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Title: Qualitative Assessment of Livestock Faeces and Sewage for Antibiotic Resistant Strains of E. coli in Roma, Lesotho

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Research Article

Qualitative Assessment of Livestock Faeces and Sewage for Antibiotic Resistant Strains of *E. coli* in Roma, Lesotho

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ABSTRACT

Sewage water (SW) and fresh livestock faeces: Chicken (ChNF), Sheep (SF), and Cattle (CF) were taken randomly from the farm of the National University of Lesotho aseptically during the 2012 academic year. Twenty ml of sewage water or 20 grams of fresh animal faeces was dissolved in 80 ml of sterilized Ringer's solution and serially diluted. A 0.1 ml of sample suspension at 10^{-4} dilution was spread plated on Nutrient Agar (NA) and Eosine Methyl Blue (EMB) and plates were incubated for 24-48h at 24 and 37°C, respectively. A disc diffusion method was used to test the reaction of selected strains of *Enterobacteriaceae* to nine antibiotics. In total, 24 strains under *Enterobacteriaceae* were isolated from the respective sources and the number of each that were *Escherichia coli,* as confirmed by the biochemical tests were: SW (7 and 3); ChNF (5 and 3); SF (8 and 6) and CF (4 and 2). Thirteen *E. coli* strains fermented lactose and were Catalase positive, while only one *E. coli* strain from SW was non-lactose fermenter and Catalase negative. The lethal potencies, as number of isolates sensitive to the 9 antibiotics, ranked as: Colistin = Amikacin (24/24) > Kanamycin (23/24) > Chloramphenicol (22/24) > Cefotaxime (21/24) > Sulphafurazole (7/24) > Methicillin (1/24) > Penicillin G = Rifampicin (0/24). Sensitivity of the *E. coli* strains ranked as follows, Colistin = Amikacin = Kanamycin (14/14) > Chloramphenicol (13/14) > Cefotaxime (12/14) > Sulphafurazole (6/14) > Methicillin (1/14) > Penicillin G = Rifampicin (0/14). This result showed that the development of antibiotic resistant *E. coli* strains, which can pass through food chain to humans and other organisms if stringent control measures are not taken. Strict quarantine procedures have to be applied to control such potential health risks.

Keywords: *Escherichia Coli*; Antibiotics; Sewage water; Entrobacteriaceae; Resistance.

INTRODUCTION

The faecal *E. coli* as an exclusive inhabitant of the gastrointestinal tract of animals, its presence in a variety of clinical, animal husbandry, food and water source samples has great implications on public health issues (Edberg et al., 2000). Detection of *Escherichia coli* indicates the presence of faecal material from warm-blooded animals, and the possible presence of disease producing bacteria, such as *Salmonella, Shigella,* and *Vibrio* (WHO, 1997, Narins, 2003).

Strains of *E. coli* are host specific with various characteristics: Including human, animals such as cattle, sheep, pig and chicken are potential *E. coli* contamination sources to the environment. The entero-aggregative (EAgg) and entero-invasive (EI) *E. coli* are the most common causative agents of food and water-borne human diarrhea worldwide (Fairbrother and Nadeau, 2006). Verotoxigenic *E. coli* (VT) attack only pigs, cattle, dogs and cats. Other groups: enterotoxigenic (ET) in pigs, sheep, goats, cattle, dogs and horses; enteropathogenic (EP) in rabbits, dogs, cats and horses; and enterohaemorrhagic *E. coli* (EH) in cattle and goats also causes diarrhea in humans (Debnath et al*.,* 1990; Fairbrother and Nadeau, 2006). In birds, *E. coli* causes a disease known as Colibacillosis which can be manifested as airsacculitis, pericarditis and septicemia and causes death (Biswas et al*.,* 2006).

In developing countries, the incidence of enteric diseases due to ET is estimated to be about 650 million cases per year, resulting in 800,000 deaths, primarily in children below five years of age (Turner et al*.,* 2006).

Mutational transformation of *E coli* has produced multi drug resistant strains to antibiotics (Narins, 2003). The bases for resistant development in *E. coli* often could be a change in structure or production of enzymes (Narins, 2003). Infection by antibiotic-resistant strains of pathogenic bacteria can have severe health implications for the sickened individual with less treatment options and development of more virulent strains (Barza and Travers, 2002).

