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Source Attribution of *Salmonella* and *Escherichia coli* Contaminating Lake Victoria fish in Kenya

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Abstract

Escherichia coli and *Salmonella* are major contaminants of fish from Lake Victoria but the sources and possible reservoirs of these microbes have not been determined. Fish and environmental samples were collected from five locations in Kenya, along with human specimens and analysed for *E. coli* and *Salmonella* spp. *E. coli* was the most common isolate recovered in humans but it was lowest in freshly caught fish. *Salmonella* spp was recovered in soil, sundried fish, humans and domestic animals. Enterotoxigenic *E. coli* were detected among humans but no *E. coli* virulence genes were detected among fish isolates. Soil was the possible source of contamination of fish, based on closeness of *Salmonella* isolates recovered from soil and a 38% misclassification of *E. coli* isolates from fish as soil isolates. Phenotypic approaches employed in this study are promising tools for attibuting sources reponsible for fish contamination in the region.

Keywords: Attributing sources, Domestic animals, *Escherichia coli,* Lake Victoria *Salmonella*

Introduction

Lake Victoria is an important source of water, food, employment, and income for communities living around its shores. Fish is the main source of protein for humans and animal feed in most of these communities and it is eaten either freshly cooked or after undergoing some form of preservation or processing such as sun-drying, salting or smoking (Abila and Jensen, 1997). Low microbiological quality of fish processed or preserved in the region has been reported in these fish products (Mungai *et al.*, 2002; Ogwang' *et al.,* 2005; Sifuna *et al*., 2008). Fish from Lake Victoria have previously been banned from the lucrative markets in the European Union (EU) because of contamination by pathogens (Abila, 2003; Ogwang' *et al*. 2005).

In East Africa the identification of food safety risks associated with fish have generally relied on traditional methods such as counts of total and faecal coliforms, and presence or absence of pathogens such as *Salmonella* spp. However, the ecology, prevalence, and resistance to stress of coliforms differ from those of many of the pathogenic microorganisms for which they are a proxy (Desmarais *et al*., 2002). The differences between coliforms and pathogenic enteric bacteria are so great that they limit the utility of coliforms as indicators of faecal contamination.

Foods contaminated with human or animal faeces are regarded as a risk to human health, because they are more likely to contain human-specific enteric pathogens such as *Salmonella enterica* serovar Typhi, *Shigella* spp., hepatitis A virus, and Norwalk-group viruses among others (Scott *et al*., 2002). Consequently, *E. coli* may not be a reliable indicator in tropical and subtropical environments owing to its ability to replicate in contaminated soils. The ubiquitous nature of coliforms means that their effectiveness as traditional indicators to predict the presence of human or animal waste and subsequent health risks is limited (Desmarais *et al*., 2002). Furthermore, some *E. coli* strains such as enteropathogenic (EPEC), enteroinvasive (EIEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) *E. coli* serotypes

(Donnenberg and Whittam, 2001) have been shown to be pathogenic.

The usefulness of *E. coli* as microbial indicator tools for risk assessment can be enhanced by development of testing methods and analysis techniques that detect specific sources of these organisms. Microbial source tracking (MST) is a set of techniques used to determine the sources of faecal bacteria in the environment (Wiggins, 1996; Wiggins *et al*., 1999; Guan *et al*., 2002; Simpson *et al*., 2002). This can be achieved by the characterization of isolates of a specific pathogen by either phenotypic or genotypic subtyping methods (Meays *et al*., 2004). In this approach, differences within different lineages of bacteria are usually focused on traits that may have been acquired from exposure to different host species or environments.

By employing some of these approaches in this study, it was possible to determine a relationship between different isolates and therefore establish the sources of contaminants in fish with diarrhoeagenic bacteria originating from Lake Victoria. Earlier studies on the microbial quality of fish from the lake have identified domesticated animals and humans, and soil and water as potentially important sources of bacterial contamination of fish (Mungai *et al*., 2002; Ogwang' *et al*., 2005; Onyango *et al*., 2009). There is little evidence, however, to confirm that these are the sources of contamination of fish in Lake Victoria.

