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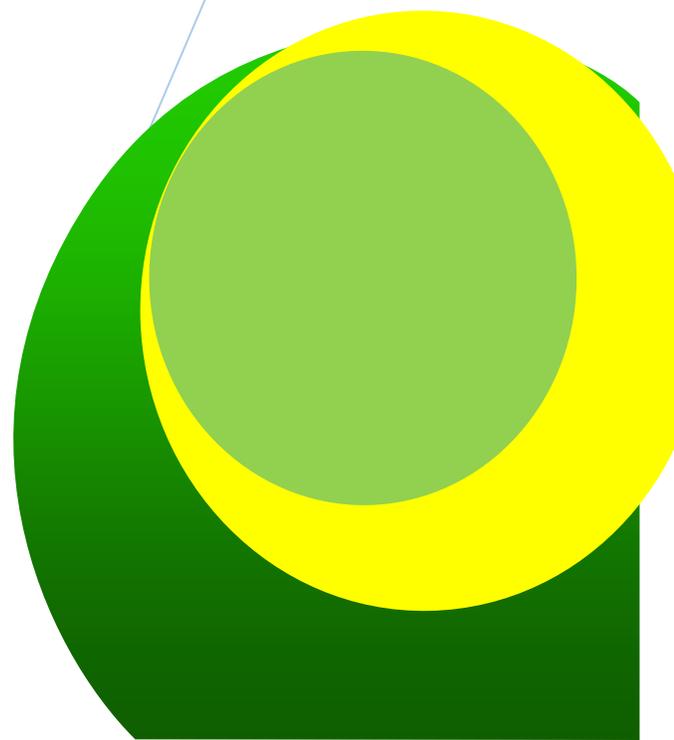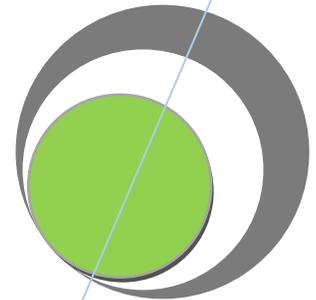
Research Article

Isolation of High Antibiotic Resistant Fecal Bacteria Indicators, *Salmonella* and *Vibrio* Species from Raw Abattoirs Sewage in Peri-Urban Locations of Nairobi, Kenya

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ABSTRACT

The research was conducted to determine indicator organisms' relationship to specific human pathogens and any presence of antibiotic resistance. Isolationof indicator organisms,*Salmonella* and *Vibrio* species was carried out using standard laboratory methods. Sensitivity to antibiotics was determined by the agar diffusion technique. The fecal bacteria load was found to be 6.2×10^6, 5.3×10^5, 2.5×10^4, 2.9×10^4, and 5.0×10^6 CFU/100 mL for fecal streptococci and 3.4×10^5, 4.1×10^3, 3.0×10^4, $2.7 \times 10^3$and 3.9×10^5MPN/100 mL for fecal coliforms in cattle wastewater, cattle sludge, goat wastewater, sheep wastewater and a mixture of goat and sheep sludge, respectively.Fecal coliforms showed the highest resistance with a mean resistance frequency of 60.8% (±25.2), followed by *Salmonella* species at 51.5% (±26.6). *Vibrio* species showed the lowest mean resistance frequency at 41.6% (±24.8). There was however no significant difference (p=0.859) in resistance among *Vibrio, Salmonella*, FS and FC isolates at *p*>0.05.There is a likelihood of slaughterhouse animals and bacteria in the intestines of these animals getting exposed to antibiotics to which the bacteria develop resistance which they can pass to human pathogens and environmental flora.

Keywords: Antibiotic resistance, Sewage, Pathogens and Abattoirs.

INTRODUCTION

Enteric bacteria from human and animal feces can be found in surface waters; the fecal bacteria are brought into aquatic environments mainly through treated or untreated wastewater release, surface runoffs and soil leaching (James *et al.,* 2003). The presence of pathogenic enteric micro-organisms in aquatic environments can be a source of disease when water is used for drinking, recreational activities or irrigation. The sanitary risk is increased if the pathogenic enteric bacteria present in waters are antibiotic resistant because human infections caused by such bacteria could be difficult to treat with drugs (Wenzel and Edmond, 2009). In addition, fecal bacteria might be able to transmit antimicrobial resistance to autochthonous bacteria through lateral transfer, when the resistance genes are carried by transferable and mobile genetic elements such as plasmids and thus contributing to the spread of antimicrobial resistance (Sayah*et al.,* 2005).