E. coli strains from different sources would differ in their biochemical characteristics (Narins, 2003). Documentation of *E. coli* variation, occurrence of antibiotics resistance strains of *E. coli* has academic, health and environmental importance. In Lesotho such documentation is lacking. The present study was therefore conducted to screen *E. coli* strains from sewage water, faeces of chicken, sheep and cattle and to assess their biochemical and cultural characteristics and antibiotics sensitivity to a range of antimicrobial chemicals which are still in use for public health medical care and animal treatment.

MATERIALS AND METHODS

Sampling site and sample collection

Samples were collected from the National University of Lesotho (NUL), Roma campus sewage water treatment pond system and animal farm. Sewage water samples were collected randomly using Scot bottles of various sites of the pond system at shallow depth (0-10cm) randomly. Fresh faeces of chicken, sheep, and cattle were collected from NUL farm using protective gloves, sterile hand fork and plastic bags. Samples were transferred immediately to the Microbiology Laboratory, Department of Biological Sciences and were processed immediately for microbiological analysis or kept in the fridge at 4°C for a while until processed.

Microbiological analysis: Screening and isolation of *E. coli* strains

A serial dilution technique was applied to all samples collected from sewage water and animal faeces. Twenty grams of fresh animal faeces (sheep, chicken and cattle) were dissolved in 80ml of Ringer's solution to make a stock suspension. From each sample, one ml of a suspension was transferred into sterile test tubes containing 9 ml of saline solution. Eosine Methylene (EMB) and Nutrient Agar (NA) plates were spread plated with 0.1ml of stock suspension of each sample and culture plates were incubated in an inverted position at 37°C and 24°C, respectively for 24-48 hours. Colonies with various cultural characteristics were picked and used for further analysis. The pigmentation, size, shape, margins, elevations, configurations, opacity, and consistency of bacterial colonies as they grow on agar medium were noted.

BIOCHEMICAL CHARACTERIZATION OF *E. COLI* STRAINS

Carbon utilization test

An IMViC reaction tests and additional biochemical tests namely: Catalase, hydrogen sulphide, lactose fermentation and motility (on SIM motility media) were used for identification of members of the family.

Antibiotic sensitivity test

The disk diffusion assay method was used for antibiotic sensitivity test on NA plates. Nine different types of antibiotics impregnated discs with either [Colistin ($25mg^{-l}$), Kanamycin ($30mg^{-l}$), Kanamycin ($30mg^{-l}$), Methicillin ($10mg^{-l}$), Penicillin G ($1.5mg^{-l}$), Cefotaxime ($5mg^{-l}$), Sulphafurazole ($30mg^{-l}$), Rifampicin ($2mg^{-l}$), Chloramphenicol ($30mg^{-l}$)] were used against *E. coli* strains isolated from all samples. Standard concentrations (10^8 cells ml^{-l}) of individual selected *E. coli* strains were spread plated on the NA plates prior to the application of antibiotic discs. Four different antibiotics discs were used per plate and the experiment was done in triplicate. Culture plates inoculated with each strain of *E. coli* without antibiotics discs served as control. Plates were then incubated at 37°C for 24 - 48 hours. The formation of clear zone around the discs was indication of susceptibility reaction of *E. coli* strains to antibiotics. The clear zone formation was measured in millimeters (mm) using a ruler. Clinical and Laboratory Standards Immediate (CLSI) guidelines were used to interpret the zone diameter (CLSI, 2005).

STATISTICAL ANALYSIS

In this semi-quantitative and qualitative analysis, the antibiotic susceptibility pattern of *E. coli* strains was analyzed by percentage representation and the analysis of variance (ANOVA) for significant difference between treatments at P=0.05 was computed using SPSS, version 10.0, 1999.

RESULTS

Biochemical Test

Bacterial isolates that were Indole (+), Voges Proskauer (-), Methyl red (+) and Simmons citrate (-) were identified as *E. coli* strains (Table 1).

