ANTIBIOGRAM, METAL TOLERANCE AND PLASMID PROFILES OF PATHOGENS ISOLATED FROM WASTEWATERS AND SLUDGE OF ABATTOIRS IN NAIROBI, KENYA

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DECLARATION

This thesis is my original work and has not been presented for any award in any other University or Institution.

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DEDICATION

This work is dedicated to my late dad, S. Nyamboya and to all mortals on whose shoulders I stood after he went to his final resting place.

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ACRONYMS AND ABBREVIATIONS

APHA	American Public Health Asssociation
CFU	Colony Forming Unit
DCA	Deoxycholate Citrate Agar
FC	Fecal Coliforms
FS	Fecal Streptococci
HM	Heavy Metals
HSD	Honestly Significant Difference
MAR	Multiple Antibiotic Resistance
MPN	Most Probable Number
SS	Salmonella-shigella agar
TCBS	Thiosulfate Citrate Bile Salts
TSI	Triose Sugar Iron
NCCLS	National Committee for Clinical Laboratory Standards
UV	Ultra-Violet
WHO	World Health Organization

ABSTRACT

Because of the prevalence of antibiotic resistant pathogenic bacteria, infectious diseases are becoming more difficult and expensive to treat. In Kenya, high levels of flouroquinone resistance of a bacterium, *Neisseria gonorrhea* have been widely reported. Antimicrobial resistance may be coded on plasmids, mutational events or on transposons. A growing body of evidence indicates that metal tolerance and antibiotic resistance are often found together in many clinical isolates and that metal and antibiotic resistance is closely associated. Besides having clinical consequences, resistant bacteria of animal origin may be the source of determinants of resistance for the possible transfer to human strains. Most of the previous studies have concentrated on clinical isolates from human and animal stools while studies targeting environmental isolates are limited. This study aimed at identifying fecal indicators and pathogens recovered from wastewaters used to clean animal carcass and sludge used as manure, from cattle slaughterhouse in Kayole and the separate sheep and goat slaughterhouses both in Kiamaiko, Nairobi and to determine susceptibility of isolated bacteria against 13 antibiotics and 6 heavy metals, in addition to studying plasmid profiles. Isolation of fecal indicators and pathogens was carried out using standard laboratory methods. Sensitivity to antibiotics was determined by the agar diffusion technique on Mueller-Hinton agar. Heavy metals tolerance was determined by well diffusion and tube dilution methods. Plasmid DNA was isolated using the alkaline lysis method. 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, 2.7×10^3 and 3.9×10^5 MPN/100 mL for fecal coliforms in cattle wastewater, cattle sludge, goat wastewater, sheep wastewater and a mixture of goat and sheep sludge, respectively. Pearson correlation analysis showed positive correlation between prevalence of fecal streptococci and fecal coliforms (r=0.931 at 0.01 level). *Vibrio* and *Salmonella* species were more frequently detected in samples which also showed high incidence of indicator organisms. There was high resistance to lincomycin (90 %), ampicillin (80 %) and methicillin (72.5 %) and low resistance to chloramphenicol (22.5 %). Another potential environmental threat noticed was heavy metal tolerance of the indicator organisms and pathogens to nickel, mercury, copper, zinc, lead and cobalt. Results of the test of toxicity in solid media agreed with those in liquid, however, inhibitory concentrations in solid media were much higher compared to those in liquid. Lead and nickel were the least toxic metals. Mercury was the most toxic component for all bacteria, followed by cobalt and then copper. Among the 40 bacterial isolates studied only 18 (45%) harbored between 1 - 2 kb plasmid DNA bands which ranged in size from 4kb to 10kb. The results showed the dynamics of resistance development in warm blooded animals usually used or consumed by humans. This study provided data on the level and risks of microbiological contamination as well as baseline data for the future assessment and monitoring of pollution levels of wastewaters. With regard to the high contamination level of the wastewaters and sludge with resistant bacteria as revealed in this study, treating of wastewaters and sludge is recommended, before using or discharging them into the environment and also proper use and disposal of antimicrobial agents. The metal-antibiotic double resistance detected in this study call for intervention measures to curb the potential health hazard that heavy metal pollution pose in the environment.

CHAPTER ONE

INTRODUCTION

1.1 Background

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, uncoordinated and often not available (Tatsuya, 2010). Lack of veterinary services of 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. 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 animal wastes may contain high

concentrations of these organisms. Animal waste can therefore contaminate human and animal drinking water sources and even soil when used as manure (Christina *et al.,* 2012).

The introduction of heavy metals in various forms in the environment can cause considerable modifications in the structure and function of microbial communities. In the last decade a number of studies have reported that antibiotic resistant bacteria may be present in the environment through co- or cross-resistance to metals or co-regulation of resistance pathways (Akinbowale *et al.,* 2007). Plasmids are known to carry resistance to antibiotics and heavy metals (Berg *et al.,* 2005). Heavy metals (iron, zinc, manganese, cobalt, copper, nickel) are essential micronutrients for bacteria since they are incorporated into enzymes and cofactors, however they are toxic in high concentrations because of binding to enzymes and DNA and by production of oxygen radicals through the Fenton reaction (Lopez-Maury *et al.,* 2002). Trace metals are significant contaminants in many aquatic systems, due in part to anthropogenic sources such as industrial and mining inputs. An organism's expression of a novel gene coding for drug resistance in remote communities has implications for the developed world. Once a resistant organism is introduced into a population, it is rapidly disseminated (Wenzel and Edmond, 2009).

In Kenya, there has been widespread resistance of *Neisseria gonorrhea* bacterium to quinolones (Laqace-Wiens *et al*., 2012). Senior officials in the ministry of health and doctors in Kenya have warned that misuse of drugs is making them ineffective as treatments for various diseases and ailments. Antibiotics are easily available over the counter with buyers not having to submit a doctor’s prescription. This easy availability

means many people are either overdosing or under dosing or simply buying antibiotics when they don't need them leading to widespread bacterial resistance to antibiotics.

1.2 Statement of the problem and justification

Animal wastewaters and sludge constitute an ideal environment for the emergence of new pathogenic and resistant bacterial strains by the acquisition of different virulence and resistance determinants (Watkinson *et al.*, 2007). Continued surveillance of the antimicrobial susceptibility profiles of food-borne and waterborne bacteria has been recommended strongly for identifying emerging antimicrobial resistant phenotypes (WHO, 2004). Transmission may occur through consumption of vegetables or water contaminated by feces from carriers. Besides having clinical consequences, resistant bacteria of animal origin may be the source of determinants of resistance for the possible transfer to human strains (Dick *et al*., 2005).

Several previous studies have concentrated on clinical isolates from humans, animals and some environmental samples (Bii *et al*., 2005; Sang *et al*., 2011; Laqace-Wiens *et al*., 2012). It is assumed that the clinical strains are representative of what could be found in the natural environment of the same geographical area. Similar studies on wastewater and sludge are however 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. With respect to animal health, environmental state and food safety concern for human consumption it is important to control the susceptibility of bacterial isolates from wastewaters and sludge to antimicrobial agents. It is also important to monitor the level of contamination and the resultant impacts on the survival and

establishment of indigenous flora and fauna especially animals and human beings. 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 study also aimed to investigate if there is a relationship between heavy metal and antibiotic resistance.

1.3 Research questions

i. What is the level of bacterial contamination in the raw animal wastewaters and sludge from the slaughterhouses in Nairobi?
ii. What is the variation in antimicrobial susceptibility pattern of the bacterial isolates from the raw animal wastewaters and sludge?
iii. What is the tolerance pattern of bacterial isolates isolated from the raw animal wastewaters and sludge to six selected heavy metals?
iv. What is the variation in plasmid profile pattern among the bacterial isolates?

1.4 Research hypotheses

i. The raw animal wastewater and sludge samples from the slaughterhouses in Nairobi are significantly contaminated with the bacteria.
ii. The bacterial isolates from the raw animal wastewaters and sludge exhibit same susceptibility to selected antimicrobial agents.
iii. The bacterial isolates from the raw animal wastewaters and sludge exhibit same tolerance pattern to six selected heavy metals.
iv. Plasmid pattern is different among the isolates from the wastewaters and sludge.

1.5 Objectives

1.5.1 General objective

To determine the level of microbial contamination in raw animal wastewaters and sludge of abattoirs in Nairobi, the plasmid profile and susceptibility of isolated bacteria against 13 antibiotics and 6 heavy metals.

1.5.2 Specific objectives

i. To quantify fecal bacteria indicators and detect pathogenic bacteria in the samples of sludge and raw animal wastewaters collected from three selected abattoirs in Nairobi.
ii. To determine the antimicrobial susceptibility pattern of the bacterial isolates from raw animal wastewaters and sludge.
iii. To determine the tolerance of bacterial isolates from raw animal wastewaters and sludge to six selected heavy metals.
iv. To determine plasmid profile of resistant and sensitive bacterial isolates using plasmid DNA fingerprinting technique.

1.6 Significance of the study

The findings on the microbial load of the wastewater samples will be crucial for the surveillance, prevention and control of many water borne diseases. The information obtained from studies of metal tolerance and antimicrobial resistance profiles will be important in: a) detecting patterns of change in tolerance b) implementing control measures on the use of antimicrobial agents and metals and c) preventing the spread of multi-drug-resistant strains of bacteria. The study on plasmid profile will enable better

understanding of effective clinical management of pathogens. The results obtained will be used to estimate possible risks of infection resulting from drinking contaminated water, the ingestion of foods obtained from plants irrigated with contaminated water.

CHAPTER TWO

LITERATURE REVIEW

2.1 Indicators of fecal contamination of water

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 are used 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 possible sewage contamination indicators have their advantages and disadvantages. Studies have demonstrated a number of deficiencies in the use of fecal coliforms as indicator organisms in marine waters in USA (Health Protection Agency, 2003). The validity and usefulness of the indicator concept depend on the existence of a constant quantitative relationship between the indicator organisms and the pathogens they monitor (Warrington, 2001). Fecal coliforms (FC) rather than fecal streptococci (FS) are usually used as indicators of fecal pollution. Their presence in water is a confirmatory test of recent fecal contamination (Kacaniova *et al*., 2009). It has been shown that the presence of *Salmonella* sp. is not necessarily correlated with great concentration of the fecal

pollution indicators (Gabutti, 2004) and therefore it would be advisable to always perform the detection of *Salmonella* sp. beside the traditional indicators. The fecal pollution indicators are therefore being substituted by more specific indicators such as *Escherichia coli*. A number of studies have shown that fecal streptococci may be ideal indicators (Martines *et al.,* 2009).

The Annapolis protocol identified fecal streptococci (for temperate marine and fresh water), *E. coli* (for temperate fresh waters) and sulphite-reducing *Clostridium perfringens* (for temperate, tropical marine and fresh waters) as primary microbial indicators (Baquero *et al.,* 2008). Routine monitoring experiments provided guidelines based on geometric mean of at least five samples per 30-day period during swimming season (Crossan, 2005). It was proposed that fecal streptococci can be used in determining the extent of fecal contamination in recreational fresh waters and marine waters (Roy *et al.,* 2003). The guideline given was 33 FS/100 mL for recreational water and 35 FS/100 mL for marine waters. When looking for contamination with *Salmonella*, nearly all the time, 67 % of the organism was isolated from shellfish with fecal coliforms levels <300/100 μL which supported the view that low levels of fecal coliforms do not necessarily indicate the absence of *Salmonella* (Baquero *et al.,* 2008).

2.2 Reuse of wastewater

The demand for water is continuously increasing in arid and semi-arid countries. Therefore, water of higher quality is preserved for domestic use while that of lower quality is recommended for irrigation. Municipal wastewater is less expensive and considered an attractive source for irrigation in arid and semi-arid countries (Mohammad

and Mazahreh, 2003). The interest in reusing wastewater for irrigation is rapidly growing in most countries. Moreover, irrigation with municipal wastewater is considered an environmentally sound wastewater disposal practice that helps to minimize the pollution of the ecosystem subjected to contamination by direct disposal of wastewater into surface or groundwater (Berg *et al*., 2005). In addition, wastewater is a valuable source for plant nutrients and organic matter needed for maintaining fertility and productivity of arid soils (Maciedo *et al*., 2010). However, the reuse of wastewater for irrigation may potentially create environmental problems if not properly treated and managed. When wastewater is used continuously as the sole source of irrigation water for field crops in arid regions, excessive amounts of nutrients are simultaneously applied and their accumulation in the soil may cause unfavorable effects on productivity and quality of crops and soil as well as groundwater by leaching in coarse textured soils (Chari *et al*., 2011). Consequently, management of irrigation with wastewater should consider the nutrient content in relation to the specific crop requirements and the concentrations of plant nutrients in the soil and other soil fertility parameters (Edge and Hill, 2005). In many areas of developing countries, untreated wastewater flows through channels into rivers where it is diverted by subsistence farmers to small plots of vegetables and salad crops, grown for nearby urban markets (Dick *et al*., 2005). Such vegetables include carrots, lettuce, cabbage and others which are easily consumed raw as salad. This may pose danger to the human health since the untreated wastewater usually has microbial contaminants (WHO, 2004).

2.3 Metal toxicity

Deposited metal elements may be remobilized and released back into water (Soltan, 2006) and these heavy metals (HM) are persistent pollutants. Unlike organic pollutants, metal

elements cannot be degraded but they accumulate throughout the food chain resulting in potential human health risks (Kacaniova *et al.,* 2009; Stawarzt *et al.,* 2009) and ecological disturbances (Lovley, 2000). The concentration and toxic effects of HM in grass of polluted areas is influenced by the distance from the emission source (Palacios *et al.,* 2002; Alonso *et al.,* 2003; Liu, 2003). During the last few years a number of studies have examined effects of HM on soil fertility (Lee *et al.,* 2002; Liu, 2003; Nwaugo et *al.,* 2008), plant vegetative growth (Singh *et al.,* 2008), forage quality, environmental pollution and water quality (Liu, 2003). The toxicity and fate of HM depend on their chemical form and concentration, which is mainly attributed to the sensitivity of enzymes to these compounds. It is well established that, depending on concentrations, HM can be stimulatory or inhibitory to a wide array of enzymes (Le *et al*., 2005) or even toxic in biochemical reactions. This is mostly due to their chemical binding to enzymes which results in disruption of enzyme structure and activities.

At high concentrations, HM are toxic and cause ecological problems and damage to human health (Argese *et al.,* 2005). Exposure of animals and humans to toxic elements is a factor that could be closely related to higher cancer prevalence (Stawarzt *et al.,* 2009), increased mortality of animals and humans (Formicki *et al.,* 2008; Korenekova *et al.,* 2008; Skalicka *et al.,* 2008; Kacaniova *et al.,* 2009), reduced fertility with reduced numbers of offspring and decreased weight of visceral organs in weaned animals (Kalafova *et al.,* 2008). However trace levels of many HM may be required for activation or functioning of many enzymes and co-enzymes (McDonald, 2003). In ruminants, HM ingested with contaminated feedstuffs may cause changes in ruminal microbial communities and thus in the digestive processes of the ruminant fore-stomachs.

