia laboratory pvt. Ltd Mumbai India), incubated at 37^{0}C overnight (18-24h) for maximum recovery of the organisms. Isolates in selenite F medium were subcultured onto both MacConkey agar (Oxoid No. 3 CM 115 Basingstoke, England) and

Deoxycholate Citrate Agar (DCA) (Himedia Laboratories pvt Mumbai India) by streaking technique and incubated at 37^0C for 18-24h .

Colonies from DCA were transferred to Nutrient broth (CDH JO 0003) and preserved at 4^0C in a refrigerator for later use in genetic analysis.

Biochemical tests (IMViC – Indole, Methyl red, Voges proskaurs, Citrate), were performed on non lactose fermenting colonies to identify bacteria genus based on their biochemical activities in different culture media. Isolates from Triple Sugar Iron (TSI) which showed indication to be *Salmonella* were subcultured in Methyl red medium and incubated at 35^0C for 48 ± 2h . Five to six drops of Methyl red indicator was then added to 5ml of 48 h glucose phosphate broth culture. Voges Proskauer (VP) test was performed by transferring 1ml of 48 hr culture isolate to a culture test tube and broth incubated for additional 48 h at 35^0C. 0.6 ml α - naphthol was added to mixture and vortexed. 0.2 ml of 40% KOH solution was later added to the mixture and then vortexed. The results were read after 4 h.

Serotyping of *Salmonella* strains was done according to Kauffman -White scheme using somatic 'O' and flagella 'H' antigens, (sifin, Germany) for confirmation of serovar status. To obtain agglutination test for 'O' antigens of *Salmonella*, circular areas were marked off on the surface of a glass slide using a crayon pencil marker. Heavily marked circular areas containing cell suspension to inhibit running onto each other or off the slide. A drop of cells suspended in normal physiological saline (0.9% NaCl) was placed in each circle and a drop of antiserum added to each. Agglutination occurred when there was a reaction between antibodies in the serum and their homologous antigens on cell wall of bacteria. The 'H' antigen test was done in a test tube following the same procedure as in

'O'antigen. The serovars were grouped according to their 'O' antigen reaction, (Kauffman *et al.,* 1966). Those *Salmonella* isolates which share 'O' antigen 2 were grouped as group A, those which share 'O' antigen 4 were grouped in B, those which share 'O' antigen 6 and 7 were grouped in C_1 and D, respectively according to Brenner *et al.,* (1998).

3.5 Genetic characterization of *Salmonella* species

3.5.1 *Salmonella* DNA extraction for Polymerase Chain Reaction (PCR)

Pure isolates obtained from a series of subcultures in selective medium DCA, and stored in nutrient broth (CDH JO 0003) were picked from solid agar plates using heat sterile chromium inoculation wire loop and reconstituted in 200μl of 0.9% NaCl solution ready for DNA extraction.

Pure isolates of 1.5ml were spinned at 15,000 x g for 2 minutes in microcentrifuge, and supernatant removed. The pellet was re-suspended in 56μl TE buffer, 30μl of 20mg/μl proteinase K, vortexed and incubated for 1h at 37^0C. 100μl of 5M NaCl was added and vortexed. 80μl of cetyltrimethylammonium bromide (CTAB/NaCl) was then added to the mixture, vortexed and then incubated at 65^0C for 10 min . An equal volume of chloroform/isoamyl alcohol was added to the mixture and spinned at 15,000 x g for 5 min . The aqueous phase was transferred to a fresh tube and DNA extracted by adding phenol/chloroform/isoamyl alcohol and spinned at 15,000 x g for 5 min . 0.6 volume isopropanol was used to precipitate DNA and DNA precipitate washed with 70% ethanol, centrifuged and supernatant removed. The pellet was dried for 24h and re-suspended in 100μl TE buffer ready for PCR.

3.6 Typing *Salmonella* species by use of *Malic acid dehydrogenase* (*mdh)* gene sequence according to Jensen and Strauss (1993) protocol.

PCR amplification was carried out in a final volume of 50µl. The following primer sequence was used.

Primer **Oligonucleotide sequence** (New England Biolabs)

mdh F5′ - TGCCAACGGAAGTTGAAGTG - 3′

mdh R5′ - CGCATTCCACCACGCCCTTC – 3′

In 1.5ml microcentrifuge tubes the following PCR master mix according to Jensen and Strauss, (1993) with minor modification during optimization was prepared and aliquoted into respective reaction tubes.

Table 1: *mdh* PCR amplification master mix

	×1	×20
Component	**Volume for 1 reaction**	
Distilled H_20	23.8µl	476 µl
10 x PCR buffer II	5.0µl	100 µl
$MgCl_2$	3.0µl	60 µl
NTPS	8.0µl	160 µl
dh F primer	2.5µl	50 µl
dh R primer	2.5µl	50 µl
aq pol	0.2µl	4 µl
	45.0µl	**900 µl**
NA template	**5.0µl**	**100 µl**
otal/Final Volume	**50.0µl**	**1000 µl**

Forty five microlitres of PCR master mix and 5µl of DNA sample were aliquoted into 1.5µl eppendof tube. Vortexed slightly and placed into MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A.). The prepared mixture were amplified according to the following conditions: Denaturation temperature was 94^0C for 5 mins, 94^0C for 25 sec, annealing at 54^0C for 45 sec, and extension 72^0C for 1 min for 30 cycles. Post elongation 72^0C for 7 mine then 4^0C to infinity.

3.6.1 Agarose gel Electrophoresis for *mdh* gene sequence

Amplicons were loaded onto casted 2% agarose gel alongside a 50 bp DNA marker,negative control and resolved at a constant voltage of 100 for 25min prior to UV visualization.

3.7 Typing *Salmonella* species by use of ST11 – ST15 random sequence primers according to Jensen and Strauss (1993) protocol.

Primer pair	**Oligonucleotide sequence** (New England Biolabs)
ST11- 5′ F	- GCCAACCATTGCTAAATTGGCGCA – 3′
ST15 -5′R	- GGTAGAAATTCCCAGCGGGTACTGG – 3′

In 1.5µl microcentrifuge tubes the following PCR master mix according to Jansen Strauss., (1993) with minor modification during optimization was prepared and aliquoted into respective reaction tubes.

Table 2: ST11 - ST15 PCR amplification master mix

	×1	×20
Component	**Volume for 1 reaction**	
Distilled H_2O	23.8µl	476 µl
10 x PCR buffer II	5.0µl	100 µl
$MgCl_2$	3.0µl	60 µl
dNTPS	8.0µl	160 µl
ST 11 F primer	2.5µl	50 µl
ST 15 R primer	2.5µl	50 µl
Taq pol	0.2µl	4 µl
	45.0µl	**900 µl**
DNA template	**5.0µl**	**100 µl**
Total/Final Volume	**50.0µl**	**1000 µl**

Forty five microlitres of PCR master mix and 5µl of DNA sample were aliquoted into 1.5µl eppendof tube. Vortexed slightly and placed into MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A.). The prepared mixture were amplified according to the following conditions:Denaturation temperature was 94^0C for 5 min, 94^0C for 25 sec, annealing at 54^0C for 45 secs, and extension 72^0C for 1 min for 30 cycles. Post elongation 72^0C for 7 minute then 4^0C to infinity.

3.7.1 Agarose gel Electrophoresis for ST 11-ST 15 primer sequence.

Amplicons were loaded onto casted 2% agarose gel alongside a 50 bp DNA marker, negative control and resolved at a constant voltage of 100 for 25min prior to UV visualization.

3.8 Typing of *Salmonella* species by use of *sef* A gene, according to Jensen and Strauss (1993) protocol.

Primers **Oligonucleotides sequence** (New England Biolabs)

Sef 167 5′ F - AGGTTCAGGCAGCGGTTACT - 3′

Sef 478 5′ R - GGGACATTTAGCGTTTCTTG - 3′

Table 3: *Sef* 167 - *Sef* 478 PCR amplification master mix

	×1	**×20**
Component	**Volume for 1 reaction**	
Distilled H_20	23.8μl	476 μl
10 x PCR buffer II	5.0μl	100 μl
$MgCl_2$	3.0μl	60 μl
dNTPS	8.0μl	160 μl
Sef 167 5′ F	2.5μl	50 μl
Sef 478 5′ R	2.5μl	50 μl
Taq pol	0.2μl	4 μl
	45.0μl	**900 μl**
DNA template	**5.0μl**	**100 μl**
Total/Final Volume	**50.0μl**	**1000 μl**

Forty five microlitres of PCR master mix and 5µl of DNA sample were aliquoted into 1.5µl eppendof tube. Vortex slightly and place into MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A.). The prepared mixture were amplified according to the following conditions:Denaturation temperature was 94^0C for 5 mins, 94^0C for 25 secs, annealing at 54^0C for 45 se, and extension 72^0C for 1 min for 30 cycles. Post elongation 72^0C for 7 min then 4^0C to infinity

3.8.1 Agarose gel Electrophoresis for *sef A* gene sequence

Amplicons were loaded onto casted 2% agarose gel alongside a 50 bp DNA marker,negative control and resolved at a constant voltage of 100 for 25min prior to UV visualization.

3.9 Typing of *Salmonella* species by use of *fliC* gene , according to Jensen and Strauss (1993) protocol.

Primers **Oligonucleotides sequence** (New England Biolabs)

Fli 15 5′ F - CGGTGTTGCCAGGTTGGTAAT - 3′

Tym 5′ R - ACTCTTGCTGGCGGTGCGACTT- 3′

Table 4: *Fli* 15 - *Tym* PCR amplification master mix

Component	Volume for 1 reaction ×1	×20
Distilled H_20	23.8µl	476 µl
10 x PCR buffer II	5.0µl	100 µl
$MgCl_2$	3.0µl	60 µl
dNTPS	8.0µl	160 µl
Fli 15 5′F	2.5µl	50 µl
Tym 5′ R	2.5µl	50 µl
Taq pol	0.2µl	4 µl
	45.0µl	**900 µl**
DNA template	**5.0µl**	**100 µl**
Total/Final Volume	**50.0µl**	**1000 µl**

Forty five microlitres of PCR master mix and 5µl of DNA sample was aliquoted into 1.5µl eppendof tube. Vortexed slightly and placed into MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A.). The prepared mixture were amplified according to the following conditions:Denaturation temperaturewas 94^{0}C for 5 min , 94^{0}C for 25 sec, annealing at 54^{0}C for 45 sec, and extension 72^{0}C for 1 min for 30 cycles. Post elongation 72^{0}C for 7 min then 4^{0}C to infinity.