In the livestock sector, different types of farm animals are capable of carrying a wide range of zoonotic pathogens (Swai and Schoonman, 2012). Moreover, animals brought for slaughter into urban areas more often come from villages where pathogen control regimens are weak, un-coordinated and often not available. Lack of veterinary services in these livestock rearing areas poses a substantial risk of widespread occurrence of diseases in the livestock population and concurrent human exposure to these zoonotic disease agents (Swai and Schoonman, 2012). Livestock often act as non-symptomatic carriers of human pathogens such as *E. coli* 0157, *Salmonella* species and *Campylobacter* which are rarely detected during routine ante-mortem examination and their wastes may contain high concentrations of the organisms. Animal waste can therefore contaminate human and animal drinking water sources and even soil when used as manure (Christina *et al.,* 2012).

Fecal pollution of aquatic resources may lead to diseases in humans because of pathogens associated with this pollution or may affect human activities (Wery*et al.*, 2010). Fecal indicator organisms are typically used to demonstrate the potential presence or absence of groups of pathogens associated with wastewater or sewage sludge (Kator and Rhodes, 2003). Fecal coliforms, total coliforms, *E. coli*and enterococci are the bacterial indicators currently used in the assessment of water quality and health risks. These bacterial isolates often occur in the feces and intestines of warm blooded mammals including livestock. These indicator organisms are not pathogenic themselves. Organisms like fecal streptococci (FS), fecal coliforms (FC) and *E.coli* areused as indicators of fecal contamination of waters since they are easier and relatively inexpensive to enumerate and detect than the pathogens themselves(Cynthia *et al.*, 2004).

The increased re-use of wastewater raises concerns about the occurrence and survival of pathogens in the environment. For instance, the survival and recovery of *Salmonella* species from surface water, wastewater and bottled water have been investigated (Eaton *et al.*, 2005). *Vibrio* species have also been reported in drinking water, surface water and sewage. *Salmonella* species is a dangerous water borne bacterial pathogen in terms of human health and diseases (Moniruzzaman*et al.,* 2000). Various serotypes of *Salmonella* species have reportedly been responsible for water and food borne epidemics in various countries (Rosmini*et al.*, 2004)emphasizing the importance of the pathogen as a food safety concern. Water is also an important source for human infections with antimicrobial resistant *Vibrio* species (Byarugaba, 2004)

Several previous studies have concentrated on clinical isolates from humans, animals and some environmental samples. Studies on wastewater and sludge are limited. The contribution of abattoirs and associated wastewaters is rarely considered and yet abattoirs are potential sources of enteric bacteria that could possess antibiotic resistance genes. In this study, indicator organisms and pathogens such as *Vibrio* and *Salmonella* species were isolated for their conventional relationships but most importantly antibiotic resistance and environmental impact. The primary objective of this study was to determine the abundance of fecal indicators and detect *Salmonella* and *Vibrio* species in the abattoir wastewaters and sludge. The study also aimed to determine the antimicrobial resistance of these bacterial isolates.

MATERIALS AND METHODS

Sample collection and preparation

A total of thirty 100 mL samples of sludge and raw animal wastewater were collected from the three slaughterhouses in Nairobi County. Eighteen samples of wastewaters (6 samples of goat, sheep and cattle wastewaters each) and twelve samples of sludge (6 samples of cattle sludge and a mixture of goat and sheep sludge each) were obtained from all the three slaughterhouses. Samples were collected three times between 9 and 10 o'clock in the morning in the month of March 2012 and April 2012 in sterile 200 mL glass bottles and were transported to Kenyatta University laboratory in an ice cooler box for analysis. Wastewater samples that were not analyzed within four hours were stored at a temperature of 4 °C. All samples were analyzed within 24 h.

Isolation and identification of bacterial isolates

Standard microbiological methods (Mariita and Okemo, 2009) were used to isolate and enumerate FC (fecal coliforms) and FS (fecal streptococci) and to detect *Vibrio* and *Salmonella* species in the samples of wastewaters and sludge. Pigmentation of the colonies and Gram's staining followed by standard biochemical characterization [such as mortality, urease, H_2S production, glucose fermentation, indole, citrate utilization and the cytochrome oxidase tests] were used to confirm the bacterial isolates. The counts of FS were expressed in the CFU (colony forming units) and that of FC (fecal coliforms) were expressed in MPN (most probable number) and quoted as means±SD (standard deviation).