The interaction between heavy metals and antimicrobial resistance can be divided into three major types: interaction of heavy metals with antibiotic compounds, interaction of heavy metals with antibiotic resistance genes or their products and interaction of heavy metals with bacterial properties such as viability or conjugation (Nishino *et al.,* 2007). Heavy metal cations complex with antibiotic substances, for example, tetracyclines (Palm *et al.,* 2008), inhibiting their intestinal absorption. Zinc and copper affect the stability of ampicillin (Fernandez-Gonzalez *et al.,* 2005). Multidrug efflux pumps, which enable the bacterium to also export antibiotic substances, are induced by some heavy metals, specific resistance genes or their products interact with heavy metals e.g. zinc-dependent β-lactamases or tetracycline efflux-proteins of the tetracycline-metal/H+-antiporter type (Lee *et al.,* 2005; Palm *et al.,* 2008). Heavy metals can cause sub lethal damage which might increase the bacterial susceptibility to antibiotics. They may influence bacterial conjugation rates. Heavy metals can also select for antibiotic resistance genes if the latter are physically linked to heavy metal resistance genes e.g. on plasmids (Ghosh *et al.,* 2000).

2.4 Conjugal plasmids and conjugation

It is generally accepted that horizontal gene transfer by conjugation promotes the evolution and adaptation of individual bacteria and of the microbial communities in natural systems. Laboratory experiments have shown that conjugation of broad-host-range plasmids is possible between distantly related bacteria and even eukaryotes (Frauke *et al.,* 2008). A lot of studies about conjugal plasmids and conjugation in natural habitats support these findings. However, the extent of conjugation events and the involvement of different groups of a community are not known because it is generally accepted that

horizontal gene transfer by conjugation promotes the evolution and adaptation of individual bacteria and of the microbial communities in natural systems (Dick *et al*., 2005).

Plasmid mediated multidrug resistance is one of the most serious problems in the treatment of infectious diseases (Parisa *et al*., 2011). Large plasmids are known to confer drug resistance. Plasmid analysis can aid in the differentiation of isolates and has been used as an epidemiologic tool in investigating outbreak of enteric diseases (Soumik *et al*., 2010). The profiles of small plasmids can be used as a marker to identify known serotypes or to determine the emergence of any new and unknown serotypes in the future. Use of curing agents could be sufficient to remove those genes necessary for virulence and resistance because both types are frequently carried by plasmids. Plasmid profiling surveillance may be of help for the epidemiologist to relate the strains with outbreaks and their spread (Tatsuya and Mary, 2010).

2.5 Antimicrobial resistance of bacteria

Antibiotic resistance is common in pathogens, opportunistic pathogens and even non-pathogenic bacteria in various environmental conditions, such as hospitals and communities among others (Baquero *et al*., 2008). Recent studies on antibiotic resistance of bacteria showed that mortality rates doubled in cases where resistant infections were present; furthermore, the length of treatments increased often requiring the use of more expensive antibiotics or antibiotic cocktails (WHO, 2007). Hence, the prevalence of antibiotic-resistant pathogens is becoming a major public health issue all over the world. Antibiotics and antibiotic resistance genes play fundamental ecological roles in shaping

the structures of microbial communities (Martines *et al*., 2009). Horizontal gene transfer among bacteria and the overuse of antibiotics intensifies the antibiotic resistance in pathogens and environmental bacteria. The prevalence of antibiotic resistant bacteria in the environment threatens to be conducive to the emergence of antibiotic resistance in bacterial pathogens (Baquero *et al*., 2008).

A microorganism is usually categorized as clinically antibiotic resistant by applying the appropriate breakpoint in a defined phenotypic test system. Agar diffusion and broth dilution tests are two common phenotypic test systems used to determine the breakpoint (Martines *et al.,* 2009). These serial assays are used to investigate the antibiotic concentration at which bacterial growth is inhibited. However, microorganisms in environmental samples are always mixed with multiple species present, rather than a single strain. Most studies on antibiotic-resistant bacteria have been reported on single strains and these have been used to describe the status of antibiotic-resistant bacteria in environmental samples (Hu *et al.,* 2008). There is limited research on general antibiotic resistance levels of environmental samples. Nevertheless, in both agar diffusion and broth dilution test methods, exposure of bacteria to antibiotics is the most practical way to confirm antibiotic resistance. This method can be used to analyze the general level of antibiotic resistance of environmental bacteria in a mixed system.

2.6 Antibiotic susceptibility of enteric pathogens in Kenya

In Kenya, diarrheal illness is ranked 4th after malaria, upper respiratory tract infections and skin infections (Laqace-Wiens *et al*., 2012). A major outbreak of multidrug resistant *Shigella dysentriae* I, occurring concurrently with *Vibrio cholera* OI Ogawa was reported

along the coastline of Kenya (Sang *et al*., 2011). The strain of S. *dysentriae* was found to be resistant to ampicillin, tetracycline, chloramphenicol and cotrimoxazole but sensitive to gentamycin, nalidixic acid and kanamycin. Also multidrug resistant entero-aggregative *E. coli* serotype 044 associated with acute and persistent diarrhoea was reported in Kenyan children (Bii, *et al*., 2005). The increasing resistance cases, for instance the emergence of quinolone resistance in gonococcal isolates highlights the importance of continued surveillance of the antimicrobial susceptibility profiles of food-borne and waterborne bacteria for identifying emerging antimicrobial resistant phenotypes.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study focused on 3 abattoirs in Nairobi County, specifically in three slaughterhouses that are a representative of slaughterhouses in the county. These included cattle slaughterhouse in Kayole and separate sheep and goats' slaughterhouses both in Kiamaiko (Fig 3.1). These are the major cattle, sheep and goat slaughterhouses in Nairobi County. The area was chosen due to the diversity and a large number of the slaughterhouses.

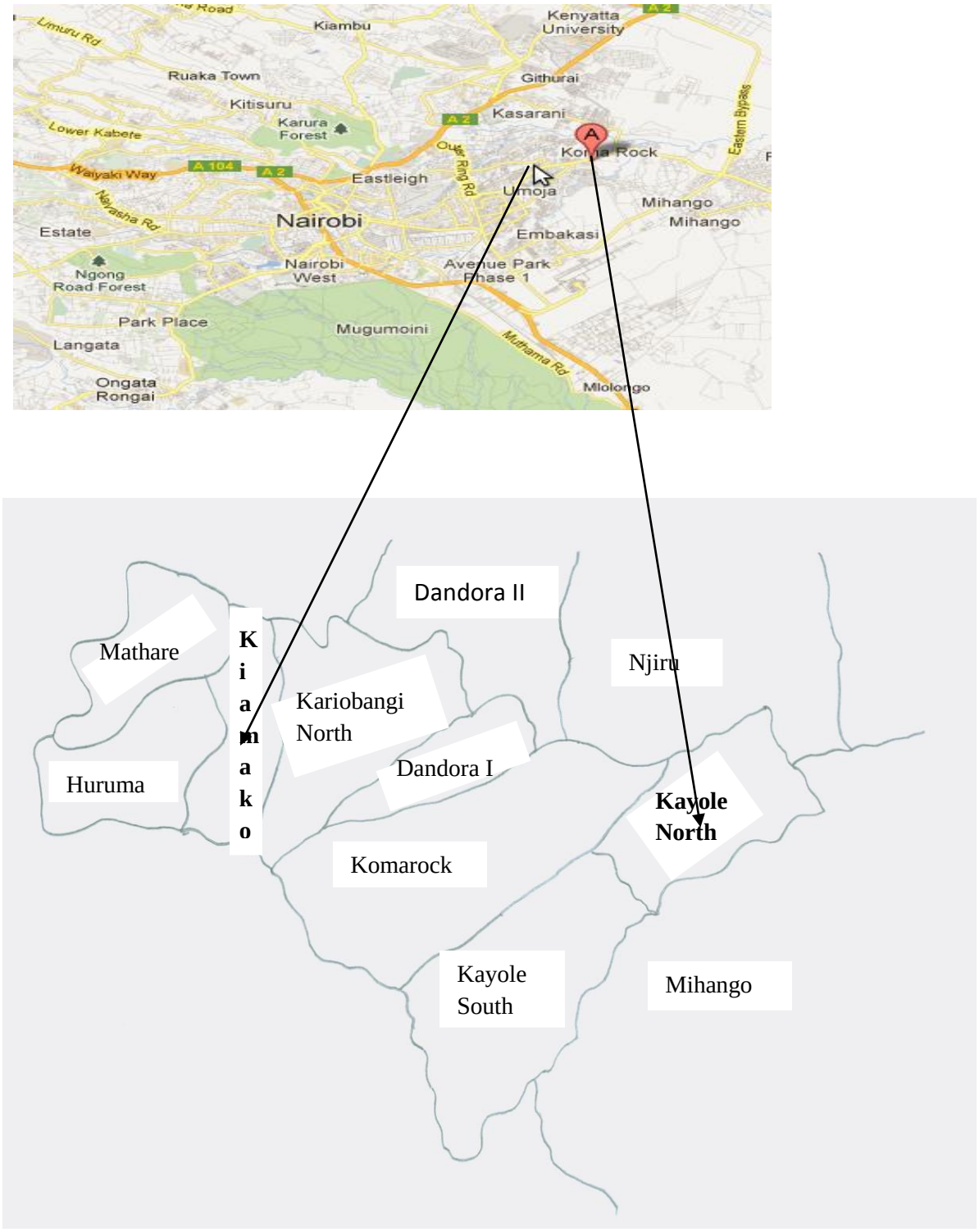

Figure 3.1: Map of Kiamaiko and Kayole slaughterouses in Nairobi County, Kenya. (Source: Google earth, 2013).

3.2 Sample collection and preparation

Data was collected as per the method applied by Amalesh *et al*., (2012). A total of thirty samples of 100 mL sludge and raw animal wastewater were collected from the cattle slaughterhouse in Kayole and Goat and sheep slaughterhouses both in Kiamako, Nairobi. 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 March 2012 and April 2012 during the rainy season, 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.

3.3 Bioassays

This section contains the methods that were used to detect and enumerate fecal coliforms, fecal streptococci, *Vibrio* species and *Salmonella* species. It also contains the methods employed to assess quantitatively the impact of metal ions on bacterial growth, antibiotic susceptibility tests and plasmid profiles of the bacterial isolates.

3.3.1 Screening for *Salmonella* species

Detection of *Salmonella* was carried out in three successive phases. In the first phase, selective enrichment was carried out using tetrathionate broth base as outlined by APHA (2003). One millilitre of each sample from different sites was mixed well with 10 mL of tetrathionate broth and the mixture was incubated for 24 h at a temperature of 35 °C. In the second phase, pour plating method was carried out using 1 mL of enriched samples.

Streaking was carried out from the same enriched samples on Salmonella-Shigella (SS) agar and MacConkey agar (Andrews and Hammack, 2003) and incubated at of 37 °C for 24 h. In the third phase, typical colonies were subjected to various standard biochemical procedures (such as mortility, indole, TSI and urease tests) using the procedure described by Mariita and Okemo (2009).

3.3.2 Screening for *Vibrio* species

Detection of *Vibrio* sp. was carried out in three successive phases: (i) Enrichment in a non-selective medium; One millilitre of each sample was enriched in sterile alkaline peptone water and dispensed in 10 mL tubes (Health Protection Agency, 2003). Incubation was carried out 35°C for 18 h. (ii) Plating out on selective medium; The streaking of the enriched samples was carried out on Thiosulfate citrate bile salts sucrose (TCBS) agar medium. The agar plates were incubated at 35 °C for 24 h for presumptive tests. To differentiate *Vibrio* sp., streaking of sucrose positive (yellow) colonies was carried out on nutrient agar according to the procedure described by Mariita and Okemo (2009) (iii) Biochemical reactions; Gram staining was carried out and observations made before carrying out the biochemical tests. First the presumptive colonies on nutrient agar were inoculated onto the TSI agar slants and incubated at 37 °C for 24 h. Simmons citrate agar slants were also inoculated and incubated at 36 °C for 72 h. The media were observed for growth and colour changes. The cytochrome oxidase test was carried out by placing a piece of filter paper in a clean petri dish and adding 3 drops of freshly prepared Oxidase reagent. A colony of the test organism was applied on the filter paper using a glass rod to observe the development of a blue purple colour. For motility test, SIM (sulphide indole mortility) media was stabbed through the centre to test for motility by

observing if there was spreading of growth from the stab line or turbidity throughout the medium. The urea agar base was used to test for urease production by examining if there was any colour change. An un-inoculated tube was used as a control.

3.3.3 Screening for fecal coliforms

The analysis of wastewaters and sludge for the presence of fecal coliforms was carried out using the multiple–tube fermentation technique (APHA, 2003). This was carried out in three steps; the presumptive, the confirmed and completed tests. Each batch was inoculated with the diluted water samples (all dilutions were carried out using sterile water blanks). In the presumptive test three series of five tubes each containing 10 mL, 1 mL and 0.1 mL portions of the sample were inoculated with lactose broth that was initially sterilized by autoclaving at 121 °C for 15 min. Pure sterile lactose broth was inoculated with sterile distilled water in the same way and used as a control. Inoculated tubes were then placed in an incubator at 37 °C for 48 h (Gyles, 2007). Sterile loop transfers were made from all tubes showing acid and gas production (of total coliform MPN) to tryptose bile broth and incubated at 44 °C for 24 h. Gas production in a fermentation tube within 24 h or less was considered as a positive reaction. The estimated number of fecal coliforms, present in 100 mL was read from a tabulated probability table using corresponding results of various combinations of positive and negative reactions from each of the three batches (APHA, 2003). For confirmation, samples considered to have a positive reaction from the tryptose broth were streaked on a plate of Eosin Methyl Blue (EMB) agar to give well isolated colonies. Incubation was carried out at 37 °C for 48 h. Development of the typical colonies on the plates was observed and a Gram stain was carried out. Completed test was carried out when two colonies considered to be of fecal

coliforms were picked and transferred to nutrient agar plates and fermentation tubes containing brilliant green lactose broth. Incubation of the agar plates and tubes was again carried out at 37 °C for 24 to 48 h. From the agar slope, a Gram stain was made to confirm the completed test. Brilliant green lactose broth was also observed for gas production.

3.3.4 Screening for fecal streptococci

Azide dextrose broth was used for the detection of fecal streptococci in the wastewaters and sludge (ISO 6222, 2000). The single strength media were inoculated with 10 mL of the samples. Tubes were then incubated in a water bath set at 45 °C and observed after 8 h for the appearance of turbidity and production of acid (indicated by the yellow colour change of the Bromothymol blue). As soon as the positive reaction appeared, a loopful of the material was transferred to a confirmatory slant (*Enterococcus* confirmatory slant). The confirmatory slants were used with the *Enterococcus* confirmatory agar, to confirm the presence of fecal streptococci (FS) in the wastewater samples. Slants of the confirmatory agar were prepared and a loopful of material from the broth streaked on the surface of the slant. The tubes were then incubated at 37°C for 12 h and examined for the presence of pinpoint colonies on the slant. A Gram stain reaction was carried out for microscopic evidence of the presence of FS. For confirmation, Kenner Fecal (KF) streptococcal agar, a selective media was used, by direct plating (pour plating). One milliliter of diluted (10^3 or 10^4) samples were used in duplicate plates. Incubation was carried out for 48 h at 35°C and all red and pink colonies counted using colony counter (and represented as CFU/100 mL) according to the procedure described by Mariita and Okemo (2009).