3.9.1 Agarose gel Electrophoresis for *fliC* gene sequence

Amplicons were loaded onto casted 2% agarose gel alongside a 50 bp DNA marker,negative control and resolved at a constant voltage of 100 for 25min prior to UV visualization.

3.9.2 Data management

(i) Fish samples were recorded at collection site (field) as D2, UH1, H, and L2 (for Dunga, Uhanya, Homa bay and Luanda Kotieno, respectively). and similarly after processing in the laboratory. The data was entered in the data sheet.

(ii) The size of each amplified DNA fragment was determined using molecular weight DNA marker extrapolation.

CHAPTER FOUR

4.0 RESULTS

4.1 Phenotypic characterization of *Salmonella* isolated from *Oreochromis niloticus*

Analysis of the fish tissue slurry indicated that the harvested fish were majorly infested with members of family *Enterobacteriacea* namely: *Salmonella, Shigella* and *E. coli.* Results obtained (section 3.4) indicated that twenty (20) bacteria isolates were phenotypically identified as *Salmonella* based on their morphological appearance and biochemical reaction on various bacteriological (differential and selective) culture media (NCCLS, 2001). Sterile selenite F broth enrichment is colourles before innoculation (fig. 2). Change of colour from colourless to orange (fig. 3) after innoculation with fish tissue slurry was due to recovery of *Salmonella.*

Phenotypic characteristic formation of creamy colonies in MacConkey Agar No. 3 (fig. 4), dome shaped colonies with a central black dot in Deoxycholate Citrate Agar (DCA) (fig. 5) and creamy colonies with Hydrogen Sulphide (H_2S) gas production in modified Salmonella - Shigella Agar in test tube (slant/butt) acted as indicator of the presence of *Salmonella spp., (S. enterica* serotype *enteritidis, S. enterica* serotype *typhimurium*, and other *Salmonella* species). These isolates were also screened on triple sugar iron (TSI). Production of acid resulting in a yellow butt with or without production of Hydrogen Sulphide (H_2S) (blackening of agar) in test tube (slant/butt) (fig. 6) indicated presence of *Salmonella spp.* , (NCCLS, 2001)

Fig. 2: Sterile Selenite F broth

Colourless sterile selenite F broth before inoculation with fish tissue slurry as in section 3.4.

Fig. 3: *Salmonella* cultures in selenite F broth

Selenite F broth after inoculation with fish slurry (section 3.4). Change of colour from colourless to orange, due to recovery of *Salmonella* from enrichment broth.

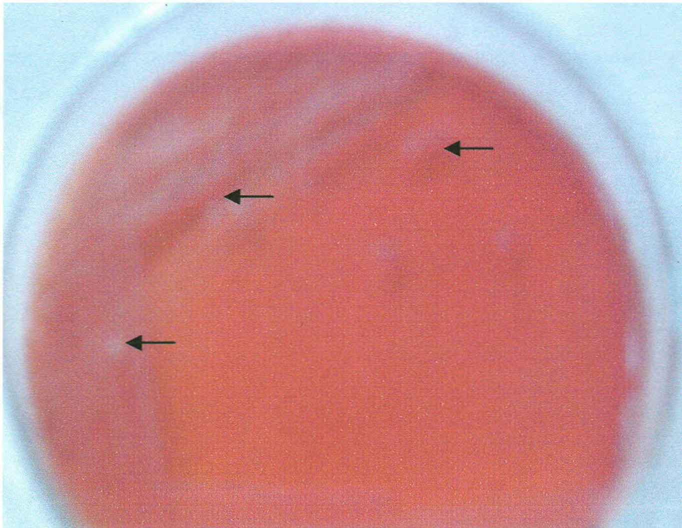


Fig. 4: *Salmonella* colonies in MacConkey Agar

Formation of 1-3 mm diameter creamy colonies (←)on MacConkey Agar No.3 which is a differential medium was indicative of presence *Salmonella* isolates.

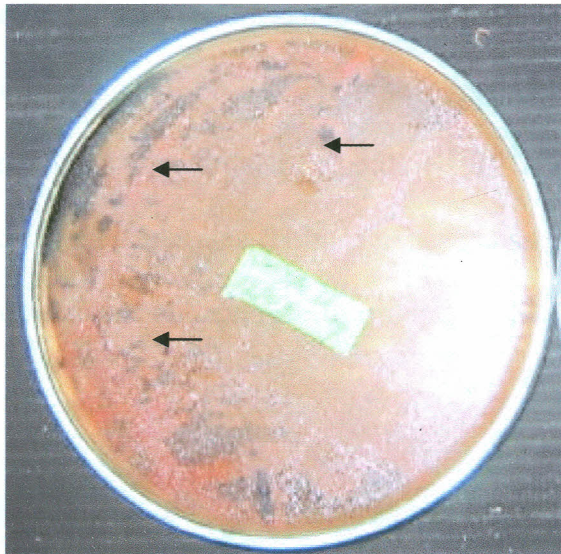


Fig. 5: *Salmonella* colonies in DCA

2-4mm diameter dome shaped colonies with a central black dot (←)on Deoxycholate Citrate Agar (Hynes), selective medium for *Salmonella* indicated presence of *Salmonella* and not *Shigella* which do not form central black dot

Fig. 6: *Salmonella* colonies in TSI (Slant/butt)

The isolates were also screned on Triple sugar iron for hydrogen sulphide gas (H_2S) production and production of acid resulting into yellow butt, with or without production of H_2S (blackening of agar) in TSI. Production of acid resulting in a yellow butt (tubes 1 ,3 ,4, and 5) with production of hydrogen sulphide (H_2S) (blackening of agar) in TSI indicated presence of *Salmonella* species. This inference is based on the fact that *Shigella* does not form acid from lactose and sucrose to produce H_2S gas in the medium within 24h.

Gram stain results indicated that 20 bacteria isolates were Gram negative, pink rods with smooth edges.

Morphological identification was followed by performing biochemical tests (IMViC) on the isolates (section 3.4). This involved, indole production test on tryptophan broth culture (Fig.7).

1 2 3

Fig. 7 : Indole production test on tryptophan broth culture

The basis of indole production test is that certain bacteria decompose tryptophan (a constituent of peptone) to indole, which reacts with p-dimethylaminobenzaldehyde to form a red coloured compound (tube 3, fig. 8), a positive test. Indole was not produced when 0.2ml-0.3ml kovac's reagent was added to 5ml of 24h tryptophan broth culture, a negative test result which indicated presence of *Salmonella* strains. Golden yellow colour observed at interface (tube 1 and 2) was a typical reaction of *Salmonella* strains and not *E. coli* which otherwise give indole positive results.

1 2 3

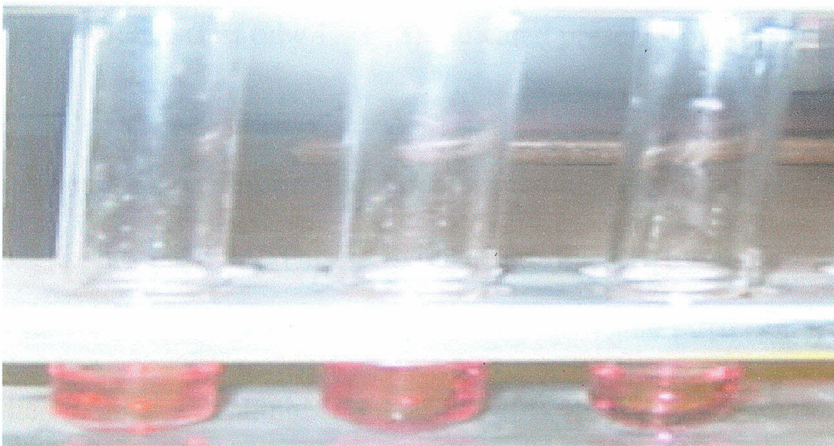


Fig. 8: Methyl red test

Methyl red test was positive. It was used to detect the maintenance of a low pH (acid production) brought about by fermentation of glucose at pH of 4.5 or less, indicated by a bright red colour throughout the medium. Bright red colour throughout medium (tube 1, 2, 3) confirmed presence of *Salmonella* strains which produces acid from glucose, mannitol and sorbitol.

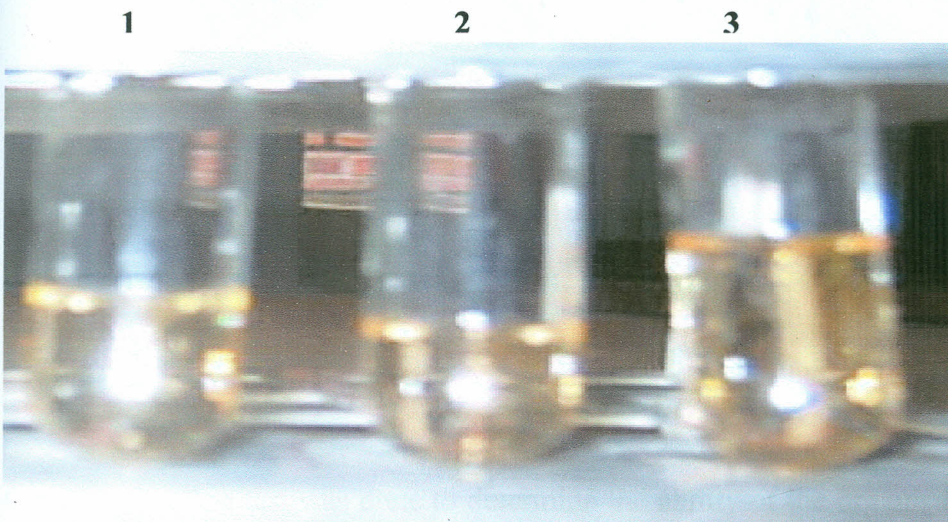


Fig. 9: Voges proskauer test

A negative VP test (test tube 1, 2, 3) indicated presence of *Salmonella.*

VP test depends on the production of acetylmethylcarbinol or butylenes glycol from dextrose in the medium. This is oxidized to diacetyl by adding alkali (KOH), which shows pink colour for positive results. Voges proskauer (VP) test was negative (tube 1 ,2 3,) when 0.6ml α - naphthol and 0.2ml of 40% KOH solutions was added respectively to 1ml of 48h VP culture and vortexed. A typical reaction of *Salmonella* species.