In Africa, environmental reservoirs and predominant modes of transmission are poorly known although various sources such as farm animals, pets and reptiles have been implicated in the transmission of *Salmonella* between animals and humans (Wall *et al*., 1996). This study investigated the distribution of *Salmonella* spp. and pathogenic *E. coli* pathotypes among samples from domesticated animals, soil, and Lake Victoria water, and from humans and fish products, using phenotypic techniques to identify possible sources of fish contaminated with the bacteria responsible for diarrhoeal infections in the study area.

Methods

Sample collection

This study targeted both rural and urban communities around Lake Victoria in the Nyanza region, western Kenya (Figure 1). Samples were collected at five fish landing beaches, namely Sirongo, Dunga, Homa Bay, Mbita town, and Luanda Konyango, a region with a 16% prevalence of diarrhoea among children (Kenya National Bureau of Statistics, 2010). Sampling was carried out over a period of two years, 2010 and 2011, with samples being collected three times a year (April, August and December). Seasonal variations were not considered in this study since fishing and fish processing by sun-drying takes place along the beaches throughout the year and was assumed to be unaffected by seasons.

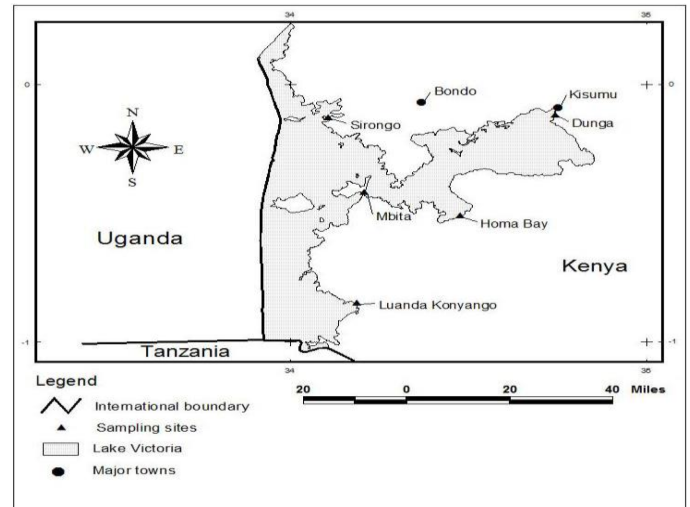

Figure 1: Location of the Nyanza Gulf of Lake Victoria, Kenya, showing the sampling sites.

Faecal samples from donkeys, goats, chicken and cattle, as well as water from the lake, fish products and soil were collected from the study sites. Human stool samples were collected from patients recruited at the Kisumu County hospital, chosen because of its location along Lake Victoria and the presence of different types of diarrhoeagenic bacteria among the patients. For ethical reasons this research proposal was presented to the Nairobi University/Kenyatta National Hospital Ethics committee - P239/06/2011 and the National Commission of Science Technology and Innovation (NACOSTI) – NCST/RRI/12/1/BS-011/39/3 for approval.

Approximately 500 g of Nile perch (*Lates niloticus*), and 150 g each of fresh and sun-dried omena/dagaa (*Rastrineobola argentea*) were purchased from fishermen at the landing sites, during fish landing and after processing or at the nearest markets. The fish were collected aseptically and placed in sterile plastic containers. Water samples were collected from each sampling site, at the shoreline, and 100 m and 150 m from the shore, by submerging and filling sterile 100-ml bottles to depth

of 30 cm below the surface. Soil samples were collected aseptically using a sterilized spoon at each point and placed into sterile containers. At least six samples of loose surface soil were collected from different points where fish were being dried; these points were 15 cm apart from each other on the beach and pooled together after collection to form a single sample.

Approximately 40 g of freshly deposited faeces from domesticated animals were aseptically collected using a sterilized spoon and put into sterile containers. The samples were collected only from animals found within 500 m of the fish landing site. Human stool specimens were collected in sterile plastic disposable stool cups containing Cary-Blair transport medium (HIMedia Lab. Pvt. Mumbai, India).