3.3.5 Antibiotic sensitivity testing

Sensitivity to antibiotics was determined by the agar diffusion technique recommended by the by the National Committee for Clinical Laboratory Standards (CLSI 2006). Bacteria were suspended in sterile 0.85% saline to a turbidity to match a Mcfarland standard diluted 1:20 and streaked on Mueller-Hinton agar (Oxoid) (Patrizia et al., 2005). The following antibiotic- impregnated disks were used: ampicillin (25µg); cotrimoxazole (25µg); streptomycin (10 µg); chloramphenicol (30µg); kanamycin (30µg); gentamicin (10µg); penicillin G (1 unit); methicillin (5µg); minocycline (30µg); lincomycin (2µg); erythromycin (15µg); tetracycline (25µg) and sulfamethoxazole (200µg). These antibiotics are commonly used in both animal and human medicine. Results were recorded as Sensitive (S), Intermediary Resistant (IR) and Resistant (R). The multiple antibiotic resistance (MAR) index values were calculated using the formula by Lee et al., (2009); a/bc (where 'a' represented the number of antibiotics the isolate was resistant to and 'b' represented the total number of antibiotics the isolate was tested against and 'c' the total number of bacterial isolates) .

3.3.6 Tolerance of bacterial isolates to heavy metals

3.3.6.1 Preparation of stock solution

The heavy metals tested were: Cu, Pb, Hg, Zn, Co and Ni. Stock solutions were prepared using distilled water. Mercury was slightly acidified by adding 2 to 4 drops of 6N HC1. The stock solutions were sterilized at a temperature of 121 °C for 15 min. The solutions were kept at a temperature of 4 °C. The glassware used was leached in 2N HNO_3 and rinsed several times with distilled water before use to avoid metal contamination (Hassen *et al.*, 2000).

3.3.6.2 Assessment of metal toxicity

In order to assess quantitatively the effects of the heavy metal on bacterial isolates, plate diffusion and tube dilution methods were used.

3.3.6.2.1 Plate diffusion method

To each plate of nutrient agar (Oxoid), 0.5 ml of the appropriate metal solution was added in a central well of 1 cm in diameter and 4 mm in depth (Konopka and Zakharova, 2000). Plates were then incubated at a temperature of 37 °C for 24 h to allow diffusion of the metal into the agar to establish a concentration gradient. On each plate, ten isolates were inoculated in radial streaks and in duplicate. Plates were then incubated at 37 °C for 48 h after which the area of growth inhibition (in mm) was measured as that from the edge of the plate to the leading edge of the growing streak. The percentage of bacterial resistance was calculated in terms of the ratio: length of the growth in mm versus length of the total inoculated streak. Each isolate was grown on nutrient agar media without metals and used as control. The range of concentrations for heavy metals used was 5-300 millimoles. Bacterial isolates that showed more than 50 % growth in 25 mM of each metal ion was considered to be resistant (Chari *et al*., 2011).

3.3.6.2.2 Tube dilution method

Concentrations of the appropriate metals compounds were prepared in tubes with a final volume of 10 ml of the nutrient broth (Oxoid). Culture medium was sterilized for 15 min at a temperature of 121 °C. Three tubes were prepared for each metallic concentration then inoculated with 200µl of an 18-hr-old cultures of the studied bacterial isolates. A positive control consisted of a metal-deficient medium inoculated with the bacterial

isolate and a negative one consisted of a metal-supplemented medium without the bacterial isolates of study. The tubes were incubated at 37 °C for 5 days. The lowest concentration of metal that completely prevented growth was termed as the minimal inhibitory concentration (MIC).

3.3.7 Plasmid profile analysis

Overnight bacterial suspensions of 10 ml were spun for 10 min in a centrifuge (1000 rpm) to pellet the cells (Maithem and Intisar, 2012). The supernatant was gently decanted, leaving 50 – 100 µl together with cell pellet which were then vortexed 5 times (10 sec) at high speed to re-suspend the cells completely. The tubes were left for 5 min then 600 µl of P1 solution (50M glucose, 25mM TrisHCL and 10mM EDTA, 50 µl RNAse) was added. The solution was mixed by vortexing for 2 – 3 sec then left for 5 min at room temperature. Thereafter, 600 µl of P2 solution (0.2 M NaOH, 1.0 % SDS) was added and the tubes mixed gently by shaking, then left for 5 min before adding 700 µl of ice cold solution P3 (3.0 M KOAc, pH 5.5). The tubes were mixed gently by shaking then centrifuged at 10000 rpm for 10 min to pellet the cell debris and chromosomal DNA. The supernatant was transferred to a fresh eppendorf tube and mixed with 100 µl of 3.0 M KOAc, pH 5.5 and mixed by turning the tubes up and down before adding 700 µl of ice cold isopropanol. The tubes were then mixed gently by turning the tubes up and down and incubated at -20°C for 30min. Centrifugation was then carried out at 10000 rpm for 30 min to pellet DNA, the supernatant was discarded then 1 ml of 70 % ethanol previously pre-cooled at a temperature of -20 °C was added and the tubes were again spun for 4 min before discarding the supernatant. The pellet was air dried and then re-suspended in 50µl of distilled water. The DNA was stored at 4°C. For gel electrophoresis, powdered agarose

(1%) was boiled in 1 x TAE buffer intermittently until the solution became a clear gel. The gel solution was allowed to cool then poured with a comb in place and allowed to solidify. The gel tray and the combs were removed. The gel was placed into the tank containing the 1 x TAE buffer. The tracking dye (Bromophenol blue, 2µl) was mixed with 1µl cyber green and 4 µl of DNA samples of the isolates and marker (1 kb) separately and then loaded into the wells. The gel was run at 120 V for 3 h and then viewed using the U-V trans-illuminator. For all the bacterial isolates, the presence and sizes of plasmid DNA was determined and photographed (Maithem and Intisar, 2012).

3.3.8 Data analysis

The data on densities of fecal indicators and prevalence of antibiotic resistance of bacterial species were analyzed using one way ANOVA at 5% significance level with SPSS computer software (version 16.0). P value of <0.05 was considered as significant. The means were separated using Tukey's Honest Significance Difference (HSD) at 5 % level.

CHAPTER FOUR

RESULTS

4.1 Enumeration of fecal bacteria indicators and detection of pathogens

The counts of FS are expressed in the CFU (colony forming units) and that of FC (fecal coliforms) in MPN (most probable number) and quoted as means±SD (standard deviation). Biochemical detection of *Vibrio* and *Salmonella*-like isolates revealed varied results.

4.1.1 Enumeration of fecal bacteria indicators

Goat and sheep sludge had the highest fecal coliform (FC) contamination with a mean density of 3.9×10^5 MPN/100 mL followed by cattle wastewater with a mean density of 3.4×10^5 MPN/100 mL (Table 4.1). Typical colonies of FC grew on EMB plate (Plate 4.1) and nutrient agar plates (Plate 4.2). The prevalence of fecal coliforms was significantly higher in goat and sheep sludge ($p = 0.004$) as compared to cattle sludge, sheep and goat wastewaters.

Table 4.1: Mean fecal bacteria densities (mean±standard deviation) in 100 mL of the wastewaters and sludge from Kayole and Kiamako abattoirs in Nairobi

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

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). Means followed by the same letters within the column indicate no significant difference according to Turkey's HSD at 5 % level.

Fecal coliforms

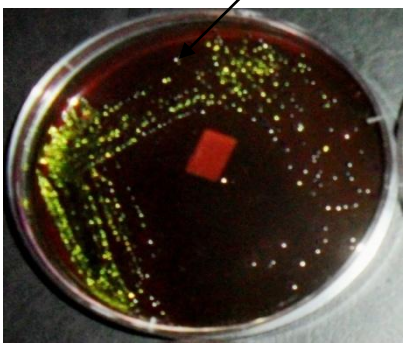

Plate 4.1: Typical colonies of fecal coliforms on EMB plate. Arrow show the colonv formed

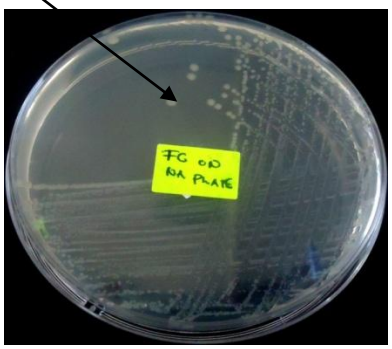

Plate 4.2: Typical colonies of fecal coliforms on nutrient agar plate. Arrow show the colony formed.

The overall mean density of fecal streptococci (FS) was higher (2.4 x 10^6 CFU/100 mL) compared to that of the fecal coliforms (1.5 x 10^5 MPN/100 mL), however there was no significant difference at $p < 0.05$. Cattle wastewater showed the highest FS density with a mean density of 6.3 x 10^6 CFU/100 mL followed by goat and sheep sludge with a mean density of 5.0 x 10^6 CFU/100 mL. The mean density of fecal streptococci from cattle wastewaters was significantly different ($p = 0.003$; $p< 0.05$) from that obtained from cattle sludge, sheep and goat wastewaters. Streptococcal species showed pink and red colonies on streptococcal KF agar plates (Plate 4.3) and cream colonies on nutrient agar plates (Plate 4.4). All the FS species stained positive following Gram stain reaction occurring in pairs and in the characteristic short to long chains (Plate 4.5). 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).

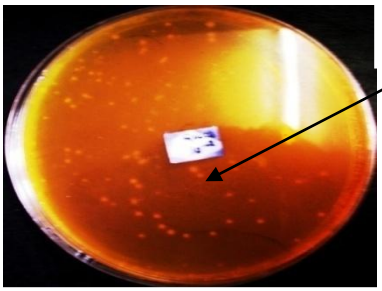

Plate 4.3: Typical colonies of FS on KF streptococcal agar. Arrow show the colony formed

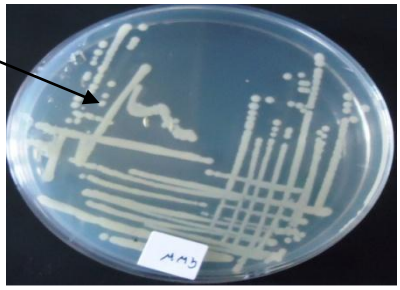

Plate 4.4: Typical colonies of FS on nutrient agar plates. Arrow show the colony formed

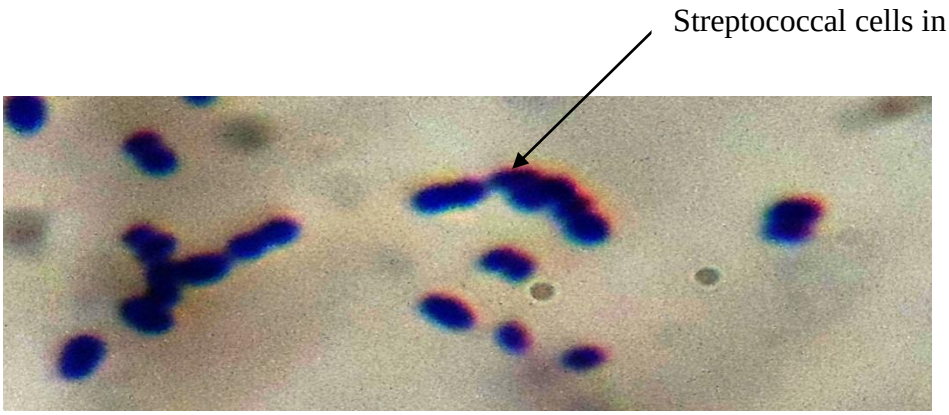

Plate 4.5: Streptococcal strains showing the characteristic short to long chains following gram stain reaction. Arrow show the colonies formed

4.1.2 Detection of *Salmonella* and *Vibrio* species

The biochemical detection of *Vibrio* and *Salmonella*-like isolates is represented in Table 4.2. Gram stain reaction of the typical colonies on Salmonella-Shigella agar (black colonies) (Plate 4.6) and MacConkey agar (white colonies) (Plate 4.7) was negative for *Salmonella*. Typical *Salmonella* colonies were cream on nutrient agar plate (Plate 4.8). The biochemical tests gave various results. The TSI agar slants inoculated with typical colonies (for hydrogen sulfide and glucose test) had yellow butts and red slants and some blackening (Plate 4.9). There was gas formation in peptone water enriched with glucose (Plate 4.10). On the SIM media, there was the spreading of turbidity from the stab line

and in some tubes there was blackening with some isolates (Plate 4.11). The *Salmonella* isolates showed typical negative reaction for urease test (Plate 4.12). There was bacterial growth and colour change from green to blue on the Simmons citrate agar slant (Plate 4.13). The cytochrome oxidase test was positive as shown by the formation of a blue-purple colour.

Table 4.2: 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	+	+	-	-	R	Y	-	-	+	+	+
Salmonella	+	-	V	-	R	R	+	+	V	+	+

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; SIM, sulfur indole motility agar; R, red; Y, yellow.

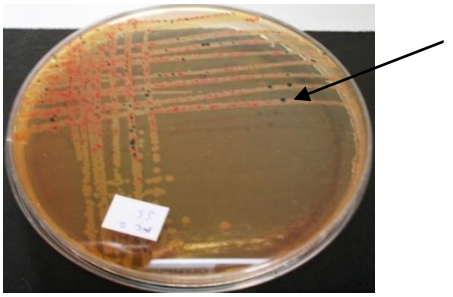

Plate 4.6: Black *Salmonella* colonies on SS agar. Arrow show the colony formed

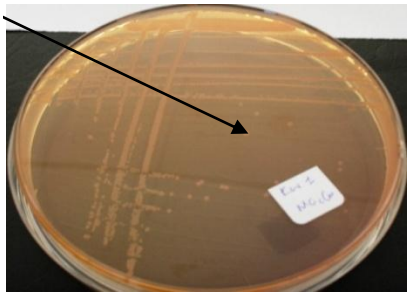

Plate 4.7: *Salmonella* colonies on MacConkey agar plate. Arrow show the colony formed

Plate 4.8: Cream *Samonella* colonies on nutrient agar plate. Arrow show the colony formed

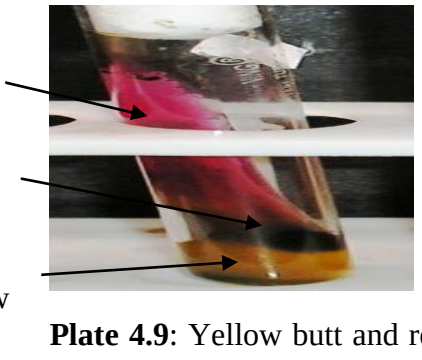

Plate 4.9: Yellow butt and red slant and some blackening on TSI slant showing the presence of *Salmonella* species

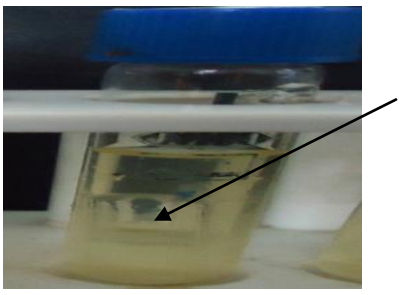

Plate 4.10: Gas production in peptone water as indicated by bubble formation in Durham inserted in tube showing presence of *Salmonella* species.