Antibiotic sensitivity test

Sensitivity to antibiotics was determined by the agar diffusion technique recommended by the NCCLS (National Committee for Clinical Laboratory Standards) (NCCLS, 2003) on Mueller-Hinton agar (Oxoid) using the following antibiotic impregnated disks: ampicillin (25 µg); cotrimoxazole (25 µg); streptomycin (10 µg); chloramphenicol (30 µg); kanamycin (30 µg); gentamicin (10 µg); penicillin G (1 unit); methicillin (5 µg); minocycline (30 µg); lincomycin (2 µg); erythromycin (15 µg); tetracycline (25 µg) and sulfamethoxazole (200 µg).

Data analysis

SPSS computer software version 16.0 was used for data entry and statistical analysis. Groups significance tests were performed using student T-test and one way ANOVA (analysis of variance) at 5% significance level and *P* value of <0.05 was considered as significant. The means were separated using Tukey's test at 5% level.

RESULTS

Prevalence of fecal bacteria indicators

Goat and sheep sludge had the highest fecal coliform (FC) contamination with a mean density of 3.9×10^5 ($\pm 3.5 \times 10^5$) MPN/100 mL followed by cattle wastewater with a mean density of 3.4×10^5 ($\pm 3.0 \times 10^5$) MPN/100 mL (Table 1). The difference in mean density of fecal coliforms from goat and sheep sludge and mean densities of fecal coliforms from cattle sludge, sheep and goat wastewaters was highly significant (p=.004) at *P*<0.01. The overall mean density of fecal streptococci (FS) was higher (2.4×10^{6a} ($\pm 3.0 \times 10^6$) CFU/100 mL) compared to that of the fecal coliforms (1.5×10^{5a} ($\pm 5.5 \times 10^6$) MPN/100 mL), however Student T-test showed no significant difference. Cattle wastewater showed the highest fecal streptococci (FS) density with a mean density of 6.2×10^6 ($\pm 5.5 \times 10^6$) CFU/100 mL followed by goat and sheep sludge with a mean density of 5.0×10^6 ($\pm 4.5 \times 10^6$) CFU/100 mL. The difference in mean density of fecal streptococci from cattle wastewaters and mean densities of fecal streptococci from cattle sludge, sheep and goat wastewaters was highly significant (*p*=.003) at P<0.01. Pearson correlation analysis indicated that prevalence of FS from all sites showed a significant positive correlation with that of FC (r = 0.931 at 0.01 level).

Table 1: Mean fecal bacteria densities (mean± standard deviation) in 100 mL of the wastewaters and sludge from Kayole and Kiamako abattoirs in Nairobi, Kenya

Sample (n=30)	Fecal streptococci	Fecal coliforms
Cattle wastewater	6.3×10^{6b} ($\pm 5.5 \times 10^6$)	3.4×10^{5ab} ($\pm 5.5 \times 10^6$)
Cattle sludge	5.3×10^{5a} ($\pm 5.5 \times 10^6$)	4.1×10^{3a} ($\pm 5.5 \times 10^6$)
Goat wastewater	2.5×10^{4a} ($\pm 5.5 \times 10^6$)	3.0×10^{4a} ($\pm 5.5 \times 10^6$)
Sheep wastewater	2.9×10^{4a} ($\pm 5.5 \times 10^6$)	2.7×10^{3a} ($\pm 5.5 \times 10^6$)
Goat and sheep sludge	5.0×10^{6ab} ($\pm 5.5 \times 10^6$)	3.9×10^{5b} ($\pm 5.5 \times 10^6$)
*Mean	2.4×10^{6a} ($\pm 3.0 \times 10^6$)	1.5×10^{5a} ($\pm 5.5 \times 10^6$)

Key: n-number of samples, fecal coliform density expressed in MPN/100 mL (most probable number) and fecal streptococci density expressed in CFU/100 mL (colony forming unit). Same letters indicate no significant difference according to Turkey's Honest Significance Difference (HSD) at 5% level.