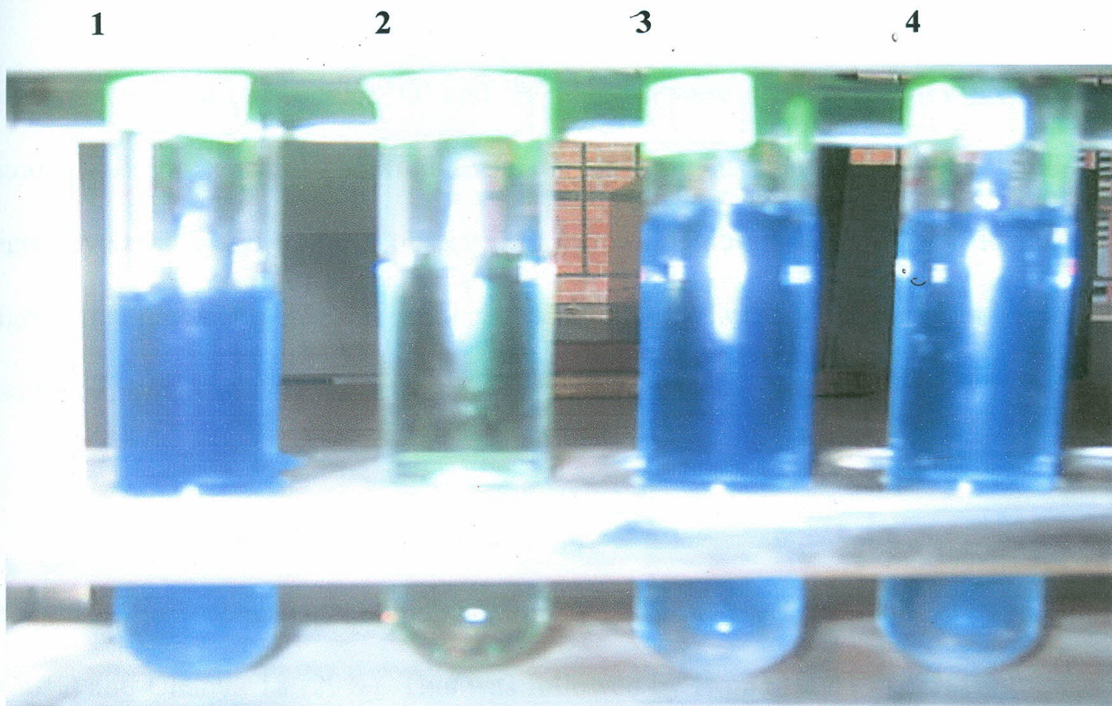


Fig. 10: Citrate utilization test

Colour change of indicator from light green (tube 2,) to blue (test tube 1, 3 &4), indicated presence of *Salmonella* strains.

Citrate utilization test was done to determine the ability of the organism to utilize citrate as a sole carbon source and an ammonium salt as the source of nitrogen. Inoculation of Koser's citrate medium was done and growth in the medium was indicated by turbidity and colour change of the indicator from light green to blue (tube 1, 3 and 4). This was due to alkaline reaction following citrate utilization, a typical reaction of *Salmonella* strains.

To verify aforementioned species identification based on morphological and biochemical tests; the isolated bacteria were subjected to Kauffmann – White scheme serological test. Serotyping was performed by seroagglutination using antisera to determine the antigenic

formula. Polyvalent *Salmonella* 'O' and 'H' antisera were used to obtain a presumptive diagnosis. The presence of polyvalent somatic 'O' antigens that detects sets of cell wall antigens type 1,4,5 and 12 were observed in form of agglutination in glass slide and 'H' antigens that detects phase 1:I and phase 2:1 and 2 were also observed indicating presence of *Salmonella* serovars.

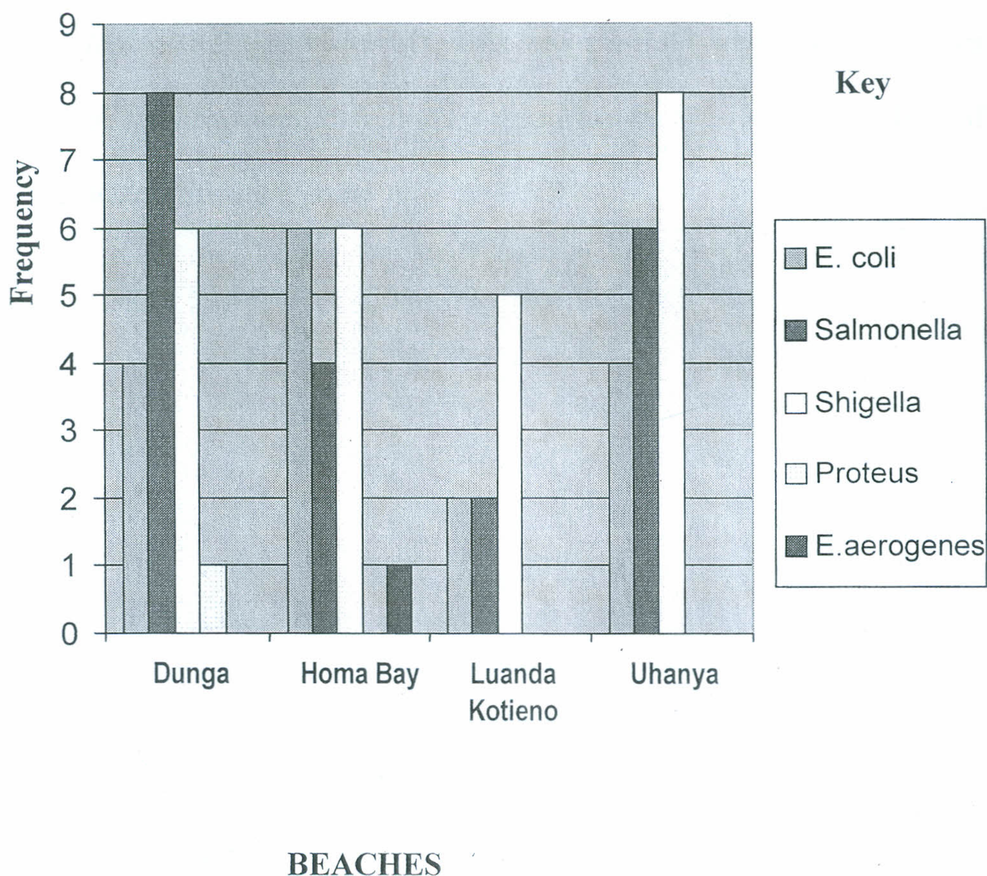
4.2 Data Analysis of Phenotypic results

The highest *Enterobacteriaceae* population isolated from the Nile tilapia was in Dunga 19 (30%) and Uhanya 18 (29%). This was followed by Homa Bay 17 (27%) and Luanda Kotieno 9 (14.2%) respectively. (Table5).

Table 5. Frequency distribution of *Enterobacteriaceae* isolated from selected beaches within Winam Gulf

	% *Enterobacterobacteriaceae* isolated						
Beaches	*E. coli spp* N=16	*Salmonella Spp.* N=20	*Shigella Spp.* n=25	*Proteus Spp.* n=1	*E. aerogenes* n = 1	Fish infested n = 63	No. of fish not infested n=57
Dunga	4 (25%)	8 (40%)	6 (24%)	1 (100%)	1(0%)	19 (30%)	11 (19.2%)
Homa Bay	6 (37.5%)	4 (20%)	6 (24%)	0(0%)	1 (100%)	17 (27)	13 (22.8%)
Uhanya	4 (25%)	6 (30%)	8 (32%)	0 (0%)	0 (0%)	18 (29%)	12 (21%)
Luanda Kotieno	2 (12.5%)	2 (10%)	5 (20%)	0 (0%)	0 (0%)	9 (14%)	21 (36.8%)
Total	**16 (100%)**	**20 (100%)**	**25 (100%)**	**1(100%)**	**1 (100%)**	**63 (100%)**	**57 (100%)**

Shigella spp. (39.6%) was the most isolated *Enterobacteriaceae* followed by *Salmonella spp.* (31.7%), *E. coli* (25.3%), *Proteus spp.*,(1.58%) and *E. aerogenes (*1.58%) respectively. Dunga beach had the highest *Salmonella spp.* 8 (40%) isolated as compared to Uhanya 6 (30%), Homa Bay 4 (20%), and Luanda Kotieno 2 (10%) (Table5). Comparatively, Uhanya had the highest *Shigella spp.* 8 (32%) isolated followed by, Dunga and Homa Bay 6 (24%), then Luanda Kotieno 5 (20%). *E. coli* was mostly isolated along Homa Bay beach 6 (37.5%), 4 (25%) in Dunga and Uhanya beach, 2 (12.5%) in Luanda Kotieno.



BEACHES

Fig. 11: Bar graph showing distribution of *Salmonella, Shigella, E. coli, E. aerogenes, Proteus* within Dunga, Homa Bay, Uhanya, and Luanda Kotieno Beaches along Winam gulf

4.3 Genotypic characterization of *Salmonella* from *O. niloticus*

Analysis of isolated *Salmonella* species revealed that, 4 (20%) were *S. enteritidis*,9 (45%) *S. enterica* serotype *typhimurium*. This phenotypic identification was confirmed by amplification of *Salmonella* house keeping gene *malic dehydrogenase* (*mdh*) that gave a band of 261 bp (fig.12). For identification of *S. enterica* serotype *typhimurium* within isolated *Salmonella* species, primer combination of *Fli*15 and *Tym* and ST11 –ST15 were used to amplify the specific genes in the *Salmonella* isolates and a band of 559 bp and 429 bp were obtained respectively for the 9 specific samples that were phenotypically positive for *Salmonella* (fig. 13 and 14). This was a confirmation of the isolates as *S. enterica* serotype *typhimurium*. *S. enteritidis* identification was genotypically confirmed by 312 bp gene amplification of *sef* A

(fig. 15)

4.4 Genotypic presentation of *Salmonella* by use of *Malic dehydrogenase* (*mdh*) gene sequence according to Jensen and Strauss (1993) protocol.