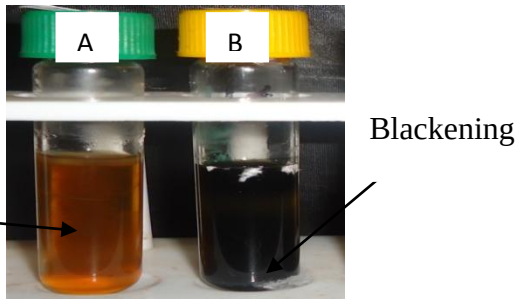

Plate 4.11: *Salmonella spp.* on SIM medium. A, arrow showing turbidity, B, arrow showing blackening.

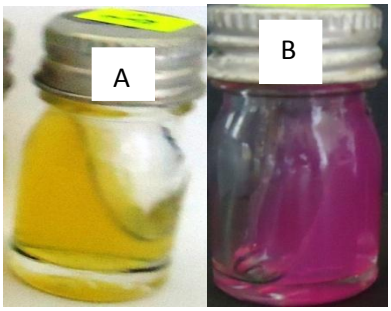

Plate 4.12: Reaction in urea agar slants. A, absence of color change showing presence of *Salmonella* sp. B, colour change showing absence of *Salmonella* sp.

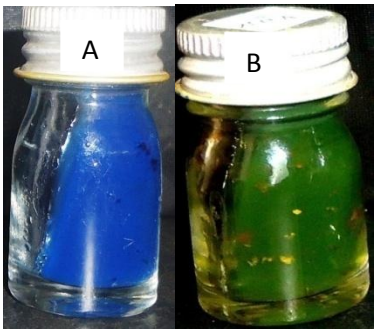

Plate 4.13: Reaction in simmons citrate agar. A, colour change from green to blue showing presence of *Salmonella* sp. B, no colour change showing absence of *Salmonella* sp.

Vibrio colonies grown on TCBS agar plates were yellow, with a diameter of 2-3 mm. Other colonies were green and varied in sizes, just as the yellow ones (Plate 4.14). The sub-cultured colonies on nutrient agar gave cream colonies (Plate 4.15). The colonies also showed gas production in peptone water enriched with glucose (Plate 4.16). The presumptive colonies gave positive results for motility test, indicated by the turbidity that spread in the SIM media (Plate 4.17). Urease test was also negative (Plate 4.18). On the TSI, the results were negative for H_2S test. The slants were red and the butts yellow (Plate 4.19). On the Simmons citrate agar, there was *Vibrio* isolate growth and colour change from green to blue in all the slants (Plate 4.20). For the cytochrome oxidase test, the presumptive colonies were positive as was indicated by the formation of a blue-purple colour. Indole test was positive as there was change of colour to pink.

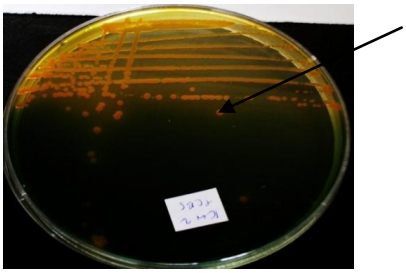

Plate 4.14: Yellow *Vibrio* colonies on TCBS agar. Arrow show the colonies formed

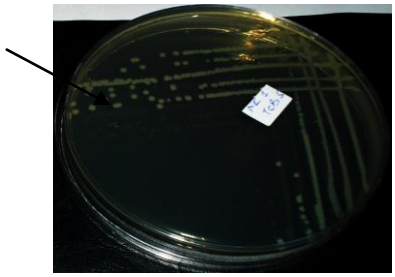

Plate 4.15: *Vibrio* colonies on nutrient agar plates. Arrow show the colonies formed

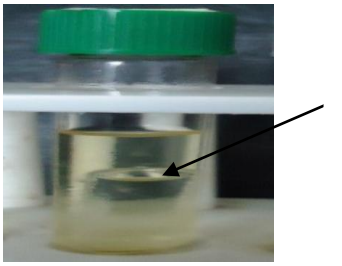

Plate 4.16: Gas production in peptone water as indicated by bubble formation in Durham inserted in tube showing presence of *Vibrio* species. Arrow show bubble formed

Plate 4.17: *Vibrio* colonies showing motility on SIM media

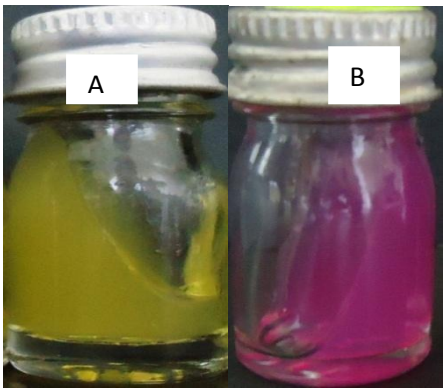

Plate 4.18: Reaction in urea agar slants. A, absence of color change showing presence of *Vibrio* sp. B, colour change showing absence of *Vibrio* sp.

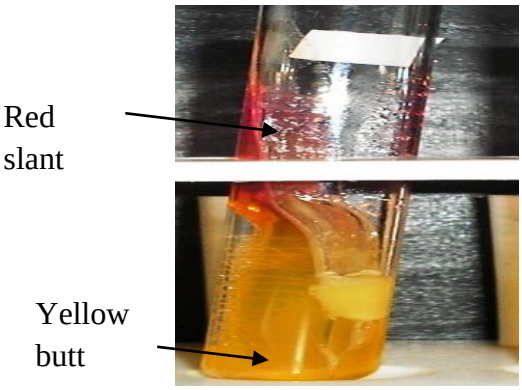

Plate 4.19: Yellow butt and red slant on TSI slants showing presence of *Vibrio* species

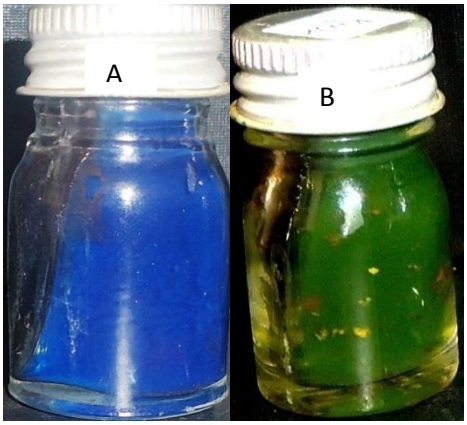

Plate 4.20: Reaction in simmons citrate agar. A, colour change from green to blue showing presence of *Vibrio* sp. B, no colour change showing absence of *Vibrio* sp.

4.1.3 Bacterial isolates

A total of 40 bacterial isolates were obtained from the animal raw wastewaters and sludge from Kayole and Kiamaiko slaughterhouses in Nairobi. The isolates identified are indicated in Table 4.3.

Table 4.3: Bacterial strains isolated from the raw animal wastewaters and sludge

Strain no	Identification*	Origin
S1	Fecal coliform	Cattle wastewater, Kayole
S2	Fecal coliform	Cattle wastewater, Kayole
S3	Fecal coliform	Cattle sludge, Kayole
S4	Fecal coliform	Cattle sludge, Kayole
S5	Fecal coliform	Goat wastewaters, Kiamaiko
S6	Fecal coliform	Goat wastewaters, Kiamaiko
S7	Fecal coliform	Sheep wastewater, Kiamaiko
S8	Fecal coliform	Sheep wastewater, Kiamaiko
S9	Fecal coliform	Goat and sheep sludge, Kiamaiko
S10	Fecal coliform	Goat and sheep sludge, Kiamaiko
S11	Fecal Streptococci	Cattle wastewater, Kayole
S12	Fecal Streptococci	Cattle wastewater, Kayole
S13	Fecal Streptococci	Cattle sludge, Kayole
S14	Fecal Streptococci	Cattle sludge, Kayole
S15	Fecal Streptococci	Goat wastewaters, Kiamaiko
S16	Fecal Streptococci	Goat wastewaters, Kiamaiko
S17	Fecal Streptococci	Sheep wastewater, Kiamaiko
S18	Fecal Streptococci	Sheep wastewater, Kiamaiko
S19	Fecal Streptococci	Goat and sheep sludge, Kiamaiko
S20	Fecal Streptococci	Goat and sheep sludge, Kiamaiko
S21	*Vibrio* sp.	Cattle wastewater, Kayole
S22	*Vibrio* sp.	Cattle wastewater, Kayole
S23	*Vibrio* sp.	Cattle sludge, Kayole
S24	*Vibrio* sp.	Cattle sludge, Kayole
S25	*Vibrio* sp.	Goat wastewaters, Kiamaiko
S26	*Vibrio* sp.	Goat wastewaters, Kiamaiko
S27	*Vibrio* sp.	Sheep wastewater, Kiamaiko
S28	*Vibrio* sp.	Sheep wastewater, Kiamaiko
S29	*Vibrio* sp.	Goat and sheep sludge, Kiamaiko
S30	*Vibrio* sp.	Goat and sheep sludge, Kiamaiko
S31	*Salmonella* sp.	Cattle wastewater, Kayole
S32	*Salmonella* sp.	Cattle wastewater, Kayole
S33	*Salmonella* sp.	Cattle sludge, Kayole
S34	*Salmonella* sp.	Cattle sludge, Kayole
S35	*Salmonella* sp.	Goat wastewaters, Kiamaiko
S36	*Salmonella* sp.	Goat wastewaters, Kiamaiko
S37	*Salmonella* sp.	Sheep wastewater, Kiamaiko
S38	*Salmonella* sp.	Sheep wastewater, Kiamaiko
S39	*Salmonella* sp.	Goat and sheep sludge, Kiamaiko
S40	*Salmonella* sp.	Goat and sheep sludge, Kiamaiko

*Isolates were identified by their morphology and biochemical properties.

4.2 Sensitivity to antimicrobial agents

4.2.1 Antibiotic resistance pattern

High percentages of bacterial isolates were resistant to lincomycin (90.0%), ampicillin (80.0%) and methicillin (72.5%) (Table 4.4). Some isolates were sensitive to gentamicin (72.5%) and chloramphenicol (75.0%). Bacterial isolates were most sensitive to chloramphenicol (75.0%), gentamicin (72.5%) and minocycline (60%). Lincomycin (5.0%), ampicillin (12.5%), tetracycline (22.5%) and sulfamethoxazole (25%) showed the lowest sensitivity. The overall mean intermediary sensitive case (8.1%) was significantly lower ($p=0.00$) compared to the mean sensitive (41.3%) and mean resistant (50.6 %) cases at $p<0.05$.

Table 4.4: Sensitivity in percentage of bacterial isolates to 13 types of antibiotics

Antibiotic (ng)	Resistance (%)	Intermediary sensitive (%)	Sensitive (%)
Ampicillin (25)	80.0	7.5	12.5
Chloramphenicol (30)	22.5	2.5	75.0
Cotrimoxazole (25)	30.0	2.5	67.5
Erythromycin (15)	50.0	7.5	42.5
Gentamicin (10)	27.5	0.0	72.5
Kanamycin (30)	37.5	17.5	45.0
Lincomycin (2)	90.0	5.0	5.0
Methicillin	72.5	7.5	20.0
Minocycline (30)	32.5	7.5	60.0
Penicillin G (1 unit)	50.0	7.5	42.5
Streptomycin (10)	32.5	20.0	47.5
Sulfamethoxazole (200)	62.5	12.5	25.0
Tetracycline (25)	70.0	7.5	22.5
[y]Mean	50.6±5.6[a]	8.1±5.6[b]	41.3±23.1[a]

[y]Means having the same letters within the row are not significantly different according to Tukey's HSD at 5 % level.

All bacterial isolates of fecal coliform origin (100%) were resistant to lincomycin and ampicillin, whereas only 30% were resistant to cotrimoxazole and minocycline (Table

4.5). 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%). 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 methicillin (80%), tetracycline and penicillin (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%. Gentamicin and chloramphenical were found to be the most effective against both *Vibrio* and FS.

Table 4.5: Sensitivity (%) of fecal coliforms, fecal streptococci, *Salmonella* and *Vibrio* species against 13 selected antibiotics

Antibiotic	Fecal coliforms (n = 10)			*Salmonella* spp. (n = 10)			Fecal streptococci (n = 10)			*Vibrio* spp. (n = 10)		
	S	IR	R	S	IR	R	S	IR	R	S	IR	R
Ampicillin	0	0	100	0	20	60	10	0	90	20	10	70
Chloramphenicol	50	0	50	80	10	10	90	0	10	80	0	20
Cotrimoxazole	70	0	30	80	0	20	70	0	30	50	10	40
Erythromycin	50	0	50	30	0	70	40	10	50	60	10	30
Gentamicin	50	0	50	80	0	20	90	0	10	70	0	30
Kanamycin	50	10	40	30	10	60	40	20	40	60	40	10
Lincomycin	0	0	100	10	0	90	10	10	80	0	10	90
Methicillin	20	0	80	20	0	80	20	0	80	20	30	50
Minocycline	60	10	30	50	10	40	60	10	30	60	10	30
Penicillin G	30	0	70	50	20	30	30	0	70	60	10	30
Streptomycin	50	10	40	60	10	30	30	20	50	50	40	10
Sulfamethoxazole	20	10	70	30	0	70	40	20	40	10	20	70
Tetracycline	20	0	80	10	20	70	30	0	70	30	10	60

S, Sensitive; IR, Intermediary resistant; R, Resistant.

With regard to cell wall morphology, Gram negative bacteria (Fecal coliforms, *Vibrio* and *Salmonella* species) appeared to be more resistant to all antibiotics compared to the Gram positive bacteria (FS) in this study (Fig. 4.1). Fecal coliforms appeared to have the highest resistance with a mean resistance frequency of 60.8%, followed by *Salmonella* species at 51.5%. *Vibrio* species showed the lowest mean resistance frequency at 41.6%. 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%, followed by FS at 42.3%. 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 *Vibrios* and FC

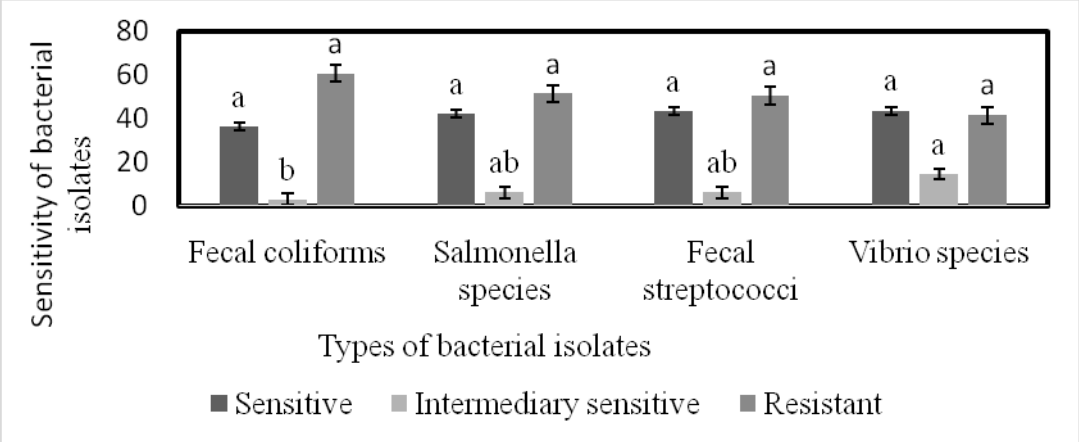

Figure 4.1: Antibiotic sensitivity pattern of bacterial isolates. Same letters on the bars indicates no significant difference of the means according to Tukey's HSD at 5% level.