A total of 543 samples were collected during the study period, made up of 170 fish samples, 180 of animal faeces and cloacal swabs; 90 of lake water, 30 of soil and 73 human stool specimens. The human stool samples came from 30 males and 43 females (ratio = 1:1.43), ranging in age from 9 months to 63 years. All the samples were transported on ice in insulated containers to the Biomedical Science and Technology laboratory at Maseno University for analysis.

Determination of Salmonella *spp. and* E. coli

The estimation of *Salmonella* and *E. coli* in water and fish samples was carried out using methods described in FAO (1992). Samples of fish (25 g) or water (25 ml) were weighed aseptically and added to 225 ml of buffered peptone water (HIMedia Lab. Pvt., Mumbai, India). Fish samples were homogenized in a stomacher 400 circulator (Seward Ltd; England) but water samples were mixed by whirling the mixture by hand for 30 seconds. This was followed by direct plating on MacConkey agar (HIMedia Lab. Pvt., Mumbai, India) for recovery of *E. coli* and then incubated at 37°C for 18-24 hours. Selenite cystene broth and tetrethionate broth (HIMedia Lab. Pvt.Mumbai, India) were used for enrichment of *Salmonella* spp. This was followed by plating on xylose lysine desoxycholate (XLD) and MacConkey agar (HIMedia Lab. Pvt., Mumbai, India), after which characteristic colonies of *Salmonella* spp were identified and recovered for further biochemical tests and API.

Animal and human faecal material was analyzed for diarrhoeagenic bacteria using methods in Kariuki *et al.* (1999). The recovery of *E. coli*, and *Salmonella* spp. was done by direct plating on selective MacConkey and XLD agar media (HIMedia Lab. Pvt. Mumbai, India) followed by incubation at 37°C for 18-24 hours. Selenite F and tetrathionate broths (HIMedia Lab. Pvt. Mumbai, India) were used for enrichment. The moisture content of the soils, recovery of *E. coli* and *Salmonella* was carried out using standard procedures (van Elsas and Smalla, 1997). Bacterial isolates were identified and confirmed using standard biochemical techniques (Ewing, 1986).

Determination of E. coli *pathotypes*

Isolates from the previous cultures, confirmed as *E. coli*, were probed for virulence factors by multiplex PCR (Panchalingam *et al.* 2012) using the PCR primers listed in Table 1. DNA from the bacterial isolates was isolated by the boiling method in which three bacterial colonies from MacConkey agar were resuspended in 500 µl of sterile DNase-free, RNase-free deionized water (GIBCO) and heated on a heating block (Techne; UK) at 100°C for 10 min. Each test was performed in volume of 20 µl containing the following PCR components: 1.25 mM dNTP (Fermentas; Thermo Scientific), PCR buffer, $MgCl_2$ (final concentration 2mM) and 20 pmol/µl of each primer (Eurofins, UK) (Table 1), 1.25U of *Taq* DNA polymerase (Bio Labs; New England), and 3µl of DNA template (50 ng). DNA isolated and purified from reference *E. coli* JM042 (EAEC), H10407 (ETEC) and E 2348/69 (EPEC) were used as controls. A multiplex PCR was performed in an Eppendorf Mastercycler (Hamburg) under the following conditions: preheating at 96°C for 4 min, followed by 35 cycles beginning with 20 secs of denaturation at 95°C, 20 secs of primer annealing at 57°C, and 1 min of elongation at 72°C, and final extension at 72°C for 7 min. The amplified PCR products were then resolved by electrophoresis in 2% agarose gels in 1 x Tris Borate - EDTA buffer (Sigma Life Science; USA) at 100 V and bands visualized by a UV transilluminator.

Table 1. The primer sequences used in this study.

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

Determination of antimicrobial susceptibility among diarrhoeagenic bacteria

Bacterial isolates were examined for antibiotic resistance using the standard Kirby-Bauer disk diffusion method. Six antibiotics, namely ampicillin (10 µg), tetracycline (30 µg), cefuroxime (30 µg), nalidixic acid (30 µg), chloramphenicol (30 µg) and gentamicin (10 µg) (Oxoid Inc, UK) were used to test for susceptibility testing. Bacterial inocula were prepared by suspending the freshly grown bacteria in 5 ml sterile 0.85% normal saline and the turbidity adjusted to that of a 0.5 McFarland standard (10^8 cfu) and plated on Mueller-Hinton medium plates (HIMedia Lab. Pvt.Mumbai, India) by swabbing in replicates for each isolate tested with the inocula and the six commercially prepared antimicrobial agent disks placed on each of the inoculated plates.

