

Isolation of *Salmonella* and *Shigella* from fish harvested from the Winam Gulf of Lake Victoria, Kenya

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Abstract

Background: Human infections caused by pathogens transmitted from fish are quite common. The aim of this study was to isolate enteric pathogenic bacteria from fish that might be transmitted to humans after the handling or consumption of such fish.

Methodology: One hundred and twenty Nile tilapia fish harvested using various fishing methods were collected from fishermen in five fish landing beaches within Winam Gulf and disinfected externally using 70% ethyl alcohol for 2 minutes then washed three times with autoclaved distilled water. Isolation of *Salmonella* and *Shigella* species from fish samples was performed using standard bacteriological procedures. Five milliliters of each fish tissue slurry was microbiologically analyzed for any *Enterobacteriaceae*. Twelve Nile tilapia collected from three open-air markets were analyzed for *Enterobacteriaceae* comparison as controls. Identification of *Salmonella* by using housekeeping genes and species-specific primers was performed.

Results: Among 120 Nile tilapia, 63 (52.5%) were infected with *Enterobacteriaceae*. Out of these, 25 (39.7%) were *Shigella* spp., 9 (14.3%) *Salmonella typhimurium*, 7 (11.1%) *S. typhi*, 4 (6.3%) *S. enteritidis*, 16 (25.4%) *Escherichia coli*, 1 (1.6%) *Proteus* spp. and *Enterobacter aerogenes* respectively. Ten fish collected from open-air markets yielded *E. coli* (50%), *S. typhimurium* (20%), *S. paratyphi* (10%) and *S. typhi* (20%).

Conclusion: Nile tilapia within Winam Gulf are infected by human enteric pathogens. *Shigella* spp., *Salmonella* and *E. coli* were the most frequently isolated, an indication that the beaches may be contaminated by untreated municipal sewage, runoff, and storm-water. *S. typhimurium*, *S. typhi* and *S. enteritidis* were the most common *Salmonella* isolates.

Key Words: Fish, Nile tilapia, *Salmonella*, *Shigella*, Winam Gulf, Kenya.

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Introduction

Human infections caused by organisms transmitted from fish or the aquatic environment are quite common depending on the season, the patients' contact with fish and related environment, dietary habits, and the immune system status of the exposed individual [1]. 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 infective source may be fish kept either for food or as ornaments [2]. However, quantification of the occurrence of these diseases is difficult because many manifestations, typically gastrointestinal illness, go unreported since the symptoms usually do not last long and are self-limiting in healthy people. Pathogenic and potentially pathogenic bacteria associated with fish and shellfish include mycobacteria, *Streptococcus iniae*, *Vibrio vulnificus*, *Vibrio* spp., aeromonads and *Salmonella* spp.

[1,3,4]. Human infections by these fish pathogens are usually through contact with infected fish while handling or with water or other constituents of an aquatic environment [2].

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 the exposed person, particularly immunosuppression and stress as in the case of HIV and AIDS (5). Fish and shellfish appear to be passive carriers of *Salmonella* that demonstrate no clinical disease and can excrete *Salmonella* spp. without apparent symptoms [6,7,8,9]. An outbreak of *Salmonella blockley* infections following consumption of smoked eel fish from four different local smoke-houses in Italy was reported in Germany (8).

Lake Victoria fisheries is dominated by two introduced species, Nile perch (*Lates niloticus*) and Nile

tilapia (*Oreochromis niloticus*), and one native cyprinid (*Rasneobola argentea*) [10]. Mitigation of environmental problems in Lake Victoria indicates five immediate microbiological pollutants, namely municipal untreated sewage, runoff, storm-water, animal waste, and maritime transport waste. Discharge of untreated municipal effluent into rivers and the lake, compounded by lack of awareness of good hygiene practices, directly contribute to the degradation of river- and lake-water quality for habitats and domestic use [10]. The effluents have significantly increased biological oxygen demand (BOD), thus significantly increasing the occurrence of water-borne diseases such as typhoid and cholera which are common in the region [10]. The effect of runoff and storm-water that collect a lot of animal, plant and human wastes from point and non-point sources and channel these to the rivers and the lake create an environment that supports microbiological pathogens [10]. These factors increase the eutrophication process thus creating a vast conducive environment for the survival of microbes which eventually infect the fish (e.g., *Salmonella* spp.) which in recent years have been isolated from various fresh and marine waters contaminated by urban sewerage [11]. It was therefore imperative to isolate enteric pathogenic bacteria from fish that might be transmitted to humans after handling, or consumption of such fish, from Winam Gulf of Lake Victoria, Kenya.

