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Using phenotypic based approaches to compare *Escherichia coli* isolates from human, livestock, fish and environmental sources within the Lake Victoria basin of Kenya

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The study compares *Escherichia coli* recovered from human, fish products, domesticated animals and the environment within the Lake Victoria basin on the basis of their antimicrobial susceptibility profiles. A total of 134 *E. coli* isolates were isolated from the collected samples. 52.2% of the *E. coli* isolates were found to be resistant to at least one antibiotic. Isolates originating from fish and soil showed the highest levels of resistance (100%). Based on the discriminant analysis (DA), most of the fish isolates were misclassified into soil category, probably due to the groups displaying similar Multiple Antibiotic Resistance (MAR) profiles. On the other hand, human isolates had the highest score of 0.55. The findings suggest that soil may be an important source of bacterial contamination of fish. Similarly resistance to antibiotics is widely prevalent among human, environment and domesticated animals within the Lake Victoria basin.

Key words: *Escherichia coli*; Environment; Discriminate; Antimicrobial resistance.

INTRODUCTION

Lake Victoria is the second largest lake in the world with an area of 68,800 km^3 and is shared between Kenya (6%), Uganda (43%) and Tanzania (51%), with a shoreline of about 3,440 km long and a catchment of 193,000 km^2 which has been estimated to have over 30 million people within the three countries (Okedi, 2005). Lake Victoria and its basin provide fresh water for domestic, industrial, agricultural and recreational use to the riparian communities. The communities in the Lake Victoria basin interact with the lake ecosystem on a daily basis in fishing as well as collecting water for domestic and commercial purposes. The state of the lake is directly linked to their livelihoods and has a major influence on water borne and related communicable diseases (Tanzarn et al., 2005).

The Lake Victoria basin has been shown to bear a great burden of diarrhoeal infections (Brooks et al., 2003; Brooks et al., 2006), and *Shigella*, *Vibrio cholerae*, *Salmonella*, diarrhoeagenic *Escherichia coli*, and *Campylobacter* are some of the pathogens that have been associated with this diarrhoeal infection. Brooks et al. (2003) has reported that drinking water from Lake Victoria (the major source of water for all use in the community under study) and also sharing of latrines between multiple households increased risk of diarrhoea. However, no attempts have been made to demonstrate

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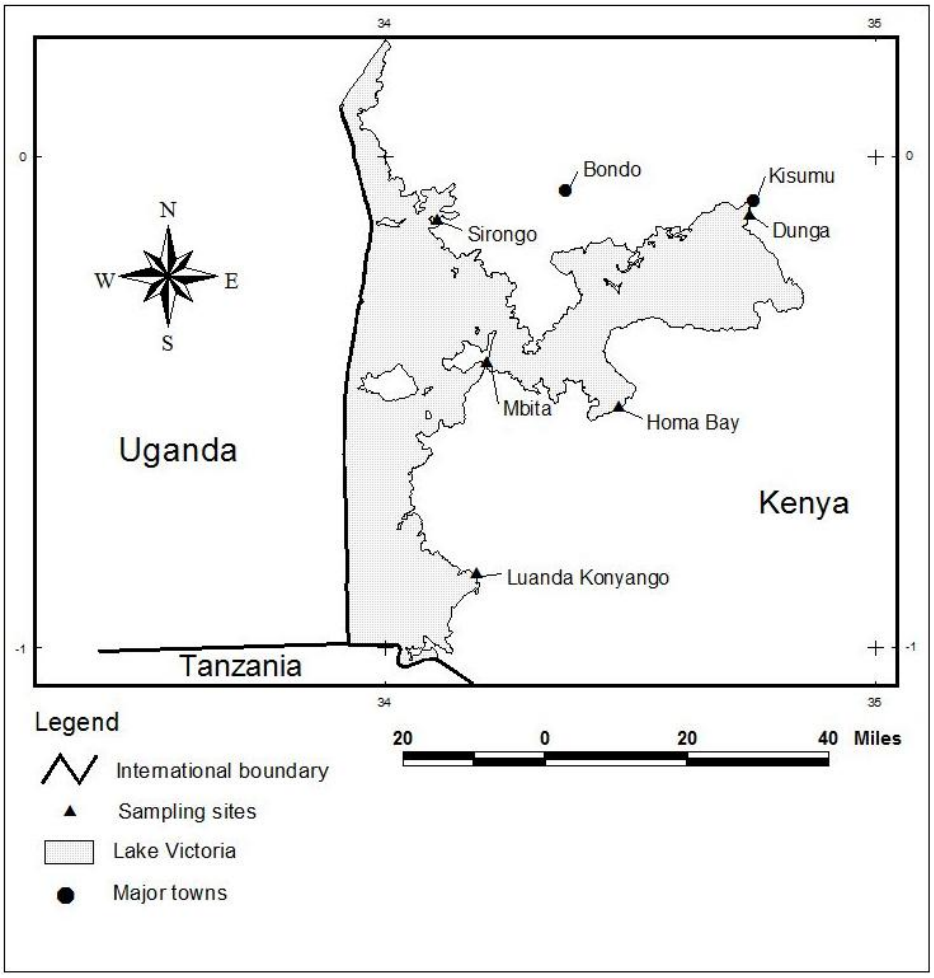