The plates were then incubated at 37°C for 18 to 20 hours. The diameters (mm) of clear zones of growth inhibition around the antimicrobial agent disks, including the 6-mm disk diameter were measured with precision calipers (Clinical and Laboratory Standards Institute (CLSI), 2008). A standard reference strain of *E. coli* (ATCC 25922) and a plate which lacked the six antimicrobials was used as a control. The breakpoints used to categorize isolates as resistant to each antimicrobial agent were those recommended by CLSI (2008).

Data analysis

The data were entered into MS-Excel Windows XP professional 2003 and analyzed by Minitab version 14. Descriptive statistics were generated to assess the occurrence and distribution of diarrheagenic bacteria based on sources and diffusion zones of bacteria among the sources sampled. Discriminate function models were generated for the different species classification groups using Minitab 14.

Results

E. coli was recovered from all sources with varying prevalences; these included 91.8% in humans, 90 % in goats, 82% in chickens and 80% in donkeys (Table 2). The lowest prevalence rates were in fresh Nile perch (7.3%) and fresh *R. argentea* (3.5%), while the prevalence of *E. coli* were slightly higher in dried *R. argentea* (15.5%).

Table 2. The occurrence (%) of *E. coli* and *Salmonella* on samples from Lake Victoria.

Source	No	*E.coli*	*Salmonella*
Lake water	90	30.0	0
Soil	30	30.0	16.7
Nile perch (fresh)	55	7.3	23.6
R. argentea (fresh)	57	3.5	0
R. argentea (dried)	58	15.5	8.6
Chicken	50	82.0	2.0
Cattle	62	69.4	0
Donkey	20	80.0	5.0
Goat	50	90.0	4.0
Human	73	91.8	6.8

The prevalence of *Salmonella* spp. was lower, with the highest rate being reported in soil samples (16.7%), followed by dried *R. argentea* (8.6%) and none were recovered from fresh *R. argentea* and cattle. The soil and human samples presented diverse *Salmonella* serovars, namely *S. enterica* ssp. *arizonae* (4.1% in human and 3.3% in soil), *S. enterica* group E (3.3% in soil) and *S. enterica* nontypable forms (10% in soil and 1.4% in human), with *S. enterica* ssp. *pullorum* (1.4%) only recovered from human samples. *Salmonella enterica* group E (5.2%) was also recovered from sundried *R. argentea*, whereas 3.2% and 3.6% of the non-typable forms were recovered from sundried *R. argentea* and Nile perch, respectively. *Salmonella enterica* ssp.*arizonae* was recovered from goats (4%), chickens (2%) and donkeys (5%), but not from *R. argentea* and Nile perch (Table 3).

Salmonella spp. infections were widely distributed across the different age groups attending the hospital. However, an *S. pullorum* infection was reported from one patient (14.3%) in the age-group 21-25 years, whereas *Salmonella enterica* (non typable) infection was recorded among the 0-5-years age group (5.9%) (Table 4). No *Salmonella* infections were recorded in the 16 - 20 and 26 - 30 year age groups, whereas the highest *Salmonella* infection rate (14.3%) occurred in the 11 – 15 and 21 – 25 year age strata. *Salmonella enterica* ssp. *arizonae* infections were reported among different age groups with varied infection ratesvarying from 5.3% (6 - 10 years) (5.3%) to 14.3 % (11-15 years).

Table 3. *Salmonella* serovars recovered (% occurrence) in samples from Lake Victoria. Dashes indicate values of zero.

Source	No.	*S. arizonae*	*S. pullorum*	Group E	Non-typable	Total
Soil	30	3.3	-	3.3	10.0	16.7
Nile perch	55	-	-	-	3.6	3.6
R. argentea (fresh)	57	-	-	-	-	-
R. argentea (dried)	58	-	-	5.2	3.4	8.6
Chicken	50	4.0	-	-	-	4.0
Donkey	20	5.0	-	-	-	5.0
Goat	50	2.0	-	-	-	2.0
Human	73	4.1	1.4	-	1.4	6.9

Table 4. *Salmonella* serovars recovered from human hospital patients in Kisumu in relation to age. Dashes indicate values of zero.