Table 1. Biochemical tests

Source of Isolate	Isolate number	Biochemical Test								Putative Organism
		Sulphide production	Simmons citrate	Methyl red	Voges Proskauer	Catalase	Lactose	Indole	Motility	
SW	SW_1	-	+	+	-	+	+	+	+	UI
	SW_2	+	-	+	-	+	+	+	+	*E. Coli*
	SW_3	-	+	-	-	+	+	-	+	UI
	SW_4	-	+	-	+	+	+	+	-	UI
	SW_5	-	-	+	-	+	+	+	+	*E. Coli*
	SW_6	-	-	-	+	+	-	-	+	UI
	SW_7	-	-	+	-	+	+	+	+	*E. Coli*
ChnF	$ChnF_1$	-	-	+	-	+	+	+	+	*E. Coli*
	$ChnF_2$	-	-	+	+	+	+	+	+	UI
	$ChnF_3$	-	-	+	-	+	+	+	+	*E. Coli*
	$ChnF_4$	+	-	-	+	+	+	+	+	UI
	$ChnF_5$	-	-	+	-	+	+	+	+	*E. Coli*
SF	SF_1	-	-	+	-	+	+	+	+	*E. Coli*
	SF_2	-	-	+	-	+	+	+	+	*E. Coli*
	SF_3	-	-	+	-	+	+	-	+	Sh. spp
	SF_4	-	-	+	-	+	+	+	-	*E. Coli*
	SF_5	-	-	+	-	+	+	+	+	*E. Coli*
	SF_6	-	-	+	-	+	+	+	+	*E. Coli*
	SF_7	-	-	-	-	+	+	-	+	UI
	SF_8	-	-	+	-	+	+	+	+	*E. Coli*
CF	CF_1	-	+	+	-	+	+	+	-	UI
	CF_2	-	-	+	-	+	+	+	-	*E. Coli*
	CF_3	-	-	+	-	+	+	+	-	*E. Coli*
	CF_4	-	+	+	-	+	+	+	-	UI

Legend: SW = Sewage water; ChnF = Chicken faeces; SF= Sheep faeces; CF = Cattle faeces; (+) = Growth/Reactivity; (-) = No growth/ No reactivity; UI = Unidentified; Sh. spp = *Shigella sp.*

Antibiotic susceptibility test

E. coli strains isolated from Sewage water (SW) were found to be highly resistant to most antibiotics used against them, followed by isolates from Chicken (ChnF) and Sheep (SF) faeces respectively (Figure 1 and Table 2).

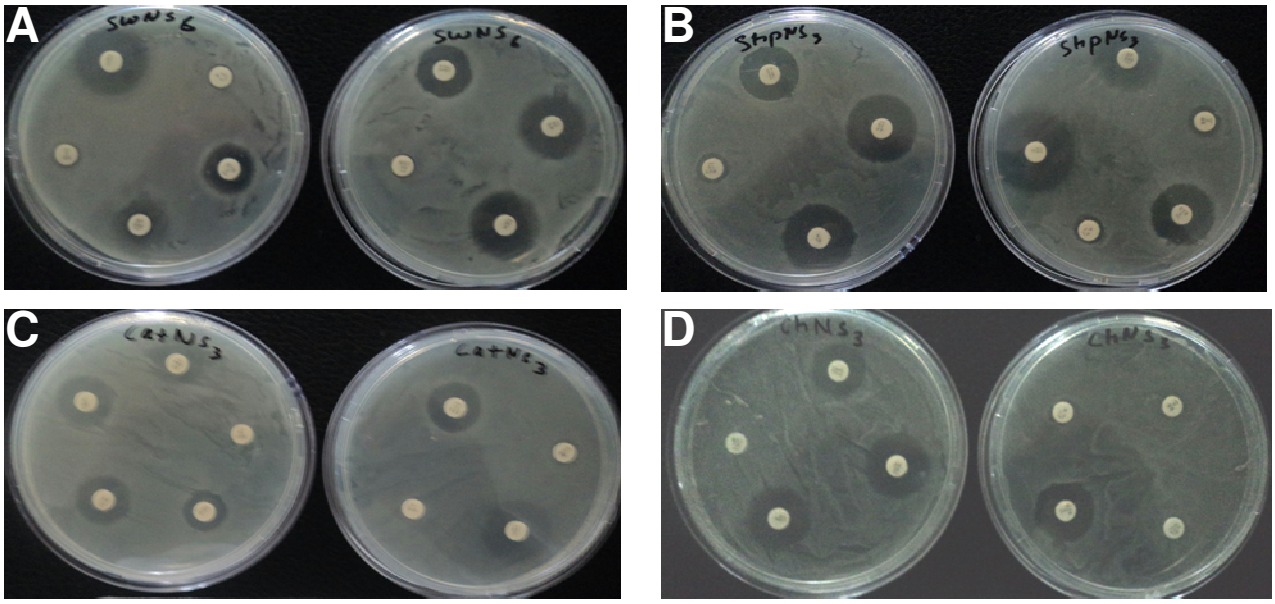

Figure 1. The effect of different antibiotics on selected E. coli strains from various sample sources:
A= Sewage water, B= Sheep faeces, C= Cattle faeces and D= Chicken faeces.