Materials and Methods

Nile tilapia fish samples were collected within a period of three months (January to March 2007) during the dry season to avoid the effects of water dilution during the rainy season. Sampling was done three times for each identified fish landing beach, i. e. Dunga, Uhanya, Homa Bay, and Luanda-Kotieno beaches (Fig. 1). Ten fish collected from fishermen were randomly selected from each fishing site during each sampling time after harvest by various fishing methods. Sampled fish were placed in sterile plastic bags and transported to the laboratory in cool boxes at 4°C within four hours from the time of collection. In the laboratory, the fish were disinfected by dipping them into 70% ethyl alcohol for two minutes, followed by three washings with autoclaved distilled water.

Fish were macerated without evisceration in sterile phosphate buffer saline (PBS) of pH 7.5 to achieve 10% w/v suspension of fish. The macerated fresh fish flesh extract was cultured in Selenite F broth/Selenite cystine broth for maximum *Salmonella* pathogen isolation. A portion of the extract was cultured in MacConkey Agar

medium to harvest coliforms and intestinal pathogens. 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) and incubated at 37°C for 18 to 24 hours. Colonies from MacConkey agar were subcultured in Xylose Lysine Deoxycholate (XLD) agar (Oxoid Basingstoke, UK) to saturation and transferred to Nutrient broth (CDH JO 0003) and preserved at 4°C in refrigeration for future use. Biochemical tests (IMViC – Indole, Methyl red, Voges proskaur's, Citrate) were performed to identify bacteria genus based on their biochemical activities in different culture Media [12,13]. Isolates identified biochemically as *Salmonella* or *Shigella* were serotyped using O and H antisera (Safoni – Germany) [12,13,14].

Figure 1. Showing the Winam Gulf and selected fishing beaches.



Genetic identification of *S. typhimurium* colonies was performed by picking pure *Salmonella* isolates from the saturated agar-disk plates using a sterile plastic inoculation loop and reconstituted in 200 µl of 0.9% sodium chloride solution for DNA isolation and analysis. The homogenate solution was then injected into an automated QIAmp Qiagen DNA extraction machine for 20 minutes (Qiagen, west Sussex, UK). The extracted *Salmonella* DNA was then used for molecular identification and analysis. The housekeeping gene determination was done using the primers (*mdh* F; 5' – TGC CAA CGG AAG TTG AAG TG – 3' R; 5' – CGC ATT CCA CCA CGC CCT TC – 3'). Forty-five microliters of PCR master mix + 5 µl QIAmp chromosomal DNA template were aliquoted into 0.2 ml eppendorf tubes. The mixture was thoroughly vortexed and then centrifuged at 10,000 g for six seconds

(Eppendorf centrifuge 5415D, Germany). The prepared mixture was then amplified using an MJ Gradient Thermocycler (PTC-225, Peltier Thermocycler, BioEnzymes, Germany). PCR was performed with the following conditions: at 94°C for five minutes for denaturation; 94°C for 25 seconds; annealing temperature of 54 °C for 45 seconds; at 35 cycles and extension temperature of 72 °C for 45 seconds; cooling at 72°C for 7 minutes then 4°C until removed. The amplicons were then loaded onto a casted 1.5% agarose gel (1.5 grams agarose powder + 100 ml of 1 × TBE buffer) with a gene marker of 50 bp, a negative control, and positive control. This was then let to run for 25 minutes at 135V after which the UV pictures were taken using the UV photo transilluminator (Gel Logic 100 Imaging System, Kodak) [15].

To differentiate between *Salmonella* spp., the following primer combinations were used: ST 11- ST 15; ST 11 (length 24) -5' -GCCAACCATTGCTAAATTGGCGCA -3' ST 15 (length 24) -5' -GGTAGAAATTCCCAGCGGGTACTGG -3'; *fliC* - *Fli 15-Tym* and *Fli 15 & Typ 04*.