Age (years)	Pathogen recovered	Infection rate (%)
0-5	*Salmonella* spp.	5.9
6-10	*S. arizonae*	5.3
11-15	*S. arizonae*	14.3
16-20	-	-
21-25	*S. arizonae*	14.3
26-30		
>30	*S. arizonae*	10.0

Distribution of ETEC infection among human with respect to age groups

In this study ETEC were recovered only from humans while ETEC, EPEC and EAEC were not recovered among cattle, donkeys, goats, chickens, fish or the environment. Among the clinical samples, ETEC was recovered in children with infection rates of 5.9% in children ≤ 5 years old and 5.3% in those aged from 6 to 10 years (Table 5) and the average infection rate in the two groups was 5.5%.

Table 5. Distribution of ETEC infection among humans in relation to age. Dashes indicate values of zero.

Age (years)	Pathogen recovered	Infection rate (%)
0-5	ETEC	5.9
6-10	ETEC	5.3
>10	-	-

According to the antimicrobial susceptibility profiles 52.2% of *E. coli* in these samples were resistant to at least one class of antibiotic and 37.3% to more than one, while 28.6% of *Salmonella* spp. was resistant to at least one antibiotic class and 9.5% to more than one antibiotic. Altogether almost half (49.7%) of the isolates recovered in this study were found to be resistant to at least one of the antibiotic

tested. Isolates from humans were resistant to the highest number of antibiotics tested. Isolates of *E. coli* recovered from soil and *R. argentea* were 100% and 90% resistant, respectively, to tetracycline. Isolates from Nile perch were100% resistant to ampicillin, followed by human isolates at 65.4%. *Salmonella* isolates from chickens, donkeys, *R. argentea* and soil were susceptible to all the six antibiotics tested but human isolates were highly resistant to ampicillin (80%) and tetracycline (60%) (Table 6).

Table 6. Rates of resistance (%) against individual antibiotics tested on different sources and types of bacteria. Tet = tetracycline; Amp = ampicillin; C = chloramphenicol; Cxm = cefuroxime; Gn = gentamicin; Na = nalidixic acid; * = % resistant to at least one antibiotic; ** = % resistant to more than one antibiotic.

Bacteria	Source	Tet	Amp	C	Na	Gn	Cxm
E. coli	Lake water	44.4	33.3	-	11.1	-	22.2
52.2%* 37.3%**	Soil	100.0	33.3	33.3	-	-	-
	Nile perch	10.0	100.0	-	-	-	-
	R. argentea	90.0	30.0	-	20.0	-	20.0
	Chicken	30.8	30.8	-	-	-	-
	Cattle	8.7	13.0	-	-	-	-
	Donkey	20.0	20.0	-	-	-	-
	Goat	18.8	12.5	-	-	-	-
	Human	75.0	65.4	36.5	36.5	9.6	15.4
Salmonella	Goat	-	100.0	-	-	-	-
28.6%* 9.5%**	Human	60.0	80.0	20.0	40.0	20.0	40.0

Presumed origins of E. coli *contaminating fish in Lake Victoria*

By subjecting the 134 *E. coli* isolates to Discriminate Analysis (DA), four possible sources of contamination of fish could be identified, namely, soil, water, chickens and humans and the average rate of correct classification (ARCC) for all isolates was 41% (Table 7). *E. coli* isolates recovered from soil were well classified (100%) followed by those from donkey (60%) and goat (56.3%) isolates. However, *E. coli* isolates recovered from chickens, water and fish were classified poorly at 15.4%, 22.2% and 23.0%, respectively (Table 7). When all isolates were reclassified into five host groups, namely livestock, fish, human, soil and water, the ARCC improved to 58.2% although the rates of misclassifying fish isolates remained the same at 38.5% as soil isolates, 15.4% as livestock isolates specifically from chicken, 15.4% as water isolates and 7.7% as human isolates.