When the sources of the isolates were considered (Table 4.6), a high percentage of cattle wastewater isolates showed resistance to ampicillin (100%), lincomycin and tetracycline (87.5%) and methicillin (75%). Most cattle sludge isolates also showed resistance to

lincomycin and ampicillin (100%) and penicillin (75%), and only 12.5% were resistant to chloramphenicol. Bacterial isolates from goat and sheep sludge mostly showed resistance to lincomycin (100 %), ampicillin (87.5%), and none of the isolates was resistant to chloramphenicol. A high percentage of the sheep wastewater isolates were resistant to lincomycin (87.5%) but none of the isolates was resistant to cotrimoxazole. Goat wastewater isolates were mostly resistant to methicillin (100%), lincomycin and tetracycline (75%) while only 12.5% were resistant to kanamycin and gentamicin. There was no significant difference (p=0.971) in resistance to antibiotics of bacterial isolates collected across the wastewaters and sludge samples.

Table 4.6: Resistance of bacterial isolates collected from wastewaters and sludge

Antibiotic (ng)	Percentage of AR among isolates in wastewaters and sludge				
	1	2	3	4	5
Ampicillin (25)	100.0	100.0	62.5	50.0	87.5
Chloramphenicol (30)	50.0	12.5	25.0	25.0	0.0
Cotrimoxazole (25)	37.5	37.5	37.5	0.0	37.5
Erythromycin (15)	50.0	50.0	50.0	50.0	50.0
Gentamicin (10)	12.5	25.0	12.5	37.5	50.0
Kanamycin (30)	50.0	25.0	12.5	62.5	37.5
Lincomycin (2)	87.5	100.0	75.0	87.5	100.0
Methicillin	75.0	62.5	100.0	62.5	62.5
Minocycline (30)	0.0	37.5	50.0	62.5	12.5
Penicillin G (1 unit)	62.5	75.0	37.5	37.5	37.5
Streptomycin (10)	25.0	37.5	50.0	25.0	25.0
Sulfamethoxazole (200)	62.5	62.5	62.5	62.5	62.5
Tetracycline (25)	87.5	62,5	75.0	62.5	62.5
[y]Mean	53.8[a]	53.4[a]	50.3[a]	48.1[a]	48.1[a]

[y]Means followed by similar letters along the row show no significant difference according to Tukey`s HSD at 5 % level. AR, antibiotic resistance; 1, cattle wastewater; 2, cattle sludge; 3, goat wastewater; 4, sheep wastewater and 5, a mixture of goat and sheep sludge.

4.2.2 Multiple antibiotic resistance of bacterial isolates

All the studied bacterial isolates exhibited resistance to more than 3 antibiotics. However, the patterns of resistance among the 40 isolates varied (Table 4.7). Of all studied bacterial isolates, 52.5% (21) showed 6 multiple antibiotic resistance (MAR) patterns (i.e. resistance to 6 of the 13 antibiotics tested). Only a small percentage of the isolates (2.5%) showed a 4 MAR pattern. None of the studied bacteria showed a 0 - 3 and 9 - 13 MAR pattern.

Table 4.7: Patterns of resistance of 40 wastewaters and sludge bacterial isolates to 13 antibiotics

No. of resisted antibiotics	Types of resisted antibiotics	No. (%) isolates
8	Tet, sulf, pen, ery, kan, gen,cot, chlo	1 (2.5)
7	Linc, amp, met, tet, ery, sulf, mino	8 (20)
	Linc, amp, met, tet, ery, pen, gen	9 (22.5)
6	Linc, amp, kan, met, sulf, pen	10 (25)
	Linc, amp, kan, tet, strep, cot	3 (7.5)
	Linc, sulf, strep, cot, mino, chlo,	6 (15.0)
	Amp, met, ery, chlo, strep, cot	2 (5)
4	Tet, kan, gen, strep	1 (2.5)

Amp, ampicillin; Linc, lincomycin; Pen, penicillin; Meth, methicillin; Ery, erythromycin; Tet, tetracycline; Cot, cotrimoxazole; Strep, streptomycin; Kan, kanamycin; Gen, gentamicin; Sulf, sulfamethoxazole; Chlo, chloramphenicol and Mino, minocycline.

All MAR index values were found to be more than 0.20, where the highest MAR index was exhibited by FC (0.40), followed by *Salmonella* sp. (0.35), Fecal streptococci (0.3) and *Vibrio* sp. (0.225) (Table 4.8).

Table 4.8: Multiple antibiotic resistance (MAR) value of bacterial isolates from wastewater slaughterhouses.

Bacterial isolate	MAR value
Fecal coliform	0.400
Salmonella species	0.350
Fecal streptococci	0.300
Vibrio species	0.225

4.2.3 Zones of inhibition

The zones of inhibition of bacterial isolates to antibiotics varied. In nutrient agar plates impregnated with lincomycin and ampicillin the diameters of the zones of inhibitions were consistently small compared to those impregnated with chloramphenicol. In some cases lincomycin and ampicillin showed complete inhibition. The diameters of the zones of inhibition of the antibiotics against the bacterial isolates are presented in appendix 2 and Plates 4.21.

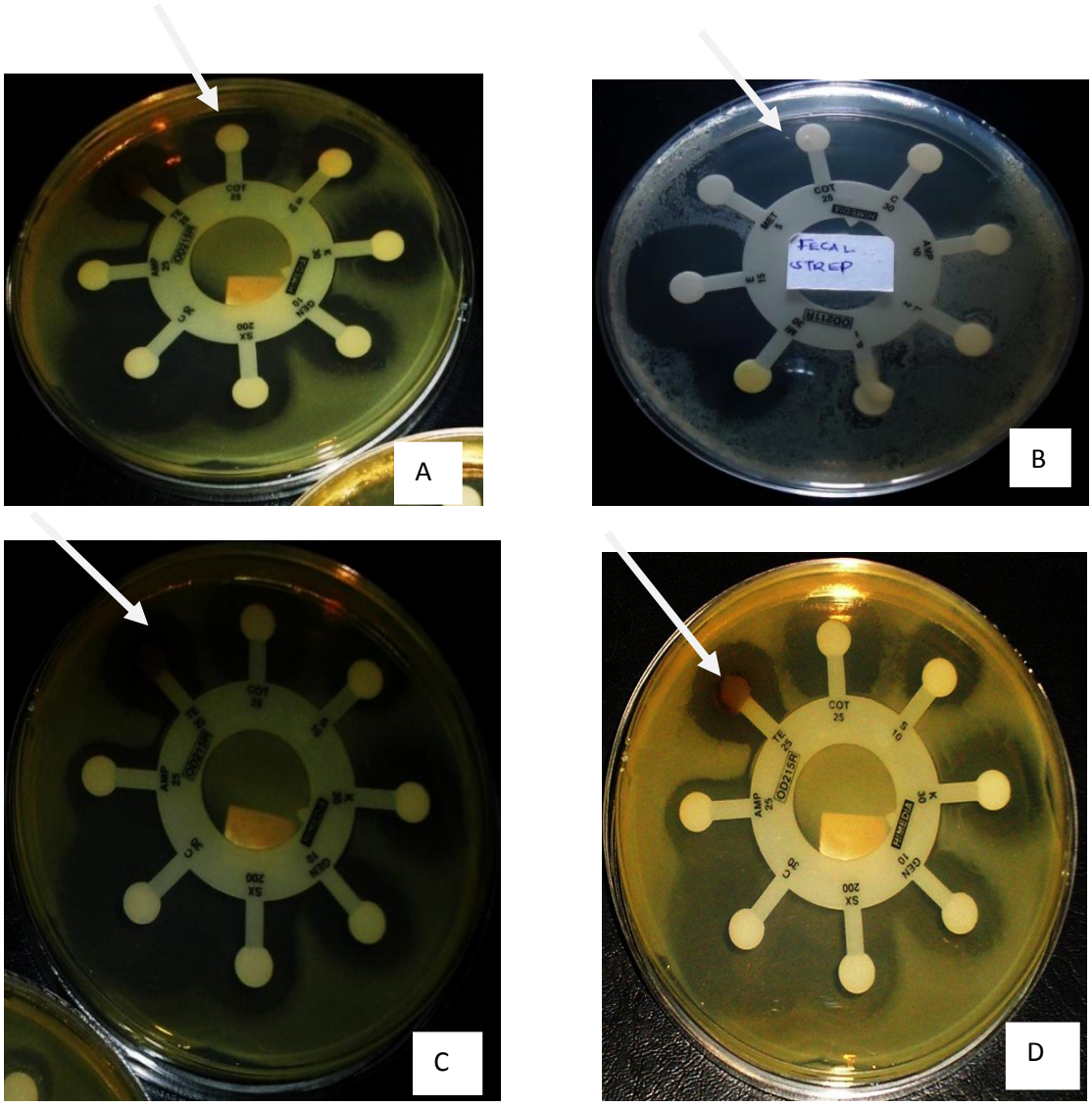

Plate 4.21: Zones of inhibition of various antibiotics against bacterial isolates A, FC; B, FS; C, *salmonella* sp. and D, *Vibrio* sp. Arrows show zones of inhibitions.

4.3 Tolerance of the bacterial isolates to heavy metals

The heavy metal tolerance of isolated bacteria was heterogeneous The tests carried out in liquid media were active at much lower concentrations compared to those in solid media

4.3.1 Tolerance of bacterial isolates in solid media

Twenty seven out of the 40 bacterial isolates obtained from the raw animal wastewaters and sludge showed resistant to multiple metal ions, however the patterns of resistance among them varied (Table 4.9). Out of the 27 resistant isolates, 5 (18.5%) showed resistance to 5 different metal ions while only 1 (3.7%) showed resistance to two different metal ions.

Table 4.9: Patterns of resistance of 27 wastewaters and sludge bacterial isolates to 6 heavy metals

No. of resisted heavy metals	Patterns of resistance	No. (%) of isolates
5	Hg, Cu, Zn, Pb, Ni	2 (7.4)
	Co, Cu, Zn, Pb, Ni	3 (11.1)
4	Co, Cu, Pb, Ni	6 (22.2)
	Zn, Cu, Pb, Ni	2 (7.4)
	Hg, Zn, Pb, Ni	3 (11.1)
	Cu, Co, Zn, Pb	1(3.7)
3	Hg, Pb, Cu	1(3.7)
	Zn, Cu, Ni	4 (14.8)
	Hg, Zn, Ni	2 (7.4)
	Pb, Zn, Ni	2 (7.4)
2	Hg, Pb	1 (3.7)

Overall, the order of toxicity of the metals was found to be Hg (more toxic) > Co > Cu > Zn > Pb > Ni (less toxic) (Figure 4.2). Resistance of the 27 bacterial isolates for each HM

was as follows; Hg 9 (33.3%), Co 11 (40.7%), Cu 18 (66.7%), Zn 19 (70.4%), Pb 21 (77.8%) and Ni 24 (88.9%). The toxic effect of the metals increased with increasing concentration. Nickel and mercury were the most tolerated and the most toxic metals respectively while zinc, lead and copper showed minimal resistance.

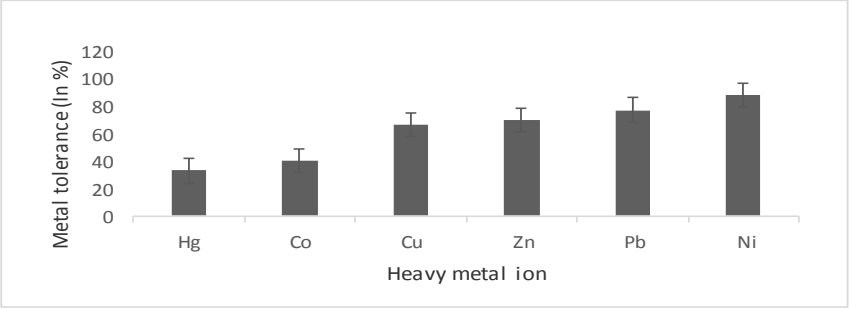

Figure 4.2: Tolerance of fecal coliforms, fecal streptococci, *Vibrio* and *Salmonella* species to 6 different heavy metal ion.

Growths of the bacterial isolates in nutrient agar plates supplemented with different concentrations of the various metals are shown in Plate 4.22.