Detection of pathogens

In Table 2, the key tests for *Vibrio* species which include indole test, a red slant and yellow butt in TSI (triose sugar iron) and citrate utilization in addition to a Gram stained Gram negative rods were observed. For the *Salmonellas*, there was mortality in SIM (sulphideindolemortility) indicating the presence of flagella in the isolated organism. The tests also resulted in the classical presentation of a yellow butt, red slant and blackening in the tubes which is indicative of hydrogen sulphide production. It is also worth noting that these pathogenic bacteria were more frequently detected in samples that showed high incidence of fecal bacteria indicators contamination, for instance cattle wastewater and goat and sheep sludge.

Table 2: Biochemical detection of *Salmonella* and *Vibrio* species.

Probable isolate	SIM			Urea	TSI						SCAN	Peptone	Cytochrome oxidase
M I B^1	urease	S	B^2	B^3	C^1	C^2	G			B^4			
Vibrio species	+ + -		-		R Y	-	-		+		+		+
Salmonella species	+ - v		-		R Y	+	+		v		+		+

Key: v-variable (+/-), M-mortility, I-indole, B^1-black, S-slope, B^2-butt, B^3-black, C^1-crack, C^2-citrate utilization, G-gas, B^4-blue, SCAN- simmons citrate agar, R-red, Y-yellow.

## Antibiotic resistance patterns

All bacterial isolates of fecal coliform origin (100%) were resistant to lincomycin and ampicillin, whereas only 30% were resistant to cotrimoxazole and minocycline (Table 3). Most fecal coliform isolates (50% and above) were however sensitive to chloramphenicol, cotrimoxazole, erythromycin, gentamicin, kanamycin, streptomycin and minocycline. *Salmonella* species on the other hand showed high resistance frequency to lincomycin (90%) and methicillin (80%). Only 10% of the *Salmonella* strains were resistant to chloramphenicol. The highest sensitivity frequency of the *Salmonella* isolates was observed with cotrimoxazole, chloramphenicol and gentamicin (80%). The highest resistance frequency among FS species isolates was related to ampicillin (90%), lincomycin and methycillin (80%) and tetracycline (70%). Gentamicin and chloramphenicol were found to be the most effective antibiotics against FS with sensitivity frequency of 90%. With *Vibrio* species, the highest resistance frequency was observed with ampicillin and sulfamethoxazole at 70% and lincomycin at 90%. Again, like with FS gentamicin and chloramphenical were found to be the most effective against *Vibrio* species.

Table 3: Sensitivity (%) of Fecal coliforms, Fecal streptococci, *Salmonella spp*. and *Vibrio spp*. against 13 selected antibiotics.

Antibiotic	FC (n=10)			*Salmonella* spp. (n=10)			FS (n=10)			*Vibrio* spp. (n=10)		
	S	IR	R	S	IR	R	S	IR	R	S	IR	R
Amp(25)	0.0	0.0	100.0	20.0	20.0	60.0	10.0	0.0	90.0	20.0	10.0	70.0
Chlo (30)	50.0	0.0	50.0	80.0	10.0	10.0	90.0	0.0	10.0	80.0	0.0	20.0
Cot (25)	70.0	0.0	30.0	80.0	0.0	20.0	70.0	0.0	30.0	50.0	10.0	40.0
Ery (15)	50.0	0.0	50.0	30.0	0.0	70.0	40.0	10.0	50.0	60.0	10.0	30.0
Gen (10)	50.0	0.0	50.0	80.0	0.0	20.0	90.0	0.0	10.0	70.0	0.0	30.0
Kan (30)	50.0	10.0	40.0	30.0	10.0	60.0	40.0	20.0	40.0	60.0	40.0	10.0
Linc (2)	0.0	0.0	100.0	10.0	0.0	90.0	10.0	10.0	80.0	0.0	10.0	90.0
Met (5)	20.0	0.0	80.0	20.0	0.0	80.0	20.0	20.0	80.0	20.0	30.0	50.0
Mino (30)	60.0	10.0	30.0	50.0	10.0	40.0	60.0	60.0	30.0	60.0	10.0	30.0
Pen (1)	30.0	0/00	70.0	50.0	20.0	30.0	30.0	30.0	70.0	60.0	10.0	30.0
Strep (10)	50.0	10.0	40.0	60.0	10.0	30.0	30.0	30.0	50.0	50.0	40.0	10.0
Sulf (200)	20.0	10.0	70.0	30.0	0.0	70.0	40.0	40.0	40.0	10.0	20.0	70.0
Tet (25)	20.0	0.0	80.0	10.0	20.0	70.0	30.0	30.0	70.0	30.0	10.0	60.0