Primer pair:

mdh – F, 5′- TGCCAACGGAAGTTGAAGTG - 3′

mdh – R, 5′ – CGCATTCCACCACGCCCTTC - 3′

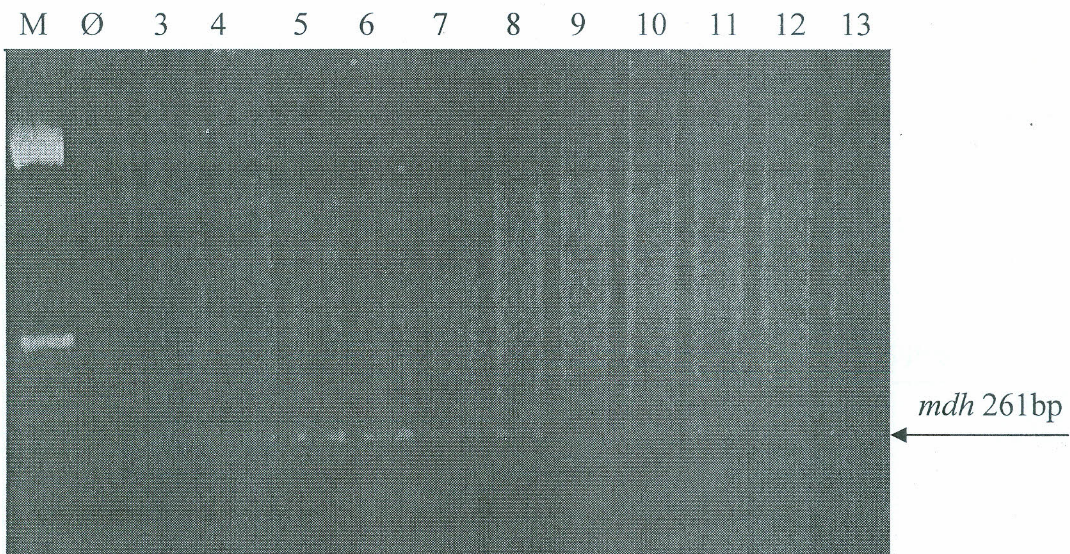


Fig. 12 : PCR gel showing *Malic cid dehydrogenase* gene products for *Salmonella*. Isolates from Dunga (Lane 3 ,4 ,5 & 6), Uhanya (Lane 7 & 8), Luanda Kotieno (Lane 10) and Homa Bay (Lanes 11 ,12 & 13). Molecular DNA maker 50bp (lane 1), Ø; negative control (lane 2).

Amplification of the housekeeping gene for *malic acid dehydrogenase (mdh*) gene with specific primers gave a band of 261 bp on 2% gel agarose gel. The isolates harboured the housekeeping gene specific for *Salmonella* and hence confirmed the isolates are *Salmonella* strains.

4.5 Genotypic presentation of *Salmonella* species by use of *fliC* gene according to Jensen and Strauss (1993) protocol .

Primers	**Oligonucleotide sequence**
Fli 15 -5′ F	- CGGTGTTGCCAGGTTGGTAAT - 3′
Tym - 5′ R -	ACTCTTGCTGGCGGTGCGACTT- 3′

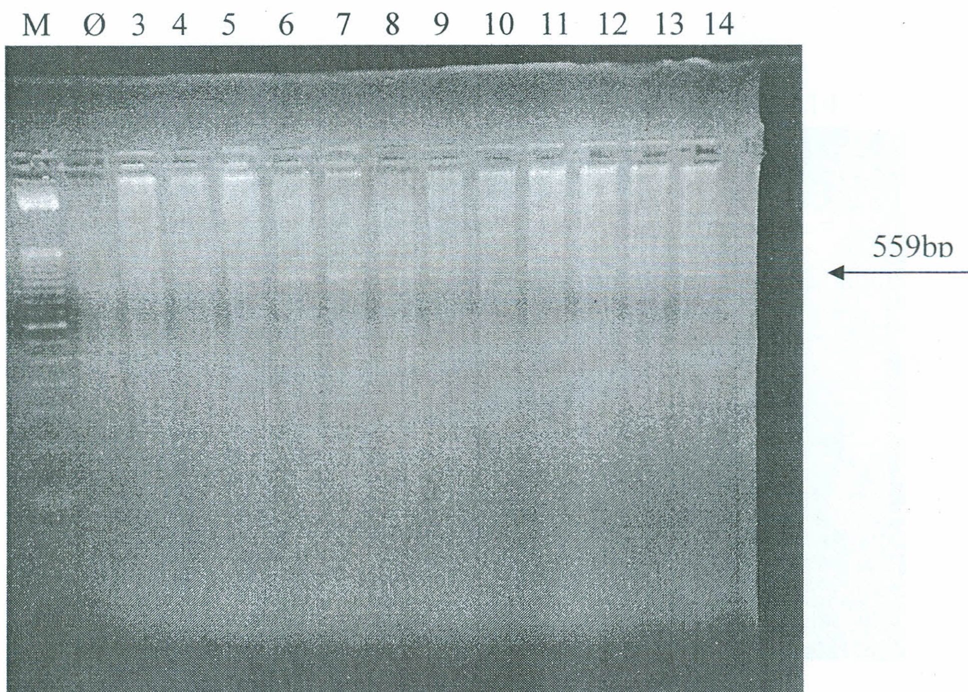


Fig. 13 : PCR gel showing *fliC* gene products for *Salmonella*. Isolates from Dunga (Lane 4 ,5 ,6 & 7), Uhanya (Lane 8 & 9), Luanda Kotieno (Lane 12) and Homa Bay (Lane 13 & 14). Molecular DNA maker 50bp (lane 1), Ø; negative control (lane 2). Amplification of *fli C* gene specific for *S. enterica* serotype *typhimurium.* with primer pair Fli 15 –Tym, gave a band of 559 bp observed on 2% agarose gel , confirming isolates as *S. enterica* serotype *typhimurium.*

4.6 Genotypic presentation of *Salmonella* strains by use of ST11 and ST15 random sequence primers according to Jensen and Strauss (1993) protocol

Primer Pair:

ST11-5′F - GCCAACCATTGCTAAATTGGCGCA-3′

ST15 - 5′R - GGTAGAAATTCCCAGCGGGTACTGG – 3′

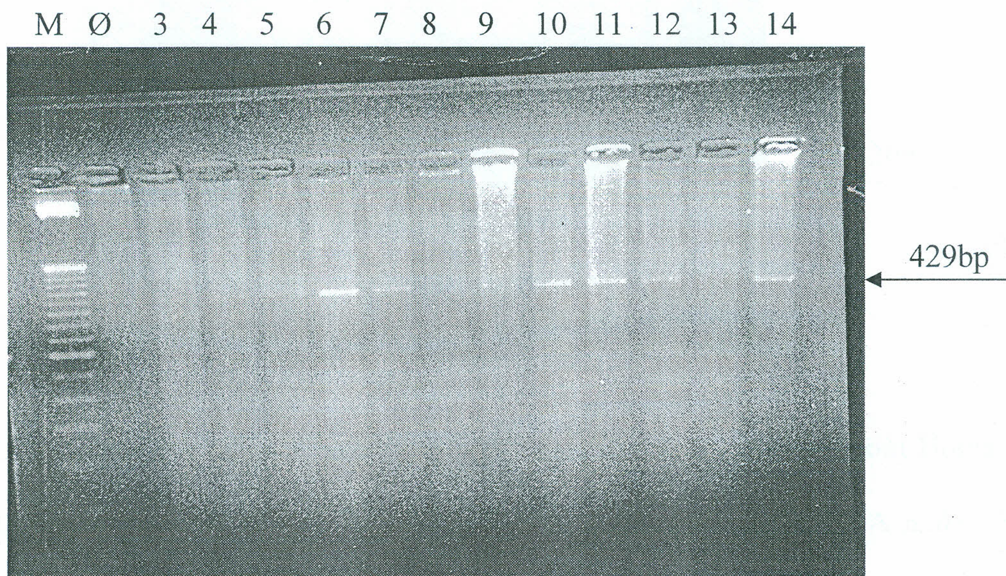


Fig. 14: PCR gel showing presence of ST11-ST15 gene pair. Isolates from Dunga (Lane 4, 5, 6 & 7), Uhanya (Lane 9 & 10), Luanda Kotieno (Lane 11) and Homa Bay (Lane 12 & 14). Molecular DNA maker 50bp (lane 1), Ø; negative control (lane 2).

Bands of 429 base pair amplified regions were observed on the gel for random sequence primers. The results indicated presence of *Salmonella* species

4.7 Genotypic presentation of *Salmonella* species by use of *sef* A gene according to Jensen and Strauss (1993) protocol

Primers	Oligonucleotides sequence
Sef 167 5′ F	- AGGTTCAGGCAGCGGTTACT - 3′
Sef 478 5′ R	- GGGACATTTAGCGTTTCTTG - 3′

M Ø 3 4 5 6 7 8 9 10 11 12 13

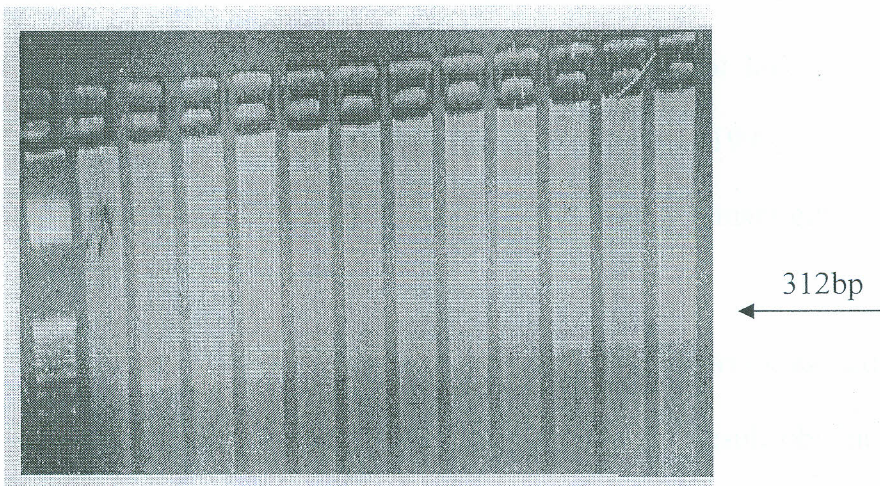


Fig. 15: PCR gel showing sefA gene products for *Salmonella*. Isolates from Homa Bay (Lane 3) Dunga (Lane 11 & 12), Uhanya (Lane 13), and. Molecular DNA maker 50bp (lane 1), Ø; negative control (lane 2).

Sef A gene is specific for *S. enterica* serotype *enteritidis* .Amplification of *Sef A* gene in 4 isolates in 2% agarose gel, gave a band of 312 bp which confirmed the isolates are *S. enterica* serotype *enteritidis*.

CHAPTER FIVE

5.0 DISCUSSION

Lake Victoria is an important source of livelihood for people living along Winam gulf. It contributes over 90% of the total annual fish production valued at 6.5 billion in Kenya (Gitonga, 2006). However, L. Victoria basin is one of the areas where environment has continued to deteriorate (Rwabinge, 2005). Microbiological pollution through wildlife, agricultural activities, forestry, agrobased industries, rural and urban settlements as surface runoff and storm water in the Kenyan catchment side of lake, have negative effects on water quality of rivers draining into lake Victoria (Crul,1995). These activities have contributed to the rising population of fish microbes within Winam gulf.