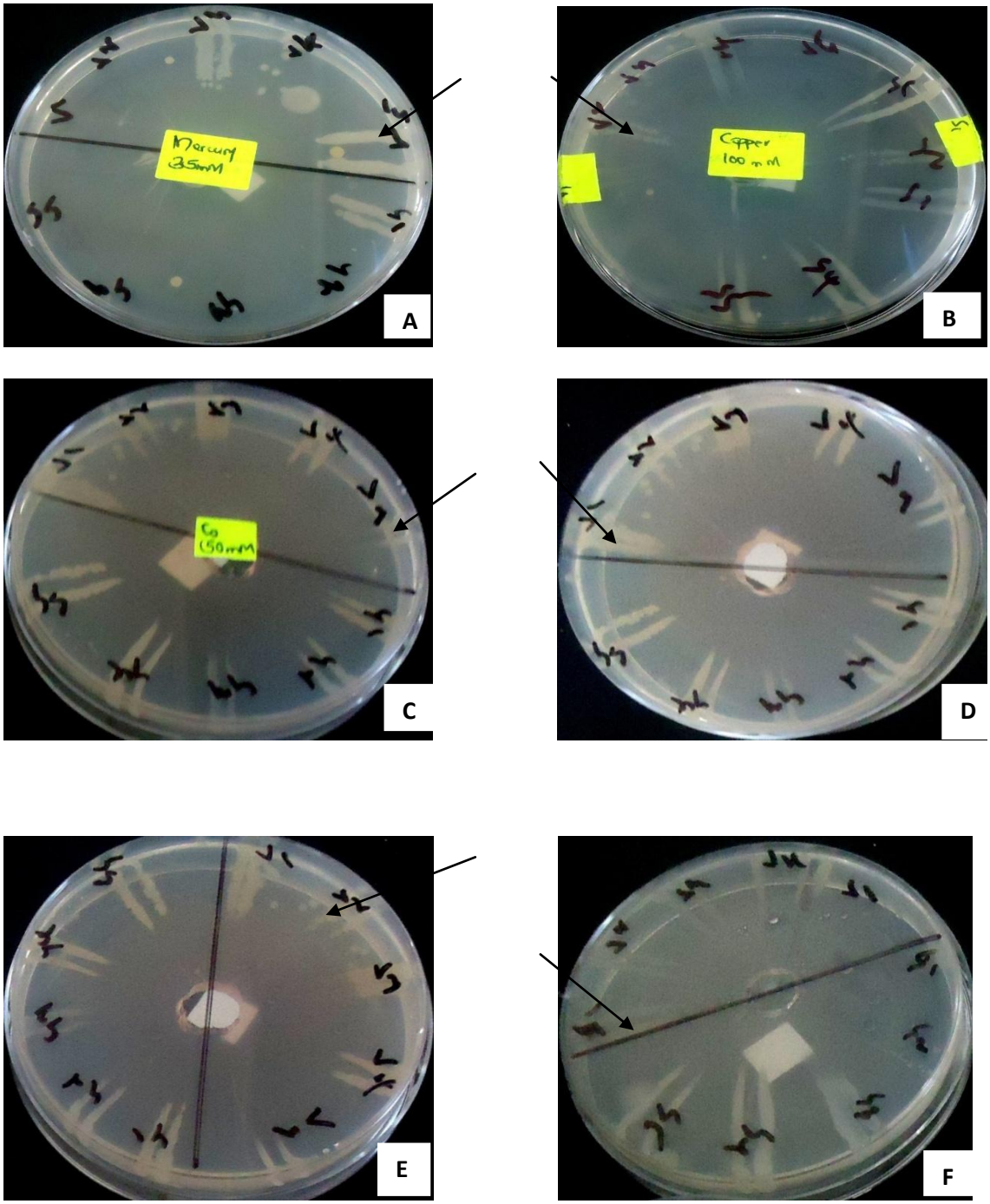

Plate 4.22: Bacterial isolates (A and B, FC; C, FS; D, *Vibrio* sp.; E and F, *Salmonella* sp.) on nutrient agar supplemented with heavy metal ions, A, mercury, B, copper, C, cobalt, D, zinc, E, lead and F, nickel. Arrows show growth of the bacterial isolates.

4.3.2 Tolerance of bacterial isolates in liquid media

All results obtained from experiments in liquid culture were expressed in minimal inhibitory concentration (MIC) (Table 4.10). All bacterial strains studied tolerated between 0.5-10 mM of Cu. The most tolerant species were essentially fecal coliform (S1, S7 and S8) and *Vibrio* (S22) strain with a MIC of 10. Tolerance was often below 5.0 mM of Co, however, some strains (17.5 %) were inhibited at up to 10 mM concentration of Co. Lead appeared less toxic, except for some bacterial strains that were found to be inhibited at lower concentration of 2.5 mM, such as fecal streptococci (S11 and S12), *Salmonella* (S39 and S40) and *Vibrio* species (S22). Most tolerance was observed at 2.5 mM and 5.0 mM concentration of Zn. Some isolates (30 %) were inhibited at higher concentration of 10 mM of zinc. Nickel appeared to be the most tolerated with quite a number of isolates inhibited at higher concentration of 10 and 15 mM. Mercury appeared as the most toxic of all metals tested (average MIC 0.05 mM of Hg). Its action affected simultaneously Gram-positive and Gram-negative bacteria.

Table 4.10: Minimal Inhibitory Concentrations (MICs) of metal ions tested against bacterial isolates from wastewaters and sludge

Strain no.	Identification	MIC (mM/liter)					
		Cu	Co	Pb	Zn	Hg	Ni
S1	Fecal coliform	10.0	1.0	10.0	2.5	0.001	2.5
S2	Fecal coliform	2.5	10.0	15.0	5.0	0.005	10.0
S3	Fecal coliform	1.0	10.0	15.0	5.0	0.1	10.0
S4	Fecal coliform	2.5	2.5	15.0	2.5	0.05	2.5
S5	Fecal coliform	2.5	1.0	10.0	2.5	0.005	15.0
S6	Fecal coliform	0.5	1.0	10.0	2.5	1.0	15.0
S7	Fecal coliform	10.0	2.5	5.0	2.5	0.005	0.5
S8	Fecal coliform	10.0	2.5	15.0	5.0	0.5	5.0
S9	Fecal coliform	1.0	1.0	5.0	10.0	1.0	5.0
S10	Fecal coliform	1.5	2.5	15.0	5.0	0.05	10.0
S11	Fecal streptococci	1.0	5.0	2.5	10.0	0.05	5.0
S12	Fecal streptococci	1.0	5.0	2.5	10.0	0.05	10.0
S13	Fecal streptococci	2.5	10.0	15.0	5.0	0.01	15.0
S14	Fecal streptococci	1.5	5.0	5.0	10.0	0.05	15.0
S15	Fecal streptococci	1.0	10.0	15.0	2.5	0.1	10.0
S16	Fecal streptococci	0.5	2.5	15.0	5.0	0.1	15.0
S17	Fecal streptococci	0.5	1.0	10.0	5.0	0.05	15.0
S18	Fecal streptococci	2.5	1.0	10.0	5.0	0.05	10.0
S19/20	Fecal streptococci	2.5	1.0	15.0	2.5	0.5	10.0
S21	*Vibrio* sp.	1.0	10.0	5.0	5.0	0.005	15.0
S22	*Vibrio* sp.	10.0	2.5	2.5	5.0	0.005	2.5
S23	*Vibrio* sp.	2.5	1.0	5.0	10.0	0.1	15.0
S24	*Vibrio* sp.	2.5	1.0	5.0	10.0	0.1	15.0
S25	*Vibrio* sp.	1.0	2.5	15.0	2.5	0.01	15.0
S26	*Vibrio* sp.	1.0	2.5	10.0	2.5	0.005	2.5
S27	*Vibrio* sp.	1.0	2.5	10.0	2.5	0.005	2.5
S28	*Vibrio* sp.	5.0	5.0	10.0	2.5	0.1	15.0
S29	*Vibrio* sp.	5.0	15.0	10.0	10.0	1.0	5.0
S30	*Vibrio* sp.	5.0	2.5	5.0	2.5	0.05	10.0
S31	*Salmonella* sp.	1.0	2.5	15.0	5.0	0.05	15.0
S32	*Salmonella* sp.	1.0	5.0	15.0	10.0	1.0	10.0
S33	*Salmonella* sp.	2.5	1.0	10.0	10.0	1.0	10.0
S34	*Salmonella* sp.	1.0	2.5	10.0	10.0	0.005	15.0
S35	*Salmonella* sp.	2.5	10.0	10.0	5.0	0.05	15.0
S36	*Salmonella* sp.	2.5	10.0	10.0	5.0	0.05	15.0
S37	*Salmonella* sp.	1.5	1.0	10.0	2.5	0.05	2.5
S38	*Salmonella* sp.	1.0	1.0	5.0	2.5	0.05	15.0
S39	*Salmonella* sp.	1.0	5.0	2.5	10.0	0.05	15.0
S40	*Salmonella* sp.	1.5	2.5	2.5	10.0	0.05	15.0

4.3.3 Interaction of metal resistance and antibiotic resistance

In this study, no particular metal resistance pattern was predictive of a particular pattern of antibiotic resistance. However, all the metal resistant isolates also showed resistant to different and multiple number of antibiotics (Table 4.11). With all six of the metals tested, there was a tendency towards a high frequency of lincomycin (77.8%), tetracycline (70.4%) and ampicillin (66.7%) resistance among all the isolates. Correlation analysis showed a positive relationship in multiple antibiotic resistance and multiple metal resistance ($r = 0.81$) among the 27 metal resistant isolates. Among the bacterial isolates, multiple metal tolerance was shown in 29.6% (8) of fecal coliforms and fecal streptococci, 18.5% (5) of *Vibrio* species and 22.2% (6) of *Salmonella* species. Equal number of the heavy metal resistant bacteria isolates, 6 (22.2%), had been isolated from a mixture of goat and sheep sludge, cattle, goat and sheep wastewater samples while only 3 (11.1%) were isolated from the cattle sludge samples.

Table 4.11: Heavy metal resistance and associated antibiotic resistance pattern of FC, FS, *Salmonella* and *Vibrio* isolated from wastewaters and sludge

Strain no.	Identification	HM resistance pattern	AR pattern
S1	Fecal coliform	Hg, Cu, Zn, Pb, Ni	Tet, sulf, pen, ery, kan, gen,cot, chlo
S2	Fecal coliform	Cu, Pb, Ni, Co	Linc, sulf, strep, cot, mino, chlo
S5	Fecal coliform	Hg, Pb, Cu	Linc, amp, met, tet, sulf, ery, mino
S6	Fecal coliform	Cu, Zn, Ni	Tet, kan, gen, strep
S7	Fecal coliform	Zn, Cu, Pb, Ni	Linc, amp, met, tet, sulf, ery, mino
S8	Fecal coliform	Cu, Pb, Ni, Co	Linc, amp, met, tet, sulf, ery, mino
S9	Fecal coliform	Hg, Zn, Ni	Linc, amp, met, tet, ery, pen, gen
S10	Fecal coliform	Cu, Zn, Ni	Linc, sulf, strep, cot, mino, chlo
S11	Fecal streptococci	Cu, Zn, Pb, Co	Linc, amp, met, tet, ery, pen, gen
S12	Fecal streptococci	Zn, Cu, Ni	Linc, amp, met, sulf, pen, kan
S13	Fecal streptococci	Hg, Cu, Pb, Zn, Ni	Tet, sulf, pen, ery, kan, gen,cot, chlo
S15	Fecal streptococci	Hg, Zn, Ni	Tet, sulf, pen, ery, kan, gen,cot, chlo
S16	Fecal streptococci	Hg, Zn, Ni, Pb	Tet, sulf, pen, ery, kan, gen,cot, chlo
S18	Fecal streptococci	Cu, Zn, Ni	Linc, amp, tet, kan, strep, cot
S19	Fecal streptococci	Cu, Co, Pb, Ni	Linc, sulf, strep, cot, mino, chlo
S20	Fecal streptococci	Cu, Co, Pb, Ni	Linc, sulf, strep, cot, mino, chlo
S21	*Vibrio spp*	Hg, Ni, Pb, Zn	Linc, amp, met, tet, ery, pen, gen
S25	*Vibrio spp*	Zn, Ni, Pb	Linc, amp, met, tet, ery, pen, gen
S27	*Vibrio spp*	Cu, Ni, Co, Pb, Zn	Linc, amp, met, tet, ery, pen, gen
S28	*Vibrio spp*	Hg, Pb	Linc, amp, met, tet, sulf, ery, mino
S30	*Vibrio spp*	Zn, Ni, Pb	Amp, met, ery, chlo, strep, cot
S31	*Salmonella spp*	Co, Cu, Ni, Pb	Linc, amp, met, sulf, pen, kan
S33	*Salmonella spp*	Co, Pb, Zn, Ni, Cu	Linc, amp, met, tet, sulf, ery, mino
S34	*Salmonella spp*	Hg, Ni, Zn, Pb	Linc, amp, tet, kan, strep, cot
S35	*Salmonella spp*	Cu, Pb, Ni, Zn	Linc, amp, met, tet, ery, pen, gen
S37	*Salmonella spp*	Cu, Co, Pb, Ni	Linc, amp, met, sulf, pen, kan
S40	*Salmonella spp*	Cu, Co, Pb, Ni, Zn	Linc, amp, tet, kan, strep, cot

Amp, ampicillin; Linc, lincomycin; Pen, penicillin; Meth, methicillin; Ery, erythromycin; Tet, tetracycline; Cot, cotrimoxazole; Strep, streptomycin; Kan, kanamycin; Gen, gentamicin; Sulf, sulfamethoxazole; Chlo, chloramphenicol and Mino, minocycline; AR, antibiotic resistance; HM, Heavy Metal

4.4 Plasmid profile patterns

Among the 40 bacterial strains obtained from the cattle, goat and sheep wastewaters and sludge, only 18 (45%) harbored plasmid DNA bands which ranged in size from 4kb to 10kb (Appendix 3, Table 4.12). In this study 6 different plasmid profiles were detected

and designated 1-6. The distribution of the profiles among the 18 plasmids containing bacterial isolates was as follows, profile 1 in 27.8 % isolates, profile 2, 3 and 6 in 5.6 % of the isolates (for each profile), profile 4 in 33.3 %, profile 5 in 22.2 % isolates. Up to 89.5 % of plasmid containing isolates were resistant to at least 6 different antibiotics. Most (68.4 %) of the plasmid containing isolates were also resistant to multiple metal ions. No particular plasmid profile was predictive of a particular pattern of antibiotic and metal resistance.

Table 4.12: Antibiogram, heavy metal resistance and plasmid pattern of FC, FS, *Salmonella* and *Vibrio* isolated from wastewaters and sludge

Strain no.	Heavy metal resistance pattern	Antibiotic resistance pattern	Plasmid (Profile)
S1	Hg, Cu, Zn, Pb, Ni	Tet, sulf, pen, ery, kan, gen,cot, chlo	None detected
S2	Cu, Pb, Ni, Co	Linc, sulf, strep, cot, mino, chlo	None detected
S3	-	Linc, amp, met, sulf, pen, kan	6 kb (1)
S4	-	Linc, amp, met, sulf, pen, kan	None detected
S5	Hg, Pb, Cu	Linc, amp, met, tet, sulf, ery, mino	None detected
S6	Cu, Zn, Ni	Tet, kan, gen, strep	8 kb, 6 kb (2)
S7	Zn, Cu, Pb, Ni	Linc, amp, met, tet, sulf, ery, mino	6 kb (1)
S8	Cu, Pb, Ni, Co	Linc, amp, met, tet, sulf, ery, mino	8 kb, 10 kb (3)
S9	Hg, Zn, Ni	Linc, amp, met, tet, ery, pen, gen	None detected
S10	Cu, Zn, Ni	Linc, sulf, strep, cot, mino, chlo	10 kb (4)
S11	Cu, Zn, Pb, Co	Linc, amp, met, tet, ery, pen, gen	4 kb (5)
S12	Zn, Cu, Ni	Linc, amp, met, sulf, pen, kan	4 kb (5)
S13	Hg, Cu, Pb, Zn, Ni	Tet, sulf, pen, ery, kan, gen,cot, chlo	None detected
S14	-	Linc, amp, met, tet, ery, pen, gen	None detected
S15	Hg, Zn, Ni	Tet, sulf, pen, ery, kan, gen,cot, chlo	None detected
S16	Hg, Zn, Ni, Pb	Tet, sulf, pen, ery, kan, gen,cot, chlo	10 kb (4)
S17	-	Linc, sulf, strep, cot, mino, chlo	6 kb (1)
S18	Cu, Zn, Ni	Linc, amp, tet, kan, strep, cot	10 kb (4)
S19	Cu, Co, Pb, Ni	Linc, sulf, strep, cot, mino, chlo	10 kb (4)
S20	Cu, Co, Pb, Ni	Linc, sulf, strep, cot, mino, chlo	None detected
S21	Hg, Ni, Pb, Zn	Linc, amp, met, tet, ery, pen, gen	6 kb (1)
S22	-	Linc, amp, met, tet, ery, pen, gen	None detected
S23	-	Linc, amp, met, sulf, pen, kan	None detected
S24	-	Linc, amp, met, sulf, pen, kan	6 kb (1)
S25	Zn, Ni, Pb	Linc, amp, met, tet, ery, pen, gen	10 kb (4)
S26	-	Linc, amp, met, sulf, pen, kan	None detected
S27	Cu, Ni, Co, Pb, Zn	Linc, amp, met, tet, ery, pen, gen	10 kb (4)
S28	Hg, Pb	Linc, amp, met, tet, sulf, ery, mino	8 kb (6)
S29	-	Linc, amp, met, sulf, pen, kan	10 kb (4)
S30	Zn, Ni, Pb	Amp, met, ery, chlo, strep, cot	None detected
S31	Co, Cu, Ni, Pb	Linc, amp, met, sulf, pen, kan	10 kb (4)
S32	-	Linc, sulf, strep, cot, mino, chlo	None detected
S33	Co, Pb, Zn, Ni, Cu	Linc, amp, met, tet, sulf, ery, mino	None detected
S34	Hg, Ni, Zn, Pb	Linc, amp, tet, kan, strep, cot	None detected
S35	Cu, Pb, Ni, Zn	Linc, amp, met, tet, ery, pen, gen	None detected
S36	-	Linc, amp, met, sulf, pen, kan	None detected
S37	Cu, Co, Pb, Ni	Linc, amp, met, sulf, pen, kan	None detected
S38	-	Amp, met, ery, chlo, strep, cot	None detected
S39	-	Linc, amp, met, tet, ery, pen, gen	None detected
S40	Cu, Co, Pb, Ni, Zn	Linc, amp, tet, kan, strep, cot	None detected