Figure 1. Map of Lake Victoria (Kenya) showing the study sites.

the distribution, occurrence, and possible reservoirs of *E. coli* species within the Lake Victoria basin thus negligible information exist about their antimicrobial resistance patterns.

Strain discrimination or strain typing has become an important aspect for epidemiological studies. Typing methods fall into two categories; phenotypic methods and genotypic methods. Phenotypic methods are cheap and easy to use and have been used successfully in strain typing (Scott et al., 2002). They include antibiotic resistance (AR) based analysis, and immunological methods. Multiple antibiotic resistance (MAR) analysis has been used to differentiate bacteria (*E. coli* or faecal *Streptococci*) from different sources using antibiotics commonly associated with human and animal therapy, as well as animal feed (Wiggins, 1996). The use of this method is based on the underlying principle that the bacterial flora present in the gut of various types of animals are subjected to different types, concentrations, and frequencies of antibiotics, and over time, selective pressure within a specific group of animal selects for flora that possess specific "fingerprints" of antibiotic resistance (Scott et al., 2002). This study therefore aimed at discriminating *Escherichia coli* recovered from human, fish products, domesticated animals and the environment within the Lake Victoria basin of Kenya. Discriminating such indicator bacteria from different sources may make it possible to compare and determine possible reservoirs and sources of contamination.

MATERIALS AND METHODS

Study site and design

This study was conducted within the Lake Victoria basin of Nyanza province in Western Kenya, targeting both the rural and urban communities. Generally, fishing, cattle rearing and subsistence farming are the principal occupation for the rural communities within the study areas (Figure 1). The samples were collected from five fish landing beaches along Lake Victoria (Sirongo, Dunga, Homa Bay, Mbita town, and Luanda Konyango) of Western Kenya. The study sites were chosen based on fish production and proximity to an urban town and human activities. Human stool specimens were

collected from Kisumu District Hospital also in western part of Kenya. The study was based on a repeated cross-sectional study design, and took a prospective approach. The samples were collected between January 2010 and June 2012.

Sample collection and processing

Freshly deposited faeces from domesticated animals (within a radius of 500 m from the fish landing site) were picked using a sterilized spoon and placed in a sterile container (Greiner Bio-one). Water samples were collected by submerging pre-sterilized pipette to depth of 30 cm below the surface and extracting a 100 ml sample which was dispensed in sterilized 250 ml Pyrex glass bottles; three water samples were collected from each sampling site (but at different points from the shores, namely at the shores- (0, 100 and 150 m). Fish samples of 500 g each were purchased from fishermen at landing sites for freshly landed samples, whereas sundried fish products were sourced from markets. Soil samples were collected aseptically using a sterilized spoon from six points and pooled together to form a representative sample for that site. Human stool specimens were transported on Cary Blair medium (HIMedia Lab. Pvt. Mumbai, India). All the samples were transported on ice in insulated containers to Maseno University Biomedical laboratory for analyses.

Upon arrival at the laboratory in Maseno, fish samples were processed according to FAO (1992). 25 g of fish was cut and homogenized aseptically in 225 ml buffered peptone water (HIMedia Lab. Pvt. Mumbai, India) followed by direct plating onto selective media MacConkey agar (HIMedia Lab. Pvt. Mumbai, India). Soil samples were processed as described by van Elsas and Smalla (1997) briefly, two spoonfuls of the pooled soil sample were transferred into a pre-sterilized, Whirl Pak bag. 100 ml of sterile phosphate-buffered water was then added and mixed for 2 min and the mixture filtered through a pre-sterilized 28 µm - pore - size nylon filter. The filtrate was then used to recover the *E. coli* by direct plating on MacConkey agar (HIMedia Lab. Pvt. Mumbai, India). Water was processed by taking 10 ml of sample and adding it to 90 ml of buffered peptone water followed by plating on MacConkey agar as described by Anazoo and Ibe (2005). Fecal samples were processed by direct plating on MacConkey agar as described by Kariuki et al. (2002).