Phenotypic identification of *Salmonella* strains from fish tissue slurry obtained from fish collected from selected beaches along Winam gulf was done. Result obtained (section 3.4) indicated that twenty (20) bacteria isolates were phenotypically identified as *Salmonella* species based on their morphological appearance and biochemical reactions on various bacteriological (differential and selective) culture media. A number of plating media have been devised for the isolation of *Salmonella.* MacConkey agar is a differential and slightly selective media in that in addition to lactose and pH indicator, it contains an inhibitor for non enterics. Most commonly used selective media for *Salmonella* and *Shigella* are Deoxycholate Citrate Agar (DCA) ,Salmonella –Shigella Agar ,which favour growth of *Salmonella* and *Shigella* and is inhibitory to coliforms and *Proteus* .*Salmonella* do not ferment lactose, most *Salmonella* strains produce Hydrogen Sulphide (H_2S) gas ,which in the media containing ferric ammonium citrate ,forms a black spot in the centre of the creamy colonies. Primary isolation requires the use of

enrichment medium. In the present study fish tissue slurry was subjected to 'enrichment culture' , where 5ml of fish slurry was incubated in Selenite F broth for maximum recovery of *Salmonella.*

Phenotypic characteristic formation of 1-3 mm diameter creamy colonies on MacConkey Agar No. 3, 2-4mm diameter dome-shaped colonies with a central black dot on DCA (Hynes), and creamy colonies with Hydrogen Sulphide (H_2S) gas production in modified *Salmonella, Shigella* Agar in test tube (slant/butt) acted as indicator for the prescence of *Salmonella* spp. These colonies were also screened on Triple Sugar Iron (TSI) for Hydrogen Sulphide gas production and for their typical production of acid resulting to a yellow butt, with or without production of H_2S (blackening of agar) in TSI, acted as indicator for prescence of *Salmonella* spp. , (*S. enterica* Serotype *enteritidis*, *S. enterica* Serotype *typhimurium*, and other *Salmonella* spp) in test tube (slant/butt) or culture medium and not *Shigella.*

Morphological identification was followed by performing biochemical tests (IMViC) on isolates (section 3.4). This involved; fermentation of glucose with production of acid and gas, failure to produce indole, a positive methyl red reaction, a negative voges proskauer reaction and citrate utilization reaction for phenotypic characterization. Indole was not produced when 0.2-0.3ml of Kovac's reagent was added to 5ml of 24h tryptophan broth culture. Golden yellow colour observed at the interface was a typical reaction of *Salmonella* strains and not *E.coli* which otherwise give indole positive results. A positive methyl red (MR) test indicated by bright red colour throughout medium confirmed the prescence of *Salmonella* strains which produces acid from glucose. Voges Proskauer (VP) was negative when 0.6ml $\propto$ - naphthol and 0.2ml of 40% KOH solutions was added

respectively to 1ml of 48h VP culture and Vortexed. This was a typical reaction of *Salmonella* spp. Inoculation of the isolates in Koser's citrate Agar was done and a positive result of growth accompanied by colour change from green to blue was indicative of the presence of *Salmonella* spp. The test is used to determine the ability of an organism to utilize citrate as the sole carbon source and an ammonium salt as the source of nitrogen. The bacteria isolates were then subjected to Kauffmann-White Scheme serological test that was used for cell wall lipopolysacharide protein 'O' antigen and 'H' flagella protein agglutination test

Although serotyping has been a reliable method for differentiating isolated strains, it is time consuming and requires more than 150 specific serum samples (Kilger *et al.,*1993). In addition, some strains cannot be identified through serotyping due to untypeability of the isolate (rough strains) (Kilger *et al.,*1993). Phenotypic analysis is therefore used as a frontline measure supported by genotyping. Molecular methods are now used as an alternative since they provide higher power of discrimination and allow rapid identification. Molecular methods are used in differentiation of bacteria species, serotypes and strains (Jensen *et al.,*1993).

Serotyping of isolated *Salmonella* strains from this study was done up to group level. This was preceded by genotypic characterization. Genotypic characterization of *Salmonella* by use of *malic acid dehydrogenase* (*mdh*), a house keeping gene gave a band of 261bp for *Salmonella* isolates. ST11-ST15 primers were used to amplify highly conserved genes in *Salmonella* genome, a band of 429 bp confirmed that isolates were *Salmonella. sefA* and *fliC* genes were also amplified for serotype identification.

Structural gene, *fliC* codes for flagellins. *fliC* alleles have conserved sequence on distal parts of the gene, making the gene of any serotype easy to amplify for species identification. Amplification of *fliC* gene gave a band of 559 bp for nine (9) isolates. This confirmed the isolates as *S.enterica* serotype *typhimurium*. Bacterial host cell attachment mechanisms is mediated by fimbriae. *S. enterica* serotype *enteritidis*, in addition to fimbriae shared between serovars, has *SEF*14 fimbriae. *SEF*14 fimbriae have a role in virulence, it is essential for binding in macrophages (Edwards *et al.,*2000). *sefA* gene encodes the most abundant subunit of *SEF*14 fimbrial shaft; of *S. enterica* serotype enteritidis. Amplification of *sefA* gene gave a band of 312 bp for four (4) isolates; this confirmed the isolates as *S.enterica* serotype *enteritidis*.

In the present study, analysis of the fish tissue slurry indicated that the harvested fish were majorly infested with *Enterobacteriaceae* namely: *Salmonella, Shigella* and *E.coli.* Results obtained (section 3.4) indicated that twenty (20) bacteria isolates were phenotypically identified as *Salmonella* based on their morphological appearance and biochemical reaction on various bacteriological (differential and selective) culture media (NCCLS, 2001). Dunga beach had the highest *Salmonella* distribution; 40% of the total *Salmonella* isolates, this was followed by *E. coli* 25% and *Shigella* 24%. The beach area is densely populated with a closed shoreline, this have led to poor water circulation in the shoreline. The effects of urbanization have serious implications on L. Victoria along Dunga beach in Kisumu city. Kisumu city is littered with pools of sewage spills from broken sewage pipes and tanks which forms part of surface runoff after heavy rainfall that ends up into the lake (Ogindo, 2005). This accounted for the high

Enterobacteriaceae distribution in Dunga. These results concur with findings of Werimo and Ouko (1999) who found *Salmonella*, *E. coli* and *Vibrio cholerae* as some of the serious pathogens associated with fish from Dunga fish landing beach. Nanyaro and Makene (1998) also identified fish landing environment to be a major source of fish quality problems in L. Victoria.

In Homa Bay beach, the highest *Enterobacteriaceae* isolate was *E. coli* (37.5%) of total *E. coli* isolates, followed by *Shigella* (24%) and *Salmonella* (20%). *E. coli* is a faecal coliform that is specific to faecal material from humans and other warm blooded animals. High *E. coli* distribution in Homa Bay provides definite evidence of faecal pollution (Ogindo, 2005). Pollution at Homa Bay is due to untreated sewage effluents disposed directly into the lake due to dilapidated sewage treatment system. These results are typical of what was found in previous studies by Shewan, 1961; Banwart, 1981; Huis in't Veld; 1996; Huss, 1994, who isolated *Salmonella, Shigella,* and *E. coli* from fish harvested from waters subjected to human sewage pollution or terrestrial runoff.

Uhanya beach had the highest distribution of *Shigella spp.* (32%), followed by *Salmonella* (30%) and *E. coli* (25%). Conditions at this beach are poor, lacking portable water supply in addition to high latrine density in overcrowded nuclear settlements. The beach area has a closed shoreline, thus water circulation in the shoreline is poor leading to high levels of *Enterobacteriaceae* counts.

However, in Luanda Kotieno distribution of members of *Enterobaacteriaceae* was low in fish collected. This was attributed to the lake area being an open inshore where there is

adequate water circulation. Dilution effect on *Enterobacteriaceae* counts at the shoreline led to low levels. The area lacked papyrus reeds and other vegetation like water *hyacinth* that could lead to eutrophication and hence thriving of coliforms as experienced in other study areas.

Salmonella enterica has been considered the causal agent of a large number of enteric infections in the world (Bell *et al.*, 2002). Raw foods and cross-contamination of ready-to-eat products are the main routes of *Salmonella* transmission (Bell *et al.*, 2002).

The results of this study indicated presence of *Salmonella* in fish harvested from lake Victoria, an indication of contamination of the lake waters by the pathogen. These results were consistent with the National Agricultural Research Organization of Uganda (NARO) (1997) report which indicated the presence of certain species of *Enterobacteriaceae* like *E. coli, Salmonella* species at some fish landing sites along the shores of L. Victoria (Atyang, 1999). Previous studies by NARO (1997) established that feacal or thermo tolerant coliforms and therefore prevalence of *Salmonella* species were higher at the inshore waters where beaches are located than offshore water, especially those beaches which are densely populated with inadequate sanitary facilities. Consequently there was risk of contamination of fish during off loading with strains of bacteria than during loading offshore.

There being probability that the main source of *Salmonella* contamination in the marine environment is of human or animal origin, the different population structures of *S. enterica* serotype *typhimurium*, *S. enterica* serotype *enteritidis* may be attributed to the different rates of growth and survival of these serovars in the aquatic environment.

Salmonella serovar *typhimurium* was the clinically important serovar mostly identified in this study which attests to its capacity of adaptation and survival in this environment, as has been suggested by other authors (Dupray *et al*., 1995; Baudart *et al.,* 2000).

5.1 CONCLUSION

Nile tilapia within Winam gulf are infested with pathogenic *Enterobacteriaceae*. *Salmonella*, *Shigella spp* and *E.coli* were the highest isolates. This may result from contamination of fish during offloading with these strains of bacteria than during loading offshore. *S. enterica* serotype *typhimurium* was the highest isolate among the nontyphoidal *Salmonella. Salmonella* was isolated in all the four beaches. The highest isolates were from Dunga beach followed by Uhanya, the least isolates were recorded in Luanda Kotieno beach. Therefore Nile tilapia may have a role in transmission of Salmonellosis in the study area Poor sanitation was a major cause of pollution at the beach inshore waters. Hence landing and marketing of fish from beaches with essential sanitary facilities can reduce the risk of cross contamination and the hazard from these pathogens. The sources of *Salmonella* are poorly understood and this study provided vital data that is critical in assessing and controlling the risk associated with the presence of *Salmonella* in *Oreochromis niloticus* in the study area.

5.2 RECOMMENDATIONS

Prevention and Control Strategies

Dunga and Uhanya beaches had the highest *Enterobacteriaceae* loads.Sanitary conditions in the adjacent areas needs serious improvement. The sewage system in Homa bay and the general sanitation at Homa bay beach needs to be improved, this would ensure elimination of initial contamination of fish by enteric pathogens from the source and also improve water quality. Luanda Kotieno had low Enterobacteriaceae counts ,and hence fish from Luanda Kotieno are relatively safe for human consumption.