Among the bacterial isolates only the fecal coliforms showed more than one plasmid ranging in size from 5 kb to 10 kb (Figure 4.3). On the other hand, only one strain of *Salmonella* possessed a plasmid (Appendix III)

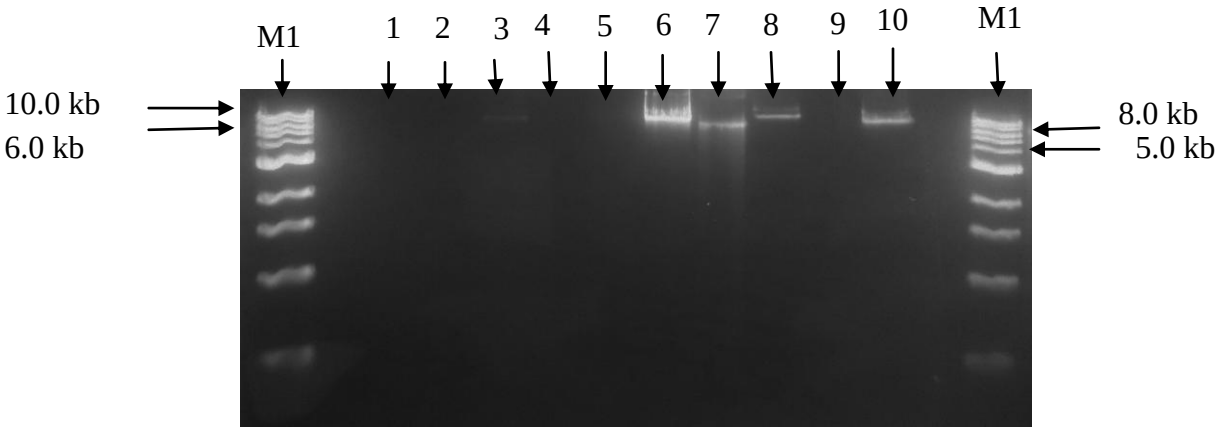

Figure 4.3: Plasmid profiles of fecal coliforms isolated from wastewaters and sludge. M1, DNA marker (1kb, GenStat); Lane 1 - 10, fecal coliforms species (S1 – S10).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

5.1.1 Bacterial population

A total of 40 bacterial isolates were identified by their morphology and biochemical properties. The five sources of bacterial contamination characterized in this study were investigated because they differ in terms of the origin of the bacterial isolates they release in the environment (cattle, goat and sheep) and in the expected exposure of these bacteria to antimicrobial selective pressure of the environment. 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 (Christina *et al*., 2012).

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* species., Aeromonas and protozoa such as *Giardia* species 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 (KF) streptococcus agar was used to enumerate FS. 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.

5.1.2 Antimicrobial resistance patterns

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 could not effective to control these bacteria since high percentages of the isolates (80%, 90% and 72.5%, respectively) were resistant to the antibiotics and only a small percentage were sensitive to them (12.5%, 5% and 20%, respectively). Bacterial resistance to some of the antibiotics resisted in this study such as erythromycin, streptomycin, penicillin, ampicillin, kanamycin, tetracycline and chloramphenicol has been reported by Roberts (2011). Nipa *et al*. (2011) observed 98% multiple resistances to 2 – 7 different antibiotics with 96.07% of the bacterial isolates resisting ampicillin in the same study.

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 resistance with a mean resistance frequency of 60.8 %, followed by *Salmonella* species at 51.5 %. *Vibrio* species showed the lowest mean resistance frequency at 41.6 %. Some *Escherichia* species and *Salmonella* species possess capsular K and Vi antigens which protect them from access by antimicrobials.

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 antibiotic contaminated animal feeds may have initiated the resistance as previously suggested (Le *et al.,* 2005). High resistance may also be attributed to the history, frequency and dose of antibiotic utilization (Aminov and Mackie, 2007). Plasmids encoding many antibiotic resistance genes are transferred between pathogenic and non-pathogenic Gram negative bacteria in several environments (Yu *et al.,* 2011). Essentially 75 % of bacterial isolates were Gram negative and they showed significantly stronger resistance to tested antibiotics compared to the Gram positive bacteria, which implied that pathogenic bacteria had many

opportunities to become resistant to antibiotics making it more difficult to prevent bacterial diseases by the use of antibiotics (Tamminem *et al.*, 2011).

5.1.2.1 Multiple antimicrobial resistances

MAR index value beyond 0.20 in every species of bacteria in the present study indicated that there has been high risk in exposure to antibiotics such as lincomycin, amoxicillin, penicillin, methicillin and tetracycline. Bacterial isolates resisted more than 3 antibiotics with one bacterial isolate resisting up to eight out of the 13 antibiotics used. Public health risk could be stressed by the occurrence of a high frequency of strains that are typically resistant to multiple antibiotics. The presence of antimicrobial agents at low concentration through leaching or continued use may lead to the development of drug-resistant isolates and multiple antibiotic resistance (MAR) in bacteria, which may result in resistance transfer to pathogenic bacteria and reduced efficacy of antibiotic treatment for human and animal diseases (Tendencia and De la Pena, 2002; Keiichi *et al*., 2012).

A number of studies have shown that the incidence of antibiotic resistance is often reduced when the use of antibiotics is restricted (Miranda and Zemelman, 2002; Vivekanadhan *et al.*, 2002, Yu *et al*., 2011). Antibiotics which are commonly used in human and veterinary medicine as well as in agriculture lead to the selection of antibiotic resistant microorganisms (Monika *et al*., 2011). Resistance to one antibiotic may contribute to selection for resistance to antibiotics of other groups. High multi-resistance incidence, similar to or higher than those found in this study, has been reported in aquatic environments (Matyar *et al*., 2007). According to Meirelles–Pereira *et al*. (2002), multiple

resistance may be coded on plasmids, mutational events or on even smaller and mobile genetic elements called transposons.

5.1.3 Tolerance of bacterial isolates to heavy metals

This study highlights the prevalent occurrence of metal resistant microbial organisms in the animal wastewaters and sludge. The microbial growth decreased with the increase in concentration of heavy metals indicating toxic effect of the heavy metals on the growth of microorganisms. The heavy metal tolerance of isolated bacteria was heterogeneous. This difference could be due to the difference in isolation conditions of the bacterial isolates and the selectivity of microbial culture techniques employed especially with regard to the nature and specificity of growth media. The tests carried out in liquid media were active at much lower concentrations compared to those in solid media probably because in liquid media, the metal is in solution hence contact with microorganisms is efficient and it is easier for bacterial isolates to take up nutrients. Chari *et al*. (2011) reported an unprecedented high level resistance of several isolates isolated from aquatic environments to heavy metals such as cobalt, lead and mercury.

Irrespective of the origin of bacteria, Hg appeared to be the most toxic and hence could be expected to significantly impact on animal microbial flora. High mercury toxicity have also been reported in the study conducted by Kumar and Kayatsha (2009). Several bacterial species have been shown to develop resistance to mercury and other HM (Singh *et al*., 2008; Parisa *et al*., 2011). The higher Hg sensitivity could be as a result of the ions reaction with the thiol groups of cysteine residues of the enzymes leading to formation of mercaptides (Zaborska *et al.*, 2004). The presence of metallothionein-like proteins in the

system of bacteria that exhibit tolerance to HM such as Hg, Cd, Zn, Ni, and Co has been reported (Robinson *et al*., 2001). Microorganisms are also known to possess a high metal affinity and can accumulate heavy metals by various mechanisms (Rehman *et al*., 2008). In this study, the resistant profiles differed among animal species. For sheep bacterial species, the sensitivity trend was in the order: Hg > Co > Cu >Zn > Pb> Ni, similar to that of goat but somewhat different from that of cattle (i.e., Hg> Cu > Co > Zn > Ni > Pb). The difference in toxicity order could be due to several factors including bioavailability, chemical form, conditions of metabolic activity and more so bacterial species (Yue *et al.*, 2007).

Since HM are all similar in their toxic mechanism, multiple tolerance are common phenomena among HM resistant bacteria. In this study the 27 bacterial isolates showed multiple resistance to the studied metal ions. This report supports the idea that metal resistance could be interrelated (Amalesh *et al*., 2012). The patterns of resistance among the 27 cultures varied probably due to the difference in the concentration of the metal ions in the wastewaters and sludge. Among the 27 metal resistant bacterial isolates, multiple metal tolerance was shown in 29.6 % (8) of fecal coliforms and fecal streptococci, 18.5 % (5) of *Vibrio* species and 22.2 % (6) of *Salmonella* species. Metal resistance may be related to the products of capsular polysaccharides often by Enterobacter group of organisms which are able to combine with metals to protect themselves from metal toxicity (Adarsh *et al*., 2007). More often the resistance is plasmid –borne and transferrable in nature leading to its spread among the sensitive aquatic bacteria including coliforms. Equal number of the heavy metal resistant bacterial isolates, 6 (22.2 %), had been isolated from goat and sheep sludge, cattle, goat and sheep wastewater samples

while only 3 (11.1 %) were isolated from the cattle sludge samples. This indicates that there could be a build-up in all the three studied abattoirs leading to an existing pool of genes with HM resistance.

No particular metal resistance pattern was predictive of a particular pattern of antibiotic resistance. However, all the metal resistant isolates also showed resistant to different and multiple number of antibiotics. Furthermore, Correlation analysis showed a positive relationship in multiple antibiotic resistance and multiple metal resistance ($r = 0.808014$) among the 27 metal resistant isolates suggesting that HM contamination of these animal wastewaters could be inducing multidrug resistance as earlier suggested by Palm *et al*. (2008). Observations made with respect to metal-antibiotic-double resistance have been reported in a study by Berg *et al*. (2010), for instance, copper tolerant bacteria were more frequently resistant to antibiotics (ampicillin, sulfonamides and chloramphenicol) compared to copper sensitive strains. High incidence of metal-antibiotic–double tolerance for penicillin and copper, ampicillin and nickel, lead and many antibiotics including β-lactams was observed and has been reported by Christina *et al*. (2012). These results show that the combined expression of antibiotic and heavy metal resistance may not be a chance phenomenon but rather a result of selection by HM presence in an environment.

5.1.4 Plasmid profile analysis

The absence of plasmids in a majority (55%) of isolates indicated low typeability and discriminatory power of these isolates. Bacterial antibiotics resistance patterns is sometimes associated with the presence of plasmids and ability of plasmids for conjugation process. Generally, plasmids which can be trans-conjugated usually possess a

high molecular weight (Zulkifli *et al*., 2009), hence the presence of plasmids that may harbor the antibiotic resistance genes in these isolates may increase their capacity to threaten human consumers since foodborne strains carrying resistant genes qualified them as potential human pathogens. However, for other isolates that were plasmid-less (55 % of isolates), they also showed the multiple antibiotics and metal resistances pattern which indicates that resistance to most of these antibiotics and metal ions could be of chromosomal origin or on mobile genetic elements that may help in the disseminations of the resistant genes to other bacteria of human clinical significance. Up to 89.5 % of plasmid containing isolates were resistant to at least 6 different antibiotics indicating the importance of presence of plasmids in MAR isolates. The relationship between certain plasmids and resistance to some antibiotics has been reported previously (Nikbin *et al.*, 2007). In this study, the results could not correlate the antibiotic and metal resistance among the bacterial isolates with a specific plasmid detected because no genetic transfer study was performed.

5.2 Conclusion

- High level of microbial contamination of the raw animal wastewaters and sludge was revealed in this study. *Vibrio* and *Salmonella* species were more frequently detected in samples which also showed high incidence of fecal bacteria indicators.
- There is a likelihood of slaughterhouse animals getting exposed to antibiotics to which they develop resistance which they could spread to the environment. 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.

- The relative effectiveness of the HM ions as inhibitors of microbial population decreased in the order: Hg (most toxic) > Co > Cu> Zn> Pb> Ni (least toxic). The antibiotic-metal double resistance revealed in this study further indicates that metal resistance could be inducing the maintenance of antibiotic resistance genes probably by increasing the antimicrobial selective pressure of the environment.
- The absence of plasmids in a majority (55%) of isolates indicated low typeability and discriminatory power of these isolates.

5.3 Recommendations

- Further studies are needed to understand better the metal and antibiotic resistant bacteria in meat, aquatic environments and sludge used as manure from other animals. The geographic scope of those studies should include other parts of the country.
- Future studies should be done with PCR and DNA sequence analysis to reveal the DNA fingerprints of each strain to enhance the process of surveillance for these organisms in similar environment and the detection of new serotypes as well as assist in environment impact assessment (EIA) study for sustainable development.
- Prior treatment of raw animal wastewaters and sludge from slaughterhouses is necessary before the wastewaters are released into the environment or the sludge used as manure in farms.
- We need to be more careful of the drastic use of antibiotics in medical practice, and also more aware of other antimicrobials that we put into the environment such as heavy metals.