Amplification of *fliC* (primer sequence; *Fli 15* (length 22) - 5' -CGGTGTTGCCAGGTTGGTAAT - 3' *Tym* (length 22) - 5' -ACTCTTGCTGGCGGTGCGACTT 3') and *sef A* - (primer sequence; *Sef 167* -*Sef 478*; *sef 167* (length 20) - 5' -AGGTTTCAGGCAGCGGTTACT - 3' *sef 478* (length 20) - 5' -GGGACATTTAGCGTTTCTTG 3') genes [16] was conducted in a total volume of 50 µl, containing 0.5 µl of Hot start Taq Polymerase (Qiagen, West Sussex, UK), 0.5 µl of each primer, 2 µl of each dNTP, 10 µl Q-Buffer (Qiagen, West Sussex, UK), 5µl of 10×Buffer (Qiagen, West Sussex, UK) and 5µl DNA, using a MJ Gradient Thermocycler (PTC-225, Peltier Thermocycler BioEnzymes, Germany) PCR. The reaction was conducted for 35 cycles of 30 seconds for denaturation at 95°C with the following conditions: building up of Hot start temperature at 95°C for 15 minutes; denaturation temperature at 94°C for one minute, for 40 cycles; annealing temperature of 55 °C for 45 seconds, 72°C for 30 seconds; extension temperature at 72° C for seven minutes then 4°C until removed. The amplicons were then loaded on to a casted 1.5% agarose gel (Eurobio, Les Ulis, France) run in TBE buffer (89mmol l⁻¹ Tris pH 8.3, 89mmol l⁻¹ borate and 2 mmol l⁻¹ EDTA). The gel was then stained with ethidium bromide solution (0.5µg ml⁻¹) and photographed under ultraviolet light (Gel Logic 100 Imaging System, Kodak) [13, 16]. All the *Salmonella*

primers used in this study were obtained from the gene bank [16].

Results

A total of 120 fish were collected for a period of three months (January to March 2007). Out of these, enteric bacteria were isolated from 63 (52.5%) samples. Analysis of the fish slurry indicated that the harvested fish were mostly infected with *Enterobacteriaceae* namely, *Shigella*, *Salmonella* and *E. coli*. The highest prevalence of *Enterobacteriaceae* isolated from the Nile tilapia was at Dunga, 19 (30%); Uhanya, 18 (29%); and Homa Bay, 17 (27%); followed by Luanda Kotieno, 9 (14.2%) respectively (Table 1).

Shigella spp., 25 (39.6%) was the most isolated *Enterobacteriaceae* followed by *Salmonella* spp. (20; 31.7%), *E. coli* (16; 25.3%), *Proteus* spp. (1; 1.58%), and *E. aerogenes* (1; 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%) (Table I). However, comparably, 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%), followed by four (25%) in Dunga and Uhanya beach and two (12.5%) in Luanda Kotieno.

Table 1. Distribution of *Enterobacteriaceae* isolates from fish along selected beaches within Winam Gulf.

Beach	Total number (%) of <i>Enterobacteriaceae</i> isolated					Total
	<i>Shigella</i> spp.	<i>Salmonella</i> spp.	<i>E.coli</i>	<i>Proteus</i> spp.,	<i>E. aerogenes</i>	
Dunga (n=19)	6 (24)	8 (40)	4 (25)	1 (100)	0	19 (30)
Uhanya (n=18)	8 (32)	6 (30)	4 (25)	0	0	18 (29)
Homa Bay (n=17)	6 (24)	4 (20)	6 (37.5)	0	1 (100)	17 (27)
Luanda Kotieno (n=9)	5 (20)	2 (10)	2 (12.5)	0	0	9 (14)
Total	25 (39.6)	20 (31.7)	16 (25.3)	1 (1.58)	1 (1.58)	63 (100)

Analysis of isolated *Salmonella* spp. revealed that nine (45%) were *S. typhimurium*, seven (35%) *S. typhi* and four (20%) *S. enteritidis*. This phenotypic

identification was confirmed by amplification of the *Salmonella* housekeeping gene *malic dehydrogenase* (*mdh*) that gave a band of 261bp (Fig. 2). For identification of *S. typhimurium* within isolated *Salmonella* species, ST 11 – ST 15, and *FliC* and *Tym* primer pairs were used to amplify the specific genes in the *Salmonella* isolates and a band of 429bp and 559bp were obtained respectively for the 9 specific samples (Fig. 3, 4). This was a confirmation of the isolates as *S.*

Figure 2. Genotypic presentation of *Malic dehydrogenase* gene. PCR gel showing band for *Malic acid dehydrogenase* gene product in selected *Salmonella* isolates. Lanes 1 and 14: M = Molecular DNA marker 50bp; lanes 2 and 15: Ø = negative control. Lanes 3, 5, 8, 11 tested isolates from Dunga beach; lanes 12, 13 tested isolates from Uhanya; lane 19 tested isolate from Luanda Kotieno; lanes 21, 22, 23 tested isolates from Homa Bay.