Key: FC – fecal coliform, FS – Fecal Streptococci, S – Sensitive, IR- Intermediary resistant, R – Resistant, Amp- ampicillin, Linc-lincomycin, Pen-penicillin, Meth-methicillin, Ery-erythromycin, Tet –tetracycline, Cot-cotrimoxazole, Strep-streptomycin, Kan-kanamycin, Gen-gentamicin, Sulf-sulfamethoxazole, Chloram-chloramphenicol and Mino-minocycline

With regard to cell wall morphology, Gram negative bacteria (Fecal coliforms, *Vibrio*species and *Salmonella* species) appeared to be more resistant to all antibiotics compared to the Gram positive bacteria (FS) in this study (Figure 1). Fecal coliforms appeared to have the highest resistance with a mean resistance frequency of 60.8% (±25.2), followed by *Salmonella* species at 51.5% (±26.6). *Vibrio* species showed the lowest mean resistance frequency at 41.6% (±24.8). There was however no significant statistical difference (p=0.859) in resistance among *Vibrio, Salmonella*, FS and FC isolates. On the other hand *Vibrio* species showed the highest sensitivity to all the studied isolates with a mean sensitivity frequency of 43.8% (±25.0), followed by FS at 42.3% (±26.2). Fecal coliforms were found to be the least sensitive to all the antimicrobial agents used. There was again no significant statistical difference (*p*=0.280) in sensitivity among all the bacterial isolates. On the other hand, there was significant difference (p=0.015) in intermediary resistance to antibiotics among *Vibrio* andfecal coliforms.
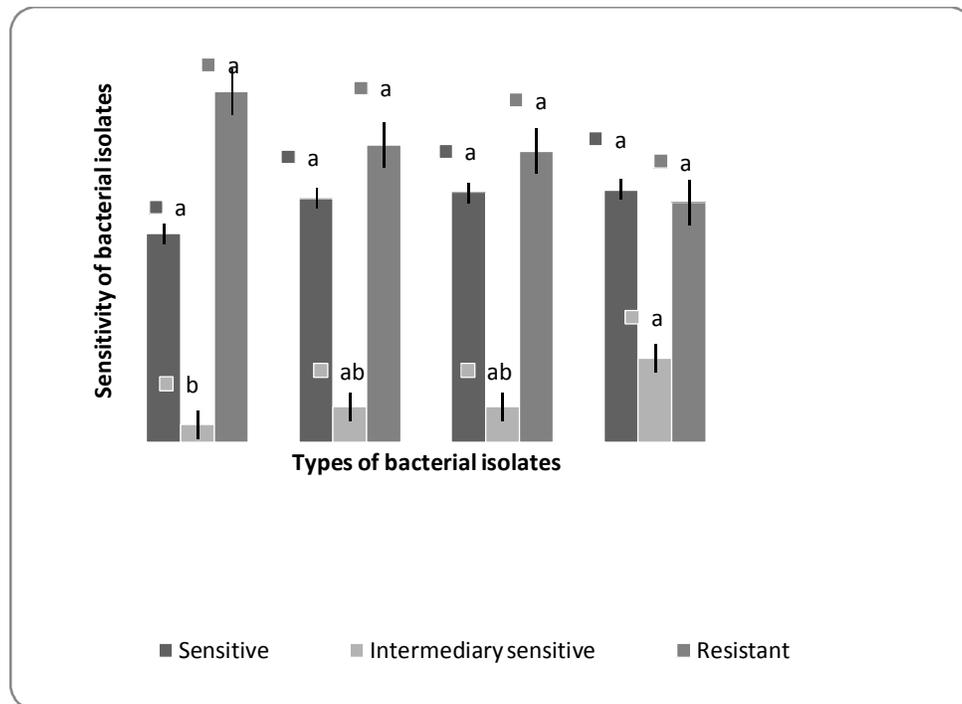
Figure 1: Antibiotic sensitivity pattern of bacterial isolates. Same letters on similar bars indicates no significant difference according to Turkey's HSD at 5% level.