Effective prevention and control programmes must involve coordinated and simultaneous actions on the problem from several directions. Hazards from *Salmonella* can be prevented by heating food enough to kill the bacteria, preventing post cooking cross contamination and prohibiting people who are ill or are carriers of *Salmonella* from working in food operations is also believed to be another control strategy. Consumers should wash hands with soap before preparing or eating food and after using bathroom. All food preparation areas should be cleaned with diluted solution of bleach and water and rinsed before and after food preparation.

Continuous development of existing surveillance measures, (control programmes, traceability of food chains) and epidemiological study is required both for foodborne pathogens, and also for sentinel organisms present in the normal flora and which may represent a reservoir of resistant strains. It is important that epidemiology of this organism be fully elucidated by use of phenotypic analysis as a 'front line' measure supported by genotyping in order to facilitate the direct implementation of an effective surveillance and control strategy. Subtyping is important epidemiologically for recognizing outbreaks of infection, detecting the cross transmission of nosocomial pathogens, determining source of infection, recognizing virulent strains of the bacteria .

REFERENCES

Abdel-Monem M.H. and Dowidar A.A. (1990). Recoveries of *Salmonella* from soil in Eastern region of Saudi Arabia Kingdon. *J. Egypt. Public Health Assoc.* 65:61- 75.

Abila R.O. and Jansen E.G. (1997). From Local to Global markets: *The fish exporting and fishing meal industries of Lake Victoria*– Structure, Strategies and Socio-economic Impacts in Kenya. IUCN Eastern Africa programme. Socio-economics of Lake Victoria fisheries: Report No.2. The World Conservation Union, Nairobi.

Acha P.N. and Szyfres B. (2003). Zoonoses and communicable diseases common to man and animals. Vol. I. Bacterioses and mycoses. 3rd ed. Scientific and Technical Publication No. 580, Pan American Health Organization, Regional Office of the WHO, Washington, USA, ISBN 92 75 31580 9, pp.384.

Alinovi A., Vecchini F. and Bassissi P. (1993). Sporothricoid mycobacterial infection – a case-report. Acta Dermato -Venereologica, 73: 146–147.

Alonso J. H., Botella M.S., Amoros I. and Rambach A. (1992). *Salmonella* detection in Marine waters using a short standard method. *Water Res*. 26: 973-978.

Angulo F.J., Johnson K.R., Tauxe R.V. and Cohen M.L. (2000). Origins, and consequences of antimicrobial resistant, nontyphoidal *Salmonella*: implication for the use of fluoroquinolones in food animals. *Microb. Drug Resist*.6:77-83

MoH. (2007). Annual Bulletin for Health Service Access, utilization and coverage, Nyanza, 2006 Dept. of HMIS.

Ansary A., Haneef R.M., Torres J.L. and Yadar M. (1992) . Plasmid and antibiotic resistance in *Aeromonas hydrophila* isolated in Malaysia from healthy and diseased fish. *J. fish Dis*. 15:191-196

Arthur G., Nduba V.N., Kariuki S.M., Kimari J., Bhatt S.M. and Gilks C.F. (2001). Trends in blood stream infections among human immunodeficiency virus – infected adults admitted to a hospital in Nairobi, Kenya, during the last decade. *Clin. Infect.Dis*. 33: 248 -56

Atyang J. (1999). A manual for Training Staff in Uganda Fish Processing Plants on Hygiene and Sanitation and application of HACCP Principles. *J. of UNU* – Fisheries Training Programme 1:43-57

Banwart G. J. (1981). *Basic Food Microbiology*. Westport Connecticut: The AVI publishing company Inc. A bridged edition.

Barker J. and Bloomfield S. F. (2000) . Survival of *Salmonella* in bathrooms and toilets in domestic homes following Salmonellosis. *J. Appl. Microbiol*. 89:137-144.

Baudart J., Lemarchand K., Brisabois A. and Lebaron P. (2000) . Diversity of *Salmonella* strains isolated from the acquatic environment as determined by serotyping and amplification of ribosomal DNA spacer regions. *Appl. Environ. Microbiol*. 66: (4);1544 –1552.

Bell C. and Kyriakides A. (2002) . *Salmonella*: a practical approach to the organism and its control in foods. Blackwell Science Ltd., Oxford, United Kingdom.

Beltran P., Musser J.M., Helmuth R., Farmer J.J., Frerichs W.M., Wachsmuth I.K., Ferris K., Wells J.G., Cravioto A. and Selander K. (1998). Toward a population genetic analysis of *Salmonella*: genetic diversity and

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relationships among strains of serotypes *S. choleraesuis, S. derby, S. dublin, S. enteritidis, S. infantis, S. newport, S. typhimurium. Proc.Natl. Acad. Sci. USA* 85:7753-7757

Bennasar A., Gloria L., Bartomeu C. and Jorge L. (1999) . Rapid Identification of *Salmonella typhimurium, S. enteritidis* and *S. Virchow* isolates by PCR based finger printing methods. *Internatl. J. Microb*. 3: 31-38.

Bern C., Martines J., Dezoysa I . and Glass R.I. (1992). The magnitude of the global problem of diarrhoeal disease: a ten-year update. Bull World Health Organization 70: 705-714

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

Bhahy M.A., Turner D.P. and Chamberlain S.T. (2000) . *Mycobacterium marinum* hand infection: case reports and review of literature. *Brit. J. Plast. Surg*., 53:161–165.

Black R.E., Brown K.H., and Becker S., (1984). Effects of diarrhoea associated with specific enteropathogens on the growth of children in rural Bangladesh. 73: 799-805

Bleiker T.O., Bourke J.E. and Burns D.A. (1996). Fish tank granuloma in a 4-year old boy. *Brit. J. Dermatol*., 135: 863–864.

Bramsnaes F. (1965). Handling of fresh fish. *In Fish as food.* Borgstrum, G.(ed.)

Brenner F.W. (1998). Modified Kauffmann-White Scheme, CDC., Atlanta,Georgia

Brooks J.T., Roger L.S., Kumar L. Wells J.G., Penelope A., Philip-Howard Vulule J.M., Hoekstra M.R., Mintz E. and Slutsker L. (2003)..Epidemiology

of periodic Bloody Diarrhoea in Rural Western Kenya. *Am. J. Trop. Med. Hyg.*, 68, 6: 671-677.

Brooks T.B., Ochieng J.B., Kumar L., Okoth G., Shapiro R.L., Wells J.G., Bird M., Bopp C., Chege W., Beatty M.E., Chiller T., Vulule J.M., Mintz, E. and Slusker L. (2006). Surveillance for Bacterial Diarrhoea and Antimicrobial Resistance in Rural Western Kenya, 1997 – 2003.

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

Cathrine M.S. (2004). Non-therapeutic, use of antimicrobial agent in animal agriculture: Implication for pediatrics. *J.American Acad, Paed.*Vol 114 No.3

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

Cheong L. (1996). Overview of the current international trade in ornamental fish with special reference to Singapore. In: *Preventing the spread of aquatic animal diseases*. (eds. B.J. Hill and T. Hastein) *Office Internatl. Des Epizooties, France* 15: 445 – 481

Cherry W.B., Hanks J.B., Thomason B.M. and Murlin. (1972). An index of pollution of surface water. *Appl. Microbiol.* 24:334-340.

Clouthier S.C., Muller K.H., Collinson S.K. and Kay W.W. (1994). Unique fimbriae like structures encoded by *SefD* of the SEF14 fimbrial gene cluster of *Salmonella enteritidis*. *Mol. Microbiol.*12:893-903.

Clouthier S.C., Muller K.H., Doran J.L., Collinson S.K. and Kay W.W. (1993). Characterization of three fimbrial genes, *SefABC,*of *S. enteritidis. J.Bacteriol.*175:2523-2533

Collier L., Albert B. and Max S. (1998). Microbiology and Microbiological Infection. *J. of Microb*. pg 969-997.

Crosa J.H., Brenner D.J., Ewing W.H. and Falkaw S. (1973). Molecular relationships among the *Salmonellae*. J. Bacteriol.115: 307 – 315

Crul R.C.M. (1995). Monographs of African great lakes; Limnology and Hydrology of L. Victoria Part 1. UNESCO/HIV-IV project comprehensive & comparative study of great lakes. A project of the fourth phase of the International Hydrological Programme of the Division of water sciences of UNESCO (UNESCO/IHP-IV Project M-5-1).

Daniel H.R., Dale D.H., Paivi M.R., Maryanne H.S., Beth C.S., Kirsten M.C., Thomas E.B. and Paul A.C. (2003). Household Contamination with *Salmonella enterica*. *Emerg. Infect. Dis*. 9: (1);120-122.

Darie H., Leguyadec T. and Touze J.E. (1993). Epidemiologic and clinical aspects of Buruliulcerin Cote-Divoire–about 124 recent cases. *Bull. Soc. Pathol. Exot*. 86: 272–276.

Davenport K. (1996). Characteristics of the current international trade in ornamental fish, with special reference to the European Union. *In: Preventing the spread of aquatic animal diseases*. (eds. B.J.Hill and T.Hastein) Review scientifique et technique. *Office International des epizooties, France* 15: 435-444

Davies R.H. and Wray C. (1996). Seasonal variations in the isolation of *Salmonella typhimurium, Salmonella enteritidis, Bacillus aureus* and *Clostridium perfringens* from environmental samples. *J. vet. Med. Ser. B* 43:119-127.

Del-Rio-Rodriguez R.E. (2002). Aerobic microflora of imported ornamental fish from Singapore and South America. *J. Fish. Vet.* 6: 1-21.

DiRita V. J. (2001). Molecular basis of *Vibrio cholerae,* pathogenesis, In E.A Groisman (ed). *Principles of bacterial pathogenesis.* Academic press, San Diego, Calif pg 457-508.

Dolzani L.E., Tonin C., Lagatolla L., Prandin and Monti-Bragadin (1995). Identification of *Acinetobacter* isolates in the *A. calcoaceticus – A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic spacer sequences. *J.Clin. Microbiol.* 33:1108-1113

Dupray, E. and A. Derrien. (1995). Influence of the previous stay of *Escherichia coli* and *Salmonella* spp. in waste water on their survival in seawater. *Water Res.* 29:1005-1011.

Edwards R.A., Schifferli D.M. and Maloy S.R. (2000). Role of *Salmonella* fimbriae in intraperitoneal infections. *Proc. Natl. Acad Sci. USA* 97:1258-1262.