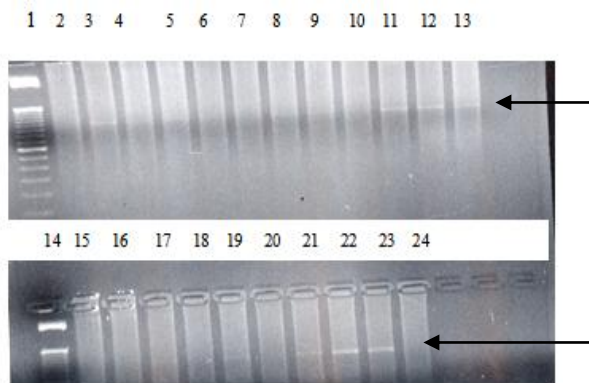


Figure 3. Genotypic presentation of *Salmonella* strains by use of ST11 and ST15 random sequence primers. PCR gel showing presence of ST11-ST15 gene pair. Isolates from Dunga (lanes 4, 5 & 6), Uhanya (lanes 7 & 8), Luanda Kotieno (Lane 9), and Homa Bay (lanes 11 & 14). Molecular DNA marker 50bp (lane 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.

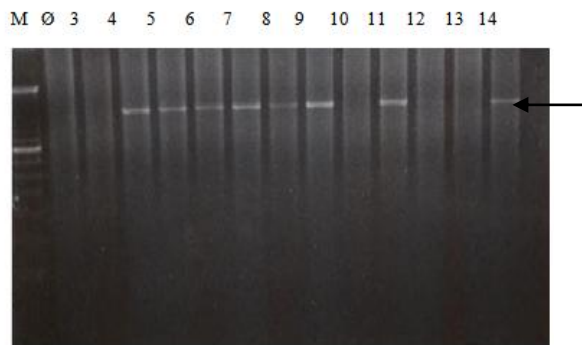


Figure 4. Genotypic presentation of *Salmonella* species by use of *fliC* gene. PCR gel showing *fliC* gene products for *Salmonella* (559bp). Isolates from Dunga (Lanes 4, 5, 6 & 7), Uhanya (Lanes 8 & 9), Luanda Kotieno (Lane 12) and Homa Bay (Lanes 13 & 14). Molecular DNA marker 50bp (lane 1), Ø; negative control (lane 2).

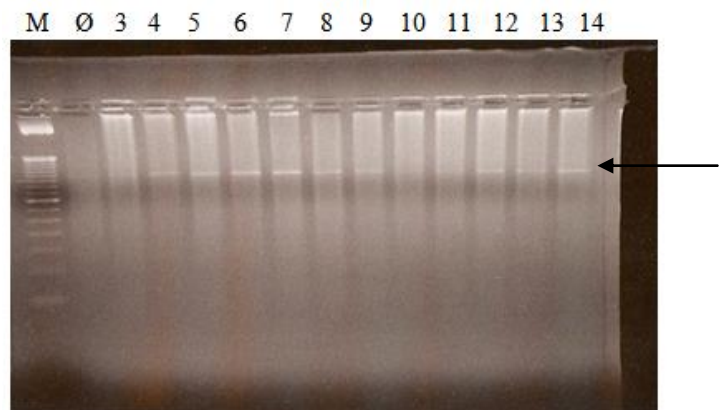
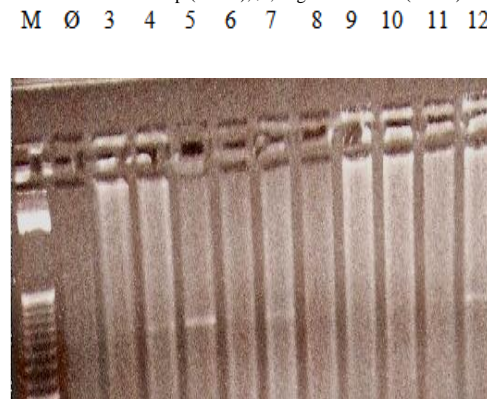


Figure 5. Genotypic presentation of *Salmonella* species by use of *sef A* gene. PCR gel showing *sef A* gene products for *Salmonella* (312bp). Isolates from Dunga (lanes 4 & 5), Uhanya (lane 7), and Homa Bay (lane 12). Molecular DNA marker 50bp (lane 1), Ø; negative control (lane 2).



typhimurium. *S. enteritidis* identification was genotypically confirmed by 312bp gene amplification of *Sef A* (Fig. 5).

Isolates of the fish collected from open-air markets displayed the following results: *E. coli*, 5 (50%), *S. typhimurium*, 2 (20%); *S. typhi*, 1 (10%); and *S. paratyphi*, 2 (20%).