All incubations were at 37°C for 18 h. Characteristic colonies were based on morphological characteristic and subjected to biochemical test triple sugar iron agar, lysine iron agar, citrate agar and indole all from HIMedia Lab. Pvt. Mumbai, India. The isolates were further confirmed to genera or species level using API 20 E (BioMerieux, France). All *E. coli* isolates were stored at - 20°C in tryptic soya broth plus 15% glycerol.

Susceptibility test with 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) was performed using the standard Kirby-Bauer disk diffusion method on Mueller Hinton (HIMedia Lab. Pvt. Mumbai, India). The plates were then incubated at 37°C for 18 to 20 h. The diameters (in millimetres) of clear zones of growth inhibition around the antimicrobial agent disks, including the 6 mm disk diameter was measured by using precision callipers (Clinical and Laboratory Standards Institute (CLSI), 2002) A standard reference strain of *E. coli* (ATCC 25922) was used as a control. The breakpoints used to categorize isolates as resistant to each antimicrobial agent were those recommended by CLSI (2002).

Data was entered in Ms Excel spread sheet Windows EP professional 2003 and analyzed by Minitab version 14. Data for the antimicrobial agent resistance of each bacterial isolate were reported as the diameter of the zone of inhibition (in millimeters). Descriptive statistics were generated to assess the distributions of the diffusion zones, and nonparametric tests (Kruskal-Wallis tests) were carried out to test for differences in diffusion zones between different groups. Discriminant function models were generated for the different species classification groups using Minitab 14. Only the linear discriminant analysis (DA) model could be performed with cross-validation. The cross-validation classification table was used to calculate the percentage of misclassified isolates and determine the average rate of correct classification.

RESULTS

Of the 134 isolates examined, 52.2% were found to be resistant to at least one antibiotic. Among the eight sources, all *E. coli* isolates originating from fish and soil sources showed (100%) resistance to at least one antimicrobial agent tested. However isolates originating from humans showed the highest level of resistance when comparing resistance to two or more antibiotics (78.8%). Apart from chicken, isolates from livestock recorded resistance levels below 22%. Among isolates recovered from cattle, no isolate recorded resistance to more than one antibiotic. Based on MAR indices calculated in this study, human isolates demonstrated the highest score of 0.55; others that scored above 0.2 were isolates from fish and soil sources. Cattle isolates recorded the lowest MAR indices of 0.04, followed by Donkey and goat isolates recording scores of 0.07 (Table 1).

As shown in Table 2, 18 distinctive antibiotic resistance patterns were observed altogether. The most frequent antimicrobial resistance observed among the isolate was that against tetracycline (21) followed by a combination of tetracycline - ampicillin (15); and ampicillin (9). The other frequent co- resistance was that against tetracycline - ampicilin - nalidixic acid (4) which was common among human isolates. Among the domesticated animals, no resistance to nalidixic acid and cefuroxime was observed. Resistance to nalidixic acid and cefuroxime was observed frequently among human isolates but also to some extent among fish and water isolates. Resistance to chloramphenicol and gentamicin was also only observed among isolates recovered from human and presented as co-resistance with other antibiotics.

Using the Kruskal Wallis test no significant differences were observed among chloramphenicol ($p = 0.075$) and gentamicin ($p = 0.11$). However, there were significant differences among the other four antibiotics tetracycline, ampicilin, nalidixic acid and cefuroxime ($p < 0.0001$). By using discriminant analysis (DA) with the 134 *E. coli* isolates, the average rate of correct classification (ARCC) for all isolates was 41% (Table 3). However when all isolates were reclassified into five host groups namely livestock, fish, human, soil and water, ARCC improved to 58.2%; and to 52.2% with cross validation (Table 4). When all isolates were reclassified into two host groups (human and non-human), ARCC rose to 78.4%; and to 76.1% with cross validation (Table 5). Soil isolates were well classified 100% followed by donkey and goat isolates at 60% and 56.3%, respectively, while chicken,

Table 1. Classification of *E. coli* sources based on percentage levels of resistance to the six antibiotics tested and Multiple Antibiotic Resistance indices.