Antibiotic Resistance Pattern

Sensitivity of *E. Coli* strains rank Colistin = Amikacin = Kanamycin (14/14) > Chloramphenicol (13/14) > Cefotaxime (12/14) > Sulphafurazole (6/14) > Methicillin (1/14) > Penicillin G = Rifampicin (0/14) (Table 3).

Table 2. Frequency of resistance of *Enterobacteriaceae* isolates from four sources to antimicrobial agents by source of sample

Sample Source	Entero Isolated	N° of *E. coli* strains	N° (%) of Isolates resistant[1]	N° (%) Isolates Resistant to:								
				CT_{25}	AK_{30}	K_{30}	MET_{10}	$P_{1.5}$	CTX_5	SF_{300}	RD_2	C_{30}
SW[b]	7	3	7 (100)[a]	0 (0.0)[g]	0 (0.0)[g]	1 (14.3)[e]	7 (100)[a]	7 (100)[a]	1 (14.3)[e]	4 (57.1)[d]	7 (100)[a]	0 (0.0)[g]
ChnF[a]	5	3	5 (100)[a]	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	5 (100)[a]	5 (100)[a]	2 (40.0)[d]	5 (100)[a]	5 (100)[a]	2 (40.0)[d]
SF[c]	8	6	8 (100)[a]	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	7 (87.5)[b]	8 (100)[a]	0 (0.0)[g]	6 (75.0)[b]	8 (100)[a]	0 (0.0)[g]
CF[b]	4	2	4 (100)[a]	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	4 (100)[a]	4 (100)[a]	0 (0.0)[g]	2 (50.0)[d]	4 (100)[a]	0 (0.0)[g]
Total	24	14	24 (100)[a]	0 (0.0)[g]	0 (0.0)[g]	1 (5.0)[f]	23 (95.8)[a]	24 (100)[a]	3 (12.5)[e]	17 (70.83)[c]	24 (100)[a]	2 (8.33)[f]

Legend: Percentage resistance of *Enterobacteriaceae* strains expressed under each column is significantly different (*P*= .05) when compared against the test antimicrobial agents used. 1Resistant to one or more antimicrobial agent; SW (Sewage water); ChnF (Chicken faeces); SF(Sheep faeces) and CF(Cattle faeces); CT25 (Colistin); AK30 (Amikacin); K30 (Kanamycin); MET10 (Methicillin); P1.5 (Penicillin G); CTX5 (Cefotaxime); SF300 (Sulphafurazole); RD2 (Rifampicin); C30 (Chloramphenicol). Subscript figure is concentration (µg) of antibiotic per disc and units per disc for Penicillin G. Entro = (*Entrobacteriaceae*).

Table 3. Frequency of *E. coli* resistance *by* sample source to antimicrobial agents

Sample Source	**N° of *E. coli* strains**	**N° (%) of Isolates resistant[1]**		**N° (%) Isolates Resistant to:**								
				CT_{25}	AK_{30}	K_{30}	MET_{10}	$P_{1.5}$	CTX_5	SF_{300}	RD_2	C_{30}
SW[b]	3	3	(100)	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	3 (100)[a]	3 (100)[a]	1 (33.3)[e]	1 (33.3)[e]	3 (100)[a]	0 (0.0)[g]
ChnF[a]	3	3	(100)	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	3 (100)[a]	3 (100)[a]	1 (33.3)[e]	3 (100)[a]	3 (100)[a]	1 (33.3)[e]
SF[c]	6	6	(100)	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	5 (83.3)[b]	6 (100)[a]	0 (0.0)[g]	4 (66.7)[c]	6 (100)[a]	0 (0.0)[g]
CF[b]	2	2	(100)	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	2 (100)[a]	2 (100)[a]	0 (0.0)[g]	0 (0.0)[g]	2 (100)[a]	0 (0.0)[g]
Total	14	14	(100)	0 (0.0)[g]	0 (0.0)[g]	0 (0.0)[g]	13 (92.9)[b]	14 (100)[a]	2 (14.3)[e]	8 (57.1)[d]	14 (100)[a]	1 (7.1)[f]

Legend: Percentage resistance of *E. coli* strains expressed under each column is significantly different (*P*= .05) when compared against the test antimicrobial agents used.