Table 7. Discriminant analysis of disc diffusion zones of *E. coli* isolates based on individual sources (N= 134, ARCC = 41%).

Source (No)	Water	Soil	Fish	Chicken	Cattle	Donkey	Goat	Human
Lake water (9)	22.2	-	15.4	-	-	-	-	9.6
Soil (3)	33.3	100.0	38.5	7.7	8.7	-	12.5	11.5
Fish (13)	-	-	23.0	15.4	4.3	-	6.2	19.2
Chicken (13)	-	-	15.4	15.4	4.3	-	-	3.9
Cattle (23)	11.1	-	-	15.4	43.6	23.0	12.5	-
Donkey (5)	-	22.2	-	23.0	13.0	60.0	12.5	9.6
Goat (16)	-	-	-	15.4	26.1	-	56.3	1.9
Human (52)	11.1	-	7.7	7.7	-	20.0	-	44.3

Discussion

The high frequency of *E. coli* in chickens and the mammalian hosts indicates that these animals are important reservoirs for *E. coli* around Lake Victoria. Generally, *E. coli* is adapted to mammals (Gordon and Cowling, 2003) which could account for the high frequencies of occurrence observed in this study. The levels of *E. coli* recovered from fish products, soil and

water were lower than those from chickens and mammals, which was expected as to survive in secondary habitats it has to overcome low nutrient availability and wide temperature fluctuations, which prevent it from sustaining a dividing population outside the animal host (Winfield and Groisman, 2003).

In fish, *E. coli* levels seem to be higher among benthic species compared to pelagic ones (Hansen *et al.*, 2008) because *E. coli* are less abundant in water than in sediments where benthic species feed (Desmerais *et al.*, 2002; Ishii *et al.,* 2007). Nile perch and *R. argentea* are not benthic fishes, which may explain the low levels of *E. coli* recovered from freshly landed fish.

No ETEC, EAEC or EIEC pathotypes were recovered sources other than humans where ETEC pathotypes were recovered. This pathotype is a common cause of childhood diarrhoea in the developing world (Nessa *et al.*, 2007) and the results of this study is further evidence of this.

Around Lake Victoria, the main reservoirs of *Salmonella* were soil, fish, livestock and humans, which reflects the fact that they are resilient microorganisms. They have complex genomic systems that enable them to adapt to harsh environmental conditions, such as extreme temperatures or pH, and in the gastrointestinal tract (Andino and Hanning, 2015). This ubiquitous distribution of *Salmonella* has been attributed to a cyclic lifestyle consisting of passage through a host into the environment and back into a new host (Thomason, 1977). The ability to survive in non-host environments for long times gives *Salmonella* an advantage over *E. coli* in long term survival in secondary habitats, thus ensuring its passage to the next host (Winfield and Groisman, 2003). All the *Salmonella enterica* serogroups recovered were of the non-typhoidal Salmonellae (NTS) which agrees with recent studies in Africa where NTS are the most common cause of infections among humans, food animals, and wildlife (Gordon and Graham, 2008; Dione *et al*., 2011; Kagambèga *et al*., 2013).

The relatively high prevalence of *Salmonella* in sundried *R. argentea* suggests that poor post-harvest handling, especially in those fish dried on the ground, is most likely responsible for this contamination. There was a close relationship between *Salmonella* serogroups recovered from soil and those recovered from fish, which suggests that soil could be an important source of *Salmonella* contamination in fish, especially those dried on the ground.

Furthermore a misclassification of 38.5% for *E. coli* isolates recorved from fish as soil isolates based on DA points to soil as the possible source of contamination of fish with pathogens. Such misclassification arises from similarities in the distribution of disk diffusion zones, which may have been influenced by the environmental interactions such as exposure or non-exposure to antibiotics within the host groups (Wiggins *et al.,* 1999; Guan *et al.,* 2002).

In Africa, the extent of antibiotic resistance among enteric bacteria originating from human, domesticated animals or environment are varied but ranges from less than 10% to over 85% (Kariuki *et al*., 1999; Kagambèga *et al*., 2013; Odwar *et al*., 2014) and the levels recorded in this study are similar to these. The use of antibiotics has been identified as an important factor in the development of resistance among microbes (Witte, 1998).

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