Discussion

Lake Victoria supports the most productive freshwater fishery in the world, with annual fish yields exceeding 300,000 tons worth US \$600 million annually [17]. Several sectors have contributed to microbiological pollution of the lake, including runoff and storm-water that contain deposits from wildlife, agriculture, urban, forestry and rural settlements. These factors have contributed to the escalating population of human-fish microbes within Winam Gulf. Most of these

beaches within Winam Gulf are found less than 50 m away from densely populated settlement areas which are prone to flooding. Lack of proper sewage amenities together with a high water table has permitted untreated sewage to enter the lake either through runoff, storm-water or by fishing forks used by people fishing in the lake. Pathogenic human *Enterobacteriaceae* deposited in the lake attach to the vegetation as well as lodge on the gills and body surfaces of fish. In this study, Dunga and Uhanya beaches are reported to have the highest *Salmonella* and *Shigella* distribution. The two beaches are densely populated and are at close proximity to Lake Victoria with a closed shoreline; thus the water circulation in the region is poor. Previous studies have shown 5.2% prevalence of diarrhoeal diseases caused by *Enterobacteriaceae* in these regions [18], which correlates well with the high *Enterobacteriaceae* population in this study. This situation is also compounded by the fact that only 61% of the total number of households (76,915/123,341) use flush toilets that sometimes run dry without water in Kisumu District as a whole. The remaining 39% (46,426 households) use either pit latrines or none at all [19].

The same scenario is observed in Homa Bay District, where there are only 1,044 pit latrines and 157 water closets shared among 67,040 households [20]. Untreated sewage effluent is disposed directly to Lake Victoria because of the debilitated sewage treatment system in the region. Thus the distribution of *E. coli* in the population (37.5%) can be seen as an indicator of faecal contamination amongst other coliforms. This observation correlates well with the diarrhoea prevalence of 8.7% of the total number of outpatients recorded in 2006 [20]. Transportation of fish in dirty fishing boats the packaging of fish in dirty baskets and plastic basins, and the display of fish on dirty nylon sheets for buyers 20 meters from the shoreline could also enhance post-harvest contamination of the fish by the fish forks. Gutting and washing of the fish using dirty water collected directly from the lake due to lack of proper municipal piped water could exacerbate the contamination process within the beaches.

In Uhanya, Bondo District, the prevalence of diarrhoea was 6.8% of the total outpatient diseases recorded in 2006 [18]. The region had the highest distribution of *Shigella* spp. (32%) followed by *Salmonella* spp. and *E. coli*. The population in the region shares the same problem affecting Dunga, Homa Bay, and Luanda Kotieno beaches. However, in Luanda Kotieno, diarrhoea prevalence was 6.8% and *Enterobacteriaceae* distribution was equally low in fish

collected from the region. This observation was attributed to the lake area being open offshore, where there is adequate mixing of lake-water with that of river-water from the Nile River, which passes a few meters way from the shoreline. The area lacks papyrus reeds and other vegetation such as water hyacinth that could lead to eutrophication and hence thriving of coliforms as experienced in other areas in this study. However, the low *Enterobacteriaceae* population observed against a high diarrhoea prevalence of 6.8% in Luanda-Kotieno could be attributed to poor fish-cooking methods before consumption practiced by the community.

In this study, it was realized that the waters of the highly populated coastal areas receive large quantities of treated and sometimes untreated wastewater discharged from human and industrial sources. In addition, since rivers and rainfall could introduce enteric pathogens from distant sources into coastal waters [21, 22], it was believed that they contributed to the lake-water pollution.

Salmonella enterica has been considered the causal agent of the largest number of enteric infections in the world [23]. Raw foods and cross-contamination of ready-to-eat products are the main routes of *Salmonella* transmission [23]. It is therefore concluded that isolation of *Salmonella* in fish harvested from the lake was an indication of contamination of the waters by the pathogen. The sources of salmonellae are poorly understood, and this study provides vital data that is critical in assessing and controlling the risk associated with the presence of salmonellae in the marine environment. Assuming that the main source of *Salmonella* contamination in the marine environment is of human or animal origin, the different population structures may be attributed to the different rates of growth and survival of these serovars in the marine environment and this necessitates a pilot study to determine the source of the isolates. It is also concluded that, *S. 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 [21, 24]. The presence of diverse enteric bacteria in the aquaculture environments suggests that strict hygiene procedures should be followed during the handling and processing of fish from the culture systems to prevent the transfer of potentially pathogenic bacteria to humans. Thus there is need for a code of practice for fish growers in tropical aquaculture systems to ensure safe food sources.

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