1Resistant to one or more antimicrobial agent; SW (Sewage water), ChnF (Chicken faeces), SF(Sheep faeces) and CF(Cattle faeces); CT25 (Colistin); AK30 (Amikacin); K30 (Kanamycin); MET10 (Methicillin); P1.5 (Penicillin G); CTX5 (Cefotaxime); SF300 (Sulphafurazole); RD2 (Rifampicin); C30 (Chloramphenicol). Subscript figure is concentration (µg) of antibiotic per disc and units per disc for Penicillin G. Entro = *(Enterobacteriaceae*).

DISCUSSION

Escherichia coli is an exclusive inhabitant of the gastrointestinal tract of animals. In this study, in total 24 strains of Entrobacteriaceae isolates, of which 14 of them are *E. coli* strains; were isolated from sheep, cattle, chicken faeces and sewage water samples. We observed antimicrobial resistance in all strains of Entrobacteriaceae when tested against several classes of antibiotics including kanamycin, methicillin, penicillin G, cefotaxime, sulphafurazole, rifampicin and chloramphenicol (Table 2). These observations are in agreement with Saurina *et al*. (2000) who studied on epidemiology and antibiotics usage patterns in human medicine in USA. Indiscriminate use of antibiotics and their contamination to the environment may have increased the selective pressure towards the development of multi drug resistant strains in the family to antibiotics (Narins, 2003).

As an indicator organism for safety and pattern of resistance study, *E. coli* strains are frequently used to set health risk standards. In this study, the *E. coli* strains distribution and diversity in sample sources has also been investigated (Table 4). The high diversity of *E. coli* in the sewage water is an indication of the genetic mix and development of strains which could be resistant to many physical and or chemical factors. *E. coli* strains isolated from chicken (ChnF) faeces were found to be highly resistant to most antibiotics used against them, followed by isolates from sewage and cattle faeces (Table 3). The high incidence of resistant *E. coli* strains in the sample sources is an indication for a huge safety risk imposed to the public health that may be entered in the food chain. Similar reports on the development of multiple drug resistance by *E. coli* strains (O157:H7) against six or seven antimicrobial agents has also been reported (Giammanco et al., 2002; Golding and Matthews, 2004). Many other reports against a variety of antimicrobial classes in VTEC indicated the development of resistance by *E. coli* strains has become public health concern (White et al., 2002; Schroeder et al., 2002; Magwira et al., 2005; Walsh et al., 2006).

In this study, the sensitivity of *E. coli* strains against different antibiotics tested is ranked in the following order as Colistin = Amikacin = Kanamycin (14/14) > Chloramphenicol (13/14) > Cefotaxime (12/14) > Sulphafurazole (6/14) > Methicillin (1/14) > Penicillin G = Rifampicin (0/14) (Table 3). This shows how severe the situation is; due to resistance development except to a few antibiotics (Colistin, Amikacin and Kanamycin), and a complete resistance to Penicillin G and Rifampicin. The results of this study corroborate to the reports made by Akond *et al* against Rifampicin, Penicillin G, Cefotaxime and Methicillin (Akond et al. 2009). Penicillin G is among the few antibiotics currently in use that could show inhibitory but not bactericidal activity (Mark et al., 1998). Resistance to these antibiotics may have been acquired via horizontal transmission of genes from sewage water contamination or due to indiscriminate use of these antibiotics in animal husbandry (Van Donkersgoed et al., 2003).

In this study, three *E. coli* strains, one from sheep ($ShpNs_4$) and two from cattle ($CatNs_2$ and $CatNs_3$) were identified as non-motile strains. The non-motile characteristic of these strains is an indication for the occurrence of gene mutation amongst *E. coli* and other members of the family Entrobacteriaceae. Despite the absence of the filamentous projections as virulent factor, the non-motile *E. coli* strains may not have lost their pathogenic character (Richards, 2011). According to the study carried out by O'Sullivan et al. (2007), a sorbitol fermenting *E. coli* O157 which is non-motile have emerged as cause of hemolytic uremic syndrome (HUS) in Europe and Australia.

From this study, it is concluded that the potential source of resistance development could be the indiscriminate use of antibiotics in animal husbandry for treatment of cattle, sheep and poultry diseases in the farm or the contamination of animals and water sources from the abandoned open sewage water treatment pond system which is closer to the farm at the NUL campus. Further detailed studies on the clinical, epidemiological and environmental impacts assessment on the existing tradition of animal medication, sewage disposal and farm management practices has to be studied, aside, to control the aggressive development of resistant *E. coli* strains and other potential pathogens. Further molecular identification of the isolated strains is recommended for new development and understanding of unidentified strains.

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