Source (n)	% Level of resistance		
	At least one antibiotic	More than one antibiotic	MAR index*
Human (52)	88.5	78.8	0.55
Fish (13)	100	38.5	0.24
Soil (3)	100	33.3	0.22
Water (9)	67	22.2	0.17
Chicken (13)	38.5	23.1	0.1
Donkey (5)	20	20	0.07
Goat (16)	18.8	12.5	0.07
Cattle (23)	21.7	0	0.04

*MAR index was calculated using the formula *a*/ (*b* - *c*); where, *a* is the aggregate antibiotic resistance score of all isolates from the sample, *b* is the number of antibiotics, and *c* is the number of isolates from the sample. Source: Krumperman (1983).

Table 2. Antibiotic resistance patterns of *E. coli* isolates from investigated sources.

Pattern	No. of isolates	Source
Tet	21	Fish, soil, water, chicken, cattle, goat, human
Tet-Amp-Na	4	Human
Tet-Amp-Na-Gn-Cxm	3	Human
Amp-C	2	Human
Tet-Amp-Na-C-Cxm	2	Human
Tet-Amp-Na-Cxm	2	Human
Tet-Na	2	Human
Tet-Na-Cxm	2	Fish, water
Amp-Cxm	1	Water
Amp-Na	1	Human
Amp-Na-C	1	Human
Na-Cxm	1	Fish
Tet-Amp-C	1	Human
Tet-Amp-Na-C	1	Human
Tet-Amp-Na-Gn	1	Human

Tet, Tetracycline; Amp, ampcillin; C, chloramphenicol; Na, nalidixic acid; Gn, gentamicin; Cxm, cefuroxime.

Table 3. Discriminant analysis of disc diffusion zones of *E. coli* isolates from various sources.

Source (n)	(%) of database isolates assigned to each source category							
	Ch(13)	Ca(23)	D(5)	F(13)	G(16)	H(52)	S(3)	W(9)
Chicken	15.4	4.3	0	15.4	0	3.9	0	0
Fish	15.4	4.3	0	23	6.2	19.2	0	0
Goat	15.4	26.1	0	0	56.3	1.9	0	0
Human	7.7	0	20	7.7	0	44.3	0	11.1
Soil	7.7	8.7	0	38.5	12.5	11.5	100	33.3
Water	0	0	0	15.4	0	9.6	0	22.2

n = 134; ARCC = 41%; Ch = chicken, Ca = cattle, D = donkey, F = fish, G = goat, H = human, S = soil, W = water.

Table 4. Discriminant analysis of disc diffusion zones of *E. coli* isolates based on classification of livestock, fish, human, soil and water sources.

Source (n)	No. (%) of database isolates assigned to each source category				
	Livestock (57)	Fish (13)	Human (52)	Soil (3)	Water (9)
Livestock	78.9	15.4	11.5	0	22.2
Fish	7	23	19.2	0	0
Human	3.5	7.7	46.2	0	11.1
Soil	10.5	38.5	11.5	100	33.3
Water	0	15.4	11.5	0	33.3

n = 134; ARCC = 58.2%; classification with cross validation ARCC = 52.2%

Table 5. Discriminant analysis of disc diffusion zones of *E. coli* isolates based on classification of human and non human sources.

Source (n)	(%) of database isolates assigned to each source category	
	Human (52)	Non human (82)
Human	73.1	18.3
Nonhuman	26.9	81.7

n = 134; ARCC = 78.4%; classification with cross validation ARCC = 76.1%.

water and fish isolates were classified poorly at 15.4%. 22.2% and 23.0%, respectively.

DISCUSSION

The aim of this study was to differentiate *Escherichia coli* recovered from various sources including the environment within the Lake Victoria basin of Kenya. A total of 134 *E. coli* isolates were recovered from eight known host sources along the shores of Lake Victoria and phenotypic approaches employed to discrimimate *E. coli* sources. The study found that other than chloramphenicol and gentamicin, all the other antibiotics tested showed significant difference among the disk diffusion zone distributions among the sources ($p < 0.0001$). There could be many factors contributing to this observation, like indiscriminate use of antibiotics by patients visiting the Kisumu District hospital where specimen were collected from, natural occurrence of some antibiotics in the environment like soil (Rysz and Alvarez, 2004) and exchange of extrachromosomal material in the environment (Andersson and Hughes, 2011).