Esaki H., Morioka A. and Ishihara K. (2004). Antimicrobial susceptibility of *Salmonella* isolated from cattle, swine, and poultry (2001 – 2002): Report from the Japanese Veterinary Antimicrobial Resistance Monitoring Program. *Antimicrob. Chemother.* 53: 266-70

Fell G., Hamouda O., Lindner R., Rehmet S., Liesegang A., Prager R., Gericke B. and Petersen L. (2000). An out-break of *Salmonella blockley* infections

following smoked eel consumption in Germany. *Epidemiol. Infect.*, 125, 9–12.

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

Foltz V. D. (1969): *Salmonella* Ecology. *J. AM. Oil Chem. Soc.* 46:222-224.

FOSRI. (1997): Food Science and Technology Research Institute. Annual Report.

Galanis E., Danilo M.A., Lo Wong., Mary E.P., Norman B., Anna C., Andrea E., Fredrick J.A. and Henrick C.W. (2006). Web-based Surveillance and Global *Salmonella* Distribution 2000 – 2002 - *Emerging infectious diseases*. www.cdc.gov/eid. Vol.12, 3: 381-388.

Gitonga N.K. (2006). Approaches to achieving safety of fish and fishery products in East Africa. Fisheries department of Kenya LVEMP.

Gomez T.M., Motrjemi Y., Miyagawa S., Kaferstein F. K. and Stohr K. (1997): Foodborne Salmonellosis. World Health Stat. Q. 50:81-9

Gooze L.M.D. (1998). Bacterial Infections associated with HIV. HIV Insight Knowledge Base Chapter. Stanford Univ. Sch. of Medicine.

Gorman R. and Catherine A.C. (2004). Characterization of *Salmonella enterica* serotype *typhimurium* isolates form food, animal sources in Republic of Ireland. *J. Clin. Microb.*vol 42, 5: 2314 – 2316.

Green S.D. and Cheesbrough J.S. (1993). *Salmonella* bacteremia among young children at a rural hospital in Western Zaire. *Ann. Trop. Paediatr.* 13:45-53.

Guarda R., Gubelin W., Gajardo J., Rohmann I.. and Valenzuela M.T. (1992). Cutaneous infection by *Mycobacterium marinum* – case report. *Revista Medica de Chile*, 120: 1027 – 1032.

Guo X., Chen J., Brackett R.E. and Beuchat L.R. (2000). Survival of *Salmonella* on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening. *Appl. Environ. Microbiol.* 67:47460-4764.

Guo X., Chen J., Brackett R.E. and Beuchat L.R. (2002). Survival of *Salmonella* on tomatoes stored at high relative humidity in soil. *J. Food Prot.* 65:274-279.

Hatha, A. A. M. and Lakshmanaperumalsamy P. (1997). Prevalence of *Salmonella* in fish and crustaceans from markets in Coimbatore, South India. *Food Microbiol.* 14:111-116.

Hayman J. (1991). Postulated epidemiology of *Mycobacterium ulcerans* infection. *Int. J. Epidemiol.*, 20: 1093-1098.

Helms M., Vastrup P. and Gerner-Smidt P. (2002). Excess mortality associated with antimicrobial drug-resistant *S. typhimurium*. *Emerg. infect. Dis.* 8:490-495.

Howgate P.F. (1982). Quality assessment and quality control. In Aitken. A., Mackie, I.M., Merrit, J.H and Windsor Crown, M.L. (eds). *Fish handling and processing*. Edinburgh, Scotland. Pg 70-72.

Huis in't veld J.H.J. (1996). Microbial and Biochemical spoilage of foods: An overview. *Internatl. J. of Food Microbiol.* 33:1-18.

Hultgren S.J. and Normark S. (1992). Chaperon assisted assembly and molecular architecture of adhesive pili. *Annu. Rev. Microbiol.* 45:383 – 415.

Huss H.H. (1994). *Assurance of seafood quality*. Food and organization Fisheries Technical paper 334. Rome: FAO

Huss H.H., Dalgaard P. and Gram L. (1997). Microbiology of fish and fish products. In Luten, J.B., Borresen, T., Oehlenschlager, J.(eds). Sea food from producer to consumer, integrated approach to quality. Amsterdam: Elsevier science publishers.

Inglis V., Abdulla S.Z., Angka S. L. and Chinabuts S. (1997). Survey of resistance to antibacterial agents used in aquaculture in five South East Asian countries. In: Diseases in Asia Aquaculture III (eds. T.W. Flegel and J.H. MacRae), Fish Health Section, Asian Fisheries Society. Manila Philippines *J.Clin.Infect.Dis*. 43:393-401

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

Jensen M.A., Webster J.A. and Strauss N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction - amplified ribosomal DNA spacer polymorphisms *Appl. Environ. Microbiol*. 59:945-952

Kakai R. and Wamola I. A.(2002). Minimizing Antibiotic Resistance to *Staphylococcus aures* in developing countries. East Afri. Med. J.

Kariuk S., Mirza N.B., Wastenson Y., Daniel S., Joseph M.G. and Olslik O. (1992). Tetracycline resistance genes in Kenyan hospital isolates of *Salmonella typhimurium*. APMIS, 100: 629-634.

Kariuki S., Oundo J.O., Muyodi J., Lowe B., Threfall E.J. and Hart C.A. (2000). Genotypes of multidrug resistant *Salmonella enterica* serotype *typhimurium* from two regions of Kenya. Kenyatta National Hospital case study. *FEMS Immuno. Med. Microb.* (29): 9-13.

Kariuki S., Revathi G., Gakuya F., Yamo V., Muyodi J. and Hart C.A. (2002). Lack of clonal relationship between non *typhi Salmonella* strain types from humans and those isolated from animals living in close contact. *FEMS. Immuno. Med. Microb.* 33: 165-171.

Kariuki S., Revathi G., Muyodi J.M., Munyalo A., Kagendo D., Murungi L. and Hart C.A. (2004). Increasing prevalence of multidrug resistant non-typhodal *Salmonella*, Kenya. *Intnatl. J. Antimicrob.* Agents 25: 38-43

Kauffman F. (1966). The bacteriology of *Enterobacteriacae*. Munksgaard, Copenhagen, Denmark.

Kayambo S. Sven E.J. (2006). *Lake Victoria.* Experience and lessons learnt. A case Study for Preliminary Risk Assessment Report. pp 431 – 446.

Kern W., Vanek E. and Jungbluth H. (1989). Fish breeder granuloma: infection caused by *Mycobacterium marinum* and other atypical mycobacteria in the human. Analysis of 8 cases and review of the literature (in German). *Med. Klin.*, 84: 578–583.

Kilger G. and Grimont P.A.D (1993). Differentiation of *Salmonella* Phase 1 flagella antigen types by restriction of the amplified *fliC* gene. *J.Clin.Microbiol.*31:1108-1110.

Korhomen T.K.,Virkola,R., Westurlund B., Holthofer H. and Parkinen F. (1990). Tissue tropism of *E.coli* adhesions in human extra intestinal infections. *Curr.Top. Immunol.Microbiol.*151:115-127.

Kourany M., Myers C.W. and Scheider C.R. (1970). Panamanian amphibians and reptiles as carrier of *Salmonella. A J., Trop. Med. Hug*. 19:632-638.

Le Minor L. and Lindberg A.A. (1984). Serology of *Salmonella. Methods Microbiol.* 15:1-141

Lepage P., Bogaerts J., Van G.C., Hitimana D.G. and Nsengumuremyi F. (1990). Multiresistant *Salmonella typhimurium* systemic infection in Rwanda. Clinical features and treatment, with Cefotaxime. *J. Antimicrob. Chemother.* 26 (suppl. A) 53-7.

Lewin C.S. (1992). Mechanisms of resistance development in aquatic microorganisms. *In: Chemotherapy in aquaculture*: from theory to reality (eds. C. Michael and D.J. Alderman) *Office International des Epizooties. Paris, France.* pg. 288-301.

Lindberg A.A. (1984). Serology of *Salmonella. Methods Microbiol.* 15: 1-141

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

Liston J. (1980). Microbiology in fishery science. In Connell, J.J. (ed). *Advances in Fish Science and Technology*. Jubilee Conference of Torry Research Oxford: Fishing News Books Ltd, Farnham, UK.

Liston J. (1992). Bacterial spoilage of sea foods. In Huss, H.H., Jakobsen, M. and Liston, J. eds. *Quality Assurance in Fish Industry*, pg.93-105 Amsterdam: Elsevier Science Publishers.

Macnah R.M. (1987). Flagella. In F.C Neidhart, J.L. Ingraham, K.B. Low, B., Magasanik, M., Schaechter, and Umbarger H.E (ed), *E. coli and S. enterica* serotype *.typhimurium:* cellular and molecular biology, vol.1. *A. soc. Microbiol.* Washington D.C., pg 70-83

Makene J. (2005). Fish quality Assurance at landing beaches and during transportation to processing plants. In: Knowledge and experiences gained from managing The Lake Victoria Ecosystem. Mallya G. Wambede, J. Kusewa, M. (eds). Published by LVEMP Regional Secretariat, Dar es Salaam.

Masette M. (1999). A comparative study of storage tissue of warm and cold water fish in view of the current market demands. A PhD thesis, United Nations University. UNU – Fisheries training programme.

Mayer B.K. and Ward D.R . (1991). Microbiology of finfish and fish processing. In Ward, D.R. and Hackney, C. (eds). *Microbiology of marine food products*. New York: Van Nostrand Reinhold.

Meier W. and Schmitt M. (1992). Resistance to antibiotics used in the treatment of fresh water fish during a ten-year period (1979 -1988). In Switzerland In: *Chemotherapy in Agriculture from theory office international des Epizootics*. Paris, France. pg 263-275.

Metz H. (1980). Water as a vector of infection: waterborne bacteria (in Germany). *Zentralbl Bakteriol Mikrobiol Hyg (B),* 172, 255–274.

Mian L.S., Maag H. and Jacal J.V. (2002). Isolation of *Salmonella* from muscoid flies at commercial animal establishments in San Bernardino country California. *J. Vector Ecol.* 27:82-85.

Michael O. and Bean P. (1999). Principles and applications of methods for DNA based typing of microbial organisms. *J.Clin.Microb.* Vol 37, 6: 1661-1669.

Minete H.P. (1986). Salmonellosis in the marine environment. A review and commentary. *Int. J. Zoonoses*, 13: 71–75.

Murugkar H.V., Rahman H., Kumar Ashok. and Bhattacharyya D. (2005). Isolation, phage typing and antibiogram of *Salmonella* from man and animals in North Eastern India. Indian *J. Med. Res. Article* 2005.

Nanyaro G. F. and Makene J. (1998). Report on studies of Fish handling in water, landing beaches, at markets and during transportation in Mwanza gulf. Fisheries management component. Lake Victoria Fisheries Organization Vol 1, pg 19-30.