DISCUSSION

A total of 40 bacterial strains were isolated and identified by their morphology and biochemical properties. Isolates were identified up to genus level only. The five sources of bacterial contamination characterized in this study were investigated because they differ in terms of the origin of the bacteria strain they release in the environment (cattle, goat and sheep) and in the expected exposure of these bacteria to antimicrobial selective pressure. Factors such as animal husbandry systems and nature of pasture grazing patterns of individual animals might have contributed to the variation in prevalence of microbes in different wastewater and sludge samples.

Fecal contamination as indicated by the finding of FS and FC could place the environment at risk for harboring microbes capable of causing human diseases. This includes certain pathogenic strains of *E. coli*, *Salmonella*, *Campylobacter*, *Aeromonas* and protozoa such as *Giardia* all of which have animal reservoirs (Santhiya*et al.,* 2011). The traditional multiple tube technique for FC detection remains useful especially when the conditions do not allow the use of membrane filter technique. The simple and time saving method of pour plating on standard Kenner Fecal (K F) streptococcus agar was used to enumerate F S. Fecal streptococci were found maximally in cattle wastewater and minimal in goat wastewater samples. Thus it appears from these findings that cattle could be the major carriers of these fecal bacteria.

Presence of *Salmonella* sp. and *Vibrio* sp.which are easily transmitted through water are of great concern. The sludge used as manure is at risk of being contaminated by *Salmonella* and *vibrio* species and thus poses a great risk to farmers and consumers of vegetables such as cabbages who use the produce to prepare

salads. These bacterial isolates are common intestinal bacteria of both animals and humans gut (Nabonita*et al.*, 2011); however contamination by *Salmonella* and *Vibrio* species may also come from public untreated water pumped into slaughterhouses or water taken by animals or cycling between the livestock and their environment or even contamination in feeds.Gagliardi and Karns, (2000) reported contamination of water used to irrigate vegetables and crops and the manure used as fertilizers with coliforms and other enteric bacteria.

In the present study, isolates were most resistant to lincomycin, ampicillin and methicillin and most sensitive to chloramphenical, gentamicin and cotrimoxazole. These results therefore show that ampicillin, lincomycin and methicillin are not effective to control these bacteria. Nipa*et al.*, (2011) observed 98% multiple resistance to 2 – 7 different antibiotics with 96.07% of the bacterial isolates resisting ampicillin in the same study. Bacterial resistance to erythromycin, streptomycin, penicillin, ampicillin, amoxicillin, kanamycin, tetracycline, oxytetracycline and chloramphenicol have been reported (Roberts, 2011). The present study demonstrated that bacterial isolates were resistant to antibiotics commonly used as feed additives (tetracycline, streptomycin and sulfonamides) or therapeutics (penicillin and tetracycline). Fecal coliforms showed the highest mean resistance frequency followed by *Salmonella* species. *Vibrio* species showed the lowest mean resistance frequancy. Some *Escherichia* species and *Salmonella* species possess capsular K and Vi antigens which protect them from access to antimicrobials. High level ofprevalence of bacterial antimicrobial resistance similar to or higher than those found in this study have been reported in various aquatic environments (Garcia-Armise*et al.*, 2011; Miranda *et al.*, 2002; Zbigniew, 2005).

The occurrence of antimicrobial agents at low concentration via leaching or continued usage may lead to the development of drug resistance, which may lead to resistance transfer to pathogenic bacteria and reduced efficacy of antibiotic treatment for animal and human diseases (Tendencia and De la Pena, 2002). Indeed the correlation between antimicrobial use and AR of commensal bacteria is well documented (Van den Bogaard and Stobberingh, 2000)and we can assume that the extent to which bacterial isolates are exposed to antibiotics before their release in the environment could be the core reason for the levels of AR observed in this study. High resistance to antibiotics may also be attributed to several other factors; first it may be that the source of drinking water for the animal had been polluted by antibiotic agents; secondly introduction of antibiotic supplemented commercial feeds may have initiated the resistance as previously suggested (Le *et al.,* 2005). High resistance may also be attributed to the history and dose of antibiotic utilization, as well as the chemical structure of the antibiotics (Aminov and Mackie, 2007).

In Conclusion *Vibrio* and *Salmonella* species were more frequently detected in samples which also showed high incidence of fecal bacteria indicators. It will be however, highly speculative to conclude that FS and FC both correlate well with these pathogenic microorganisms since the exact prevalence of the pathogenic microorganisms was not determined in the current study. There is a likelihood of slaughterhouse animals getting exposed to antibiotics to which they develop resistance which they could spread to the environment.

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