Based on the MAR indexing of *E. coli* and antibiogram patterns observed in this study, the results show that human isolates have higher rates of antimicrobial resistance which agrees with other studies conducted within the Lake Victoria basin (Brooks et al., 2001; Brook et al., 2003). However livestock showed low MAR indices as compared to other studies elsewhere (Krumperman, 1983; Sayah et al., 2005). In fact in this study, out of 23 *E. coli* isolates recovered from cattle, no multiple resistance was observed, whereas fish and soil isolates showed intermediate indices of 0.24 and 0.22, respectively. The low antimicrobial resistance profile observed among isolates recovered from cattle could be due to the fact that cattle farmers in the study site do not practice in intensive farming. Therefore the animals are not exposed to antibiotics as growth promoters. The intermediate MAR indices among fish and soil cannot be explained by this study and calls for more investigations.

However using the MAR indices, at least the *E. coli* sources could be classified into four groups, one group consisting of human isolates with the highest levels of resistance 0.55. This group consists of isolates with high levels of multiple antimicrobial resistance of over 40%. A second group that consisted of fish and soil isolates with MAR indices of 0.24 and 0.22, respectively, the group had multiple antimicrobial resistance of between 30 to 39%. The third group consisted of water, chicken, goat and donkey isolates with MAR indices of 0.17 and 0.1 for water and chicken sources and 0.07 for the latter two. These sources have multiple antimicrobial resistance ranging between 12.5 to 23.1%. The last group was that of cattle isolates with MAR indices of 0.04; this group did not record any multiple antimicrobial resistance. The low occurrence of MAR indices among livestock within the Lake Victoria basin compared to those reported in developed countries (Krumperman, 1983; Sayah et al., 2005) could be an indication of low use of antibiotics among livestock in this region. However the difference among the members of the livestock group sampled in this study could be attributed to the different environments where

the animals find food. Goats, chicken and donkey feed around the human settlements, whereas cattle graze away in the fields not close to human settlements.

According to Krumperman (1983), using MAR indexing, *E. coli* isolate can be categorized into two groups based on risks and public health concerns. Sources with MAR indices with 0.2 and above can be classified as high risk food sources whereas those below 0.2 as low risk food sources. In this study; fish products, within the Lake Victoria basin could be classified as high risk food source. The classification of fish and soil together using the MAR indices, serves to augment the finds by Abila and Jensen (1997), that poor fish handling practices such as drying fish on top of soil, and absence of acceptable sanitary condition along the fishing landing ports a long Lake Victoria may be responsible for high fish post harvest losses.

By using DA with the 134 *E. coli* isolates, the average rate of correct classification (ARCC) for all isolates was 41%. Among the eight sources, chicken, water and fish were classified poorly (15.4, 22.2 and 23.0%, respectively), most likely because of their disk diffusion zones distribution, which may have been influenced by the environmental interactions with the host groups. Most of the fish isolates were misclassified into soil category because these groups displayed similar MAR profiles and antimicrobial susceptibility inhibition zones, further supporting the earlier argument that poor fish handling and sanitary practices may be responsible of fish contamination.

When all isolates were reclassified into five groups by grouping all the domesticated animals as livestock, the ARCC improved to 52.2% with cross validation. However, fish and water were misclassified (38.1% and 33.5%). When classified into two groups namely human and non-human, the ARCC improved to 76.1%. The study demonstrates that the average rate of correct classification increased with reduction of number of groups to be discriminate analysis. Kaneene et al. (2007) also demonstrated that ARCC improved by reducing the numbers of species classifications and antimicrobial agents.

Conclusion

The results of this study confirm the occurrence of multiple antibiotic resistant *E. coli* strains from different sources within the Lake Victoria basin, which is a major public health threat worldwide. Using the two phenotypic approaches, the study was able to discriminate *E. coli* isolates from the different sources sampled in this study. The MAR indexing was able to classify the sources based on the levels of multiple resistance to antibiotics, whereas DA utilized disk diffusion zone expressed by *E. coli* isolates from the different sources. However it appears that including a large number of isolates within the database may be necessary to improve its average rate of correct classification of isolates by their sources. The findings from this study appear to point that soil may be an important source of bacterial contamination of fish products within the Lake Victoria basin and human an important reservoir of multiple antibiotic resistant *E. coli* strains.

The study therefore recommends the adoption of the two techniques to aid in risk assessment, establishment of relationships and interaction of indicator organisms like the *E. coli* among the different food sources within the Lake Victoria region. The two techniques are cheap and can easily be employed within region.

To control the continued emergence of antibiotic resistance within the Lake Victoria basin, studies with comprehensive collection of samples targeting more sources are urgently required to enhance our understanding of mechanisms involved in the dissemination of resistance to antimicrobial agents.

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