NARO – National Agricultural Research Organization Uganda (1997). A survey of sanitary and hygienic conditions of the industrial fish processing sector, from landing sites through plant to exit routes (airport) NAROSEC report.

National Committee for Clinical laboratory standards (2001). Performance standard for antimicrobial disk susceptibility test, 7th ed. Applied Standards. NCCLS documents M2-A7. NCCLS, Weyne, pa.

Nesbitt A. and Nazir B.M. (1989). *Salmonella* septiceamia in Kenyan children. *J. of Tropic. Paediatrics*. 35: 35-39.

Newaj-Fyzul A., Abiodun A., Adesiyun and Mutani A. (2005). Prevalence and antimicrobial resistance of *Salmonella* sp isolated from apparently healthy ornamental fish and pond water in Trinidad. *J. of food. Agric. and Environ.* Vol 4, 1: 27-29

M. (eds). Published by LVEMP Regional Secretariat, Dar es Salaam. pg 90-108.

Onyango D.(2006). Molecular characterization and mechanisms of multidrug resistant human *Salmonella typhimurium* strain in Western Kenya. PhD Thesis Maseno University.

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 2008; 2(2):106-111.

Oundo J.O., Muli F. and Kariuki S. (2002). Nontyphi *Salmonellae* in Children with Severe Malaria. *East Afr. Med. J.*79:633-9

Pang J., Bhutta Z.A., Finlay B.B. and Altwegg M. (1995). Typhoid, fever and other Salmonellosis: a continuity challenge. *Trends microbiol.* 3:253-255.

Parker W.F. and Mee B. J. (1982). Survival of *Salmonella adelaide* and feacal coliform in course sands of the Swan Coastal plain, Western Australia. *Appl. Environ. Microbiol.* 43:981-986.

Paul J. H.,Rose J.B., Jiang S., Ellogg C. and Shinn E.A. (1995). Occurrence of feacal indicator bacteria in surface waters and the subsurface aquifer in key Hargo, Florida. *Appl. Environ. Microbiol. 61:2235-2241*

Popoff M.Y. and Le Minor. (1997). Antigenic formula of the *Salmonella* serovars, 7th edition World Health Organization, collaborating center for reference and research on *Salmonella*. Pausteur institute, Paris, France.

Ridley A.M., Threfall J. and Rowe B. (1998). Genotypic characterization of *Salmonella enteritidis* phage types by plasmid analysis, ribotyping and Pulse Field gel electrophoresis. *J. Clin. Microbiol*. 36: 2314 -2321.

Rodrigue D.C., Tauxe E.V. and Rowe B. (1990). International increase in *Salmonella enteritidis*: a new pandemic? *Epidemiol. infection* 105:21-27

Roger H. S., Kumar L., Philips-Heward P., Wells J.G., Adrock P., Brooks J., Ackers M.L., Ochieng J.B., Mintz E.S., Waiyaki P. and Sluker L. (2001). Antimicrobial resistant bacterial diarrhoea in rural Western Kenya. *J.infect. Dis*. 183: 1701-4.

Ruiz J., Liliana C., Laura N., Dolores C., Joseph M.S., Mohammed H., Juan J.B. and Jordi V. (1999). Mechanisms of resistance to amplicillin, chloramphenicol and quinolones in multiresistant *Salmonella typhimurium* strains isolated from fish. *J. Antimicrob. Chemother*. 43: 699 - 702.

Rwabinge A.F. (2005). Ecological sanitation in the Lake Zone: Awareness creation and action. In: *Knowledge and experiences gained from managing The Lake Victoria Ecosystem*. Mallya G. Wambede, J. Kusewa, M. (eds). Published by LVEMP Regional Secretariat, Dar es Salaam. pg 624-634

Said R., Volpin G., Grimberg B., Friedenstrom S.R., Lefler E. and Stahl S. (1998). Hand infections due to non-*cholera vibrio* after injuries from St. Peter's fish (*Tilapia zillii*). *J. Hand Sur. British and European*, 23: 808–810.

Saitanu K., Chongthaleong A., Endo M., Umeda T., Takami K., Aoki T. and Kitao T. (1994). Antimicrobial susceptibilities and detection of transferable R-plasmids from *Aeromonas hydrophila* in Thailand. Asian fisheries science. 7:41- 46.

Sanyal D., Douglas T. and Roberts R. (1997). *Salmonella* infection acquired from reptilian pates. *Arch. Dis. Child.* 77: 345-346.

Seiberras S., Jarnier D., Guez S. and Series C. (2000). *Mycobacterium marinum* nodular lymphangitis. *Press. Med.*, 29: 2094–2095.

Senanayake S.N., Ferson M.J., Botham S.J. and Belinfante R.T. (2004). A child with *Salmonella enterica* serotype Para-typhi B infection acquired from a fish tank. *Med. J.Australia*, 180: 250.

Shewan J.M. (1961). The Microbiology of sea water fish. In Borgstrum, G. (ed). *Fish as food* 1:487-9

Shotts E.B.J., Vanderwork V.L. and Campbell L.M. (1997). Occurrence of R factors associated with *Aeromonas hydrophila* isolated from aquarium fish and waters. *J.Fish.Res. Board of Canada*. 33: 736 - 740.

Singh D. V. and Sanyal S. C. (1997). Enterotoxicity, haemolytic activity and antibiotic susceptibility of *Aeromonas eucrenophila* strains isolated from water and infected fish. *Indian J. Exp. Bio*. 35: 144 -147.

Sorensen N.K. and Mjelde A. (1992). Preservation of pelagic fish quality for further processing on board and ashore. In Burt, J.R. Hardy, R., Whittle, K.J. (eds). Pelagic fish: *The Resource and its Exploitation*, pg 40. Oxford: Fishing News Books.

Sugita H., Miyajima C., Fukumoto M., Koyama H. and Deguchi Y. (1989). Effect of Oxolinic acid on fecal Microflora of gold fish *(Carassius auratus)*. Aquaculture 80: 163 – 174.

Tauxe R. V. (1997). Emerging food borne diseases: an evolving public health challenge. The fish exporting and fishmeal industries of Lake Victoria - structure,

strategies and socio-economic impacts in Kenya: Annual Report, *Emerge. Infect. Dis*. 3:425-434.

Thomason B. M., Dodd D.J. and Cherry W.B. (1977). Increase recovery of *Salmonella* from environmental samples. Enriched with buffered peptone water. *Appl. Environ. Microbial*. 34:270-273.

Thorns C.J., Sojk M. G. and Chasey D. (1990). Detection of a novel fimbrial structure on the surface of *Salmonella enteritidis* by using a monoclonal antibody. *J. Clin. Microbiol*. 28:2409-2414.

Threfall E.J. (2000). Epidemic *Salmonella typhimurium* DT104 – a truly International Multiresistant clone. J. *Antimicrob. Chemother*. 44: 2166-9.

Threfall E.J. (2002). Antimicrobial drug resistance in *Salmonella*: problems and perspectiveness in food and waterborne infections. *FEMS Microbiol.Rev*.26:141-148

Threfall E.J., Fisher I.S.T., Berghold C., Gernersmidt H.P., Tscape H., Comican M., Luzzi, I., Schieder F., Wannet W., Machado J. and Edwards G. (2003). Antimicrobial drug resistance in isolates of *S. enterica* from cases of Salmonellosis in human in Europe in 2000: Results of International Multi-center surveillance. *Eurosurveilance* 8:41-45

Threfall E.J., Frost J.A., Ward L.R. and Rowe B. (1994). Epidemic in cattle and humans of *Salmonella typhimurium* DT104 with chromosomally integrated multiple drug resistance. *Vet. Rec*.134:577

Threfall E.J., Hampton M.D., Schofield S.L., Ward L.R., Frost J.A. and Rowe B. (1997). Epidemiological application of differentiating multiresistant

Salmonella typhimurium DT 104 by plasmid profile, *Comm. Dis. Rep. Rev.* 6:155-159.

Thrower S.J. (1987). Handling practices on inshore fishing vessels: Effect on the quality of finfish products. CSIRO Food Research, 47:50-57.

Todd E.C. D. (1997). Epidemiology of foodborne diseases: a worldwide review. World health Stat. Q. (50): Non-typhi *Salmonella* in children with severe malaria *East Afr.Med.J.*79: 633-9

Townsend S.M., Kramer N.E. and Edwards R.A. (2001). *Salmonella enterica* serotype typhi possesses a unique repertoire of fimbrial genes. *Infect. Immun.* 69:2894-2901

Turcotte C. and Woodward M.J. (1993). Cloning DNA nucleotide sequence and distribution of gene encoding the SEF14 fimbrial antigen of *S. enteritidis*. *J.Gen.Microbiol.* 139:1477 – 1485.

US FAD (2006). Food borne Pathogenic microorganisms and natural toxins.

Velge P., Alex C. and Barrow P. (2004). Emergence of *Salmonella* epidemic: the problems related to *Salmonella enterica* serotype *enteritidis* and multiple antibiotic resistance in other major serotypes. *Vet. Res.* 36: 267-288.

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Von-Reyn C.F., Arbeit R.D., Tosteson A.N.A., Ristola M.A., Barber T.W., Waddell R., Sox C.H., Brindle R.J., Gilks C.F., Ranki A., Bartholomew C., Edwards J., Falkingham J.O., O'Connor G.T., Jacobs N.J., Maslow J., Lahdevirta J., Buhler S., Ruohonen R., Lumio J., Vuento R., Prabhakar P. and Magnusson M. (1996). The international

epidemiology of disseminated *Mycobacterium avium* complex infection in *AIDS*. AIDS, 10: 1025–1032.

Waiyaki P.G. (1993). Bacterial drug resistance diarrhoeal diseases and laboratory diagnosis of Pulmonary tuberculosis (editorial) East Afr. Med. J. 70: 259-262.

Wallis T.S. and Eduoard E.G. (2000). Molecular basis of *Salmonella* induced enteritis. *J. Mol. Microb.* 36: (5); 997-1005.

Walsh A.L., Molyneux E.M., Kabudula M., Phiri A.J., Molyneux M.E. and Graham S.M. (2002). Bacteremia following blood transfusion in Malawian children: predominance of *Salmonella*. *Trans. Roy soc. Trop. Med. Hyg.* 96: 276-7

Ward D.R. and Baj N.J. (1998). Factors affecting Microbiological quality of sea foods. *Food Technology*. 42, 3:85-9.

White D.G., Datta A.P., McDermott S., Friedman S., Qaiyumi S., Ayers L., English S. and McDermott D.D. (2003). Antimicrobial susceptibility and genetic relatedness of *Salmonella* serovars isolated from animal derived dog treats in the USA. *J. of Antimicrob. Chemother*. 52:860-863

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