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APPENDICES

APPENDIX I

TABLES REPRESENTING DIAMETERS OF ZONES OF INHIBITION AGAINST BACTERIAL ISOLATES BY ANTIBIOTICS

a. Diameters of zones of inhibition against bacterial strains S1-S18

	Diameter of zones of inhibition (in mm)												
Strain	Amp	Linc	Pen	Meth	Ery	Tet	Cot	Strep	Kan	Gen	Sulf	Chloram	Mino
S1	0.0	9.0	15.0	0.0	5.0	0.0	0.0	5.0	8.0	17.0	0.0	25.0	8.0
S2	4.0	5.0	14.0	11.0	9.0	10.0	29.0	13.0	25.0	5.0	8.0	24.0	21.0
S3	7.0	0.0	20.0	0.0	25.0	9.0	30.0	9.0	15.0	18.0	11.0	20.0	9.0
S4	5.0	5.0	13.0	17.0	10.0	24.0	6.0	20.0	21.0	20.0	0.0	9.0	20.0
S5	3.0	6.0	12.0	5.0	17.0	13.0	36.0	21.0	10.0	0.0	20.0	23.0	22.0
S6	13.0	10.0	7.0	6.0	30.0	15.0	34.0	10.0	9.0	25.0	9.0	27.0	10.0
S7	10.0	11.0	15.0	7.0	12.0	12.0	4.0	19.0	20.0	26.0	23.0	30.0	25.0
S8	9.0	8.0	23.0	8.0	29.0	11.0	31.0	25.0	29.0	7.0	8.0	0.0	27.0
S9	9.0	0.0	32.0	5.0	13.0	21.0	32.0	11.0	16.0	19.0	12.0	35.0	15.0
S10	8.0	12.0	34.0	0.0	32.0	10.0	5.0	27.0	26.0	21.0	8.0	32.0	30.0
S11	9.0	16.0	29.0	0.0	25.0	9.0	33.0	29.0	12.0	9.0	7.0	29.0	13.0
S12	4.0	10.0	8.0	19.0	10.0	8.0	34.0	30.0	28.0	30.0	19.0	30.0	32.0
S13	5.0	10.0	30.0	5.0	27.0	23.0	7.0	8.0	10.0	32.0	5.0	26.0	12.0
S14	5.0	0.0	10.0	13.0	10.0	7.0	29.0	12.0	11.0	8.0	0.0	27.0	29.0
S15	0.0	0.0	32.0	7.0	27.0	0.0	28.0	35.0	21.0	34.0	13.0	29.0	11.0
S16	17.0	0.0	11.0	15.0	30.0	16.0	8.0	12.0	17.0	29.0	0.0	30.0	21.0
S17	0.0	9.0	31.0	9.0	0.0	5.0	27.0	36.0	10.0	28.0	18.0	32.0	10.0
S18	0.0	21.0	13.0	8.0	28.0	26.0	26.0	13.0	25.0	30.0	5.0	8.0	25.0

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.

b.Diameters of zones of inhibition against bacterial strains S19-S40

	Diameter of zones of inhibition (in mm)												
Strain	Amp	Linc	Pen	Meth	Ery	Tet	Cot	Strep	Kan	Gent	Sulf	Chloram	Mino
S19	4.0	8.0	35.0	7.0	9.0	10.0	12.0	28.0	9.0	6.0	25.0	28.0	11.0
S20	3.0	10.0	26.0	8.0	25.0	17.0	25.0	10.0	27.0	27.0	7.0	27.0	21.0
S21	20.0	0.0	14.0	18.0	8.0	9.0	9.0	29.0	14.0	25.0	14.0	29.0	22.0
S22	24.0	0.0	15.0	6.0	27.0	8.0	28.0	13.0	10.0	6.0	8.0	6.0	17.0
S23	25.0	5.0	36.0	7.0	34.0	7.0	9.0	30.0	30.0	26.0	30.0	32.0	27.0
S24	12.0	7.0	37.0	19.0	7.0	25.0	28.0	9.0	14.0	20.0	5.0	33.0	30.0
S23	0.0	10.0	34.0	0.0	6.0	9.0	8.0	32.0	11.0	25.0	29.0	7.0	9.0
S26	7.0	17.0	14.0	0.0	18.0	8.0	29.0	14.0	32.0	5.0	0.0	24.0	29.0
S27	0.0	13.0	33.0	21.0	35.0	7.0	30.0	33.0	15.0	26.0	0.0	20.0	28.0
S28	0.0	11.0	9.0	5.0	9.0	27.0	32.0	8.0	13.0	27.0	7.0	10.0	8.0
S29	0.0	10.0	32.0	7.0	10.0	0.0	35.0	34.0	33.0	0.0	8.0	19.0	26.0
S30	7.0	0.0	33.0	8.0	29.0	11.0	31.0	0.0	15.0	23.0	28.0	25.0	7.0
S31	7.0	23.0	11.0	25.0	12.0	25.0	27.0	20.0	27.0	24.0	0.0	22.0	25.0
S32	0.0	0.0	24.0	9.0	0.0	10.0	0.0	5.0	9.0	9.0	19.0	25.0	10.0
S33	6.0	9.0	35.0	8.0	27.0	9.0	29.0	25.0	35.0	27.0	6.0	30.0	27.0
S34	22.0	8.0	11.0	6.0	10.0	21.0	28.0	12.0	16.0	25.0	5.0	14.0	16.0
S35	0.0	9.0	10.0	10.0	9.0	8.0	7.0	26.0	8.0	26.0	15.0	27.0	21.0
S36	5.0	5.0	32.0	0.0	19.0	7.0	29.0	9.0	29.0	10.0	8.0	23.0	20.0
S37	20.0	4.0	9.0	7.0	8.0	23.0	29.0	24.0	15.0	28.0	8.0	11.0	11.0
S38	6.0	11.0	34.0	0.0	36.0	0.0	30.0	13.0	10.0	30.0	20.0	24.0	30.0
S39	0.0	0.0	8.0	21.0	7.0	10.0	9.0	23.0	30.0	32.0	0.0	25.0	32.0
S40	13.0	0.0	11.0	8.0	30.0	9.0	30.0	14.0	13.0	33.0	9.0	7.0	25.0

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.

APPENDIX II

TABLES REPRESENTING METAL TOLERANCE OF BACTERIAL ISOLATES TO DIFFERENT CONCENTRATIONS OF METALS

a. Mercury

Tolerance of bacterial isolates at different concentrations of mercury (in %)																				
Conc.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
5 mM	86.5	83.8	73.8	76.3	88.8	83.8	67.5	70.0	66.3	73.8	73.8	71.3	71.3	66.3	88.8	92.5	81.3	82.5	96.3	100.0
10 mM	62.5	65.0	51.3	53.8	65.0	63.8	52.5	56.3	60.0	58.8	60.0	57.5	51.3	48.8	66.3	63.8	56.3	60.0	65.0	65.0
25 mM	51.3	46.3	36.3	30.0	50.2	45.0	38.8	43.8	52.5	45.0	43.8	46.3	57.5	40.0	57.5	60.0	43.8	38.8	43.8	46.3
50 mM	38.8	42.5	27.5	23.8	22.5	26.3	13.8	18.8	15.0	20.0	26.3	25.0	30.0	25.0	37.5	42.5	27.5	23.8	33.8	35.0
100 mM	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.8	3.8	1.3	2.0	0.0	0.0	0.0	0.0
>200 mM	No growth																			
Control	100% growth																			
Conc.	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
5 mM	70.0	65.0	95.0	95.0	73.8	71.3	93.8	95.0	71.3	81.3	97.4	95.0	85.0	96.3	81.3	73.8	75.0	78.8	78.8	76.3
10 mM	63.8	48.8	66.3	63.8	57.5	57.5	62.5	66.3	58.8	53.8	68.8	68.8	72.5	67.5	61.3	65.0	57.5	53.8	63.8	58.8
25 mM	53.8	33.8	48.8	43.8	42.5	46.3	40.7	57.5	47.5	46.3	45.0	42.5	48.8	52.5	41.3	45.0	43.8	48.8	49.0	43.8
50 mM	21.3	17.5	36.3	31.3	26.3	27.5	32.5	32.5	26.3	26.3	33.8	31.3	35.0	36.3	26.3	21.3	21.3	23.8	27.5	25.0
100 mM	0.0	0.0	12.5	7.5	0.0	0.0	6.3	0.0	5.0	6.3	11.3	12.5	8.8	7.5	3.8	0.0	5.0	5.0	0.0	0.0
>200 mM	No growth																			
C	100% growth																			

S, bacterial strain; C, control; Conc, concentration of metals in millimoles.

b.Nickel

Tolerance of bacterial isolates at different concentrations of nickel (in %)																			
Conc. (mM)	S1	S2	S3	S4	S5	S6	S7	S8	S8	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
5	100% growth																			
10	100.0	97.5	98.8	96.3	96.3	93.8	95.0	100.0	100.0	96.3	96.3	98.8	95.0	97.5	97.5	97.5	100.0	98.8	100.0	97.5
25	68.8	65.0	48.8	49.0	49.8	63.8	67.5	61.3	73.8	47.8	49.8	70.0	63.8	46.3	52.5	66.3	48.5	45.0	67.5	72.5
50	40.0	35.0	37.5	36.3	36.3	31.3	32.5	35.0	40.0	32.5	36.3	43.8	31.3	33.8	41.3	38.8	31.3	32.5	38.8	32.5
100	20.0	15.0	18.8	13.8	20.0	18.8	12.5	8.8	13.8	11.3	16.3	15.0	13.8	15.0	16.3	17.5	11.3	8.8	15.0	13.0
200	7.5	2.5	1.3	0.0	7.5	1.3	0.0	0.0	0.0	0.0	1.3	0.0	0.0	2.5	0.0	5.0	0.0	0.0	0.0	5.0
>200	No growth																			
C	100% growth																			
Conc.	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
5	100% growth																			
10	98.8	96.3	98.7	100.0	97.5	100.0	92.5	96.3	98.8	93.8	96.3	97.5	100.0	97.5	98.8	91.3	96.3	93.8	95.0	97.5
25	62.5	43.8	47.5	45.0	62.5	46.3	63.8	50.0	43.8	62.5	61.3	47.5	58.8	67.5	62.5	47.5	52.5	43.0	46.3	61.3
50	40.0	33.8	38.8	30.0	30.0	31.3	47.5	36.3	41.3	37.5	42.5	36.3	33.8	25.0	30.0	36.3	32.5	35.0	32.5	31.3
100	12.5	12.5	16.3	18.8	15.0	10.0	13.8	13.8	12.5	12.5	16.3	15.0	10.0	15.0	11.3	12.5	13.8	11.3	12.5	11.3
200	0.0	0.0	3.8	1.3	0.0	0.0	0.0	1.3	0.0	2.5	0.0	0.0	0.0	2.5	0.0	0.0	0.0	2.5	0.0	2.5
>200	No growth																			
C	100% growth																			

S, bacterial strain; C, control; Conc, concentration of metals in millimoles.

c. Copper

Tolerance of bacterial isolates at different concentrations of copper (in %)																			
Conc.	S1	S2	S3	S4	S5	S6	S7	S8	S8	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
5 mM	90.0	90.0	100.0	98.8	88.8	86.3	100.0	100.0	85.0	90.0	100.0	100.0	90.0	100.0	93.8	100.0	100.0	100.0	98.8	100.0
10 mM	83.8	82.5	91.3	85.0	82.5	76.3	87.5	90.0	76.3	78.8	87.5	88.8	83.8	70.0	78.8	88.8	85.0	82.5	73.8	80.0
25 mM	56.3	55.0	45.0	40.0	48.8	50.8	65.0	52.5	43.8	60.0	59.0	41.3	53.8	41.3	42.5	48.8	45.0	56.3	61.3	58.8
50 mM	23.8	21.3	23.8	18.8	26.3	23.8	21.3	22.5	26.3	22.3	23.8	25.0	18.8	23.8	15.8	23.8	25.0	16.3	28.8	25.0
100 mM	15.0	8.8	11.3	10.0	11.3	3.8	6.3	2.5	7.5	5.0	11.3	12.5	2.3	8.8	3.8	10.0	8.8	1.3	11.3	7.5
200 mM	4.7	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3
>200	No growth																			
C	100% growth																			
Conc.	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
5 mM	92.5	91.3	95.0	100.0	91.3	90.0	100.0	100.0	95.0	98.8	100.0	98.8	98.8	98.5	97.5	90.0	97.5	100.0	100.0	100.0
10 mM	72.5	75.0	75.0	86.3	83.8	73.7	78.8	82.5	90.0	75.0	81.3	82.5	90.0	95.0	78.8	72.5	76.3	77.5	90.0	78.8
25 mM	40.0	46.3	46.3	45.0	40.0	38.8	50.0	48.8	48.8	43.8	57.5	41.3	51.3	46.3	52.5	45.0	50.0	41.3	43.8	56.3
50 mM	20.0	20.0	27.5	28.8	21.3	16.3	30.0	21.3	20.0	20.0	26.3	18.8	20.0	18.8	13.8	27.5	21.3	23.8	17.5	20.0
100 mM	3.8	6.3	11.3	8.8	2.5	1.3	12.5	8.8	6.3	8.8	11.3	7.5	8.8	3.8	2.5	11.3	6.3	11.3	3.8	2.5
200 mM	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	1.3	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
>200	No growth																			
C	100% growth																			

S, bacterial strain; C, control; Conc, concentration of metals in millimoles.

d. Zinc

Tolerance of bacterial isolates at different concentrations of zinc (in %)																				
Conc.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
5 mM	100.0	100.0	98.8	100.0	100.0	96.3	98.8	96.3	96.3	95.0	92.5	93.8	96.3	96.3	98.8	96.3	95.0	98.8	95.0	95.0
10 mM	90.0	90.0	93.8	87.5	87.5	86.3	88.8	91.3	95.0	83.8	90.0	90.0	88.8	81.3	83.8	86.3	86.3	85.0	81.3	83.8
25 mM	61.3	48.8	45.0	43.8	42.5	61.3	67.5	46.3	65.0	55.0	51.3	65.0	55.0	42.5	63.8	52.5	40.5	51.8	43.8	45.5
50 mM	28.8	28.8	30.0	25.0	32.5	26.3	26.3	30.0	33.8	27.5	30.0	38.8	27.5	28.8	38.8	35.0	28.8	23.8	33.8	30.0
100 mM	17.5	11.3	13.8	12.5	13.8	6.3	8.8	3.0	10.0	7.5	13.8	15.0	6.3	11.3	6.3	11.3	11.3	3.8	13.8	10.0
200 mM	5.5	0.0	0.0	0.0	2.5	0.0	1.3	0.0	1.3	0.0	2.5	0.0	0.0	3.0	0.0	3.8	0.0	0.0	0.0	0.0
>200	No growth																			
C	100% growth																			
Conc.	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
5 mM	88.8	93.8	98.8	100.0	100.0	100.0	100.0	100.0	95.0	96.3	98.8	96.3	100.0	100.0	93.8	91.3	100.0	100.0	98.8	97.5
10 mM	78.8	78.8	78.8	88.8	87.5	77.5	86.3	81.3	91.3	77.5	83.8	86.3	90.0	87.5	85.0	78.8	81.3	81.3	91.3	83.8
25 mM	53.8	45.0	40.0	43.8	55.0	45.0	53.8	45.0	41.3	59.0	42.5	43.8	55.0	60.0	53.8	49.7	46.3	47.3	45.3	55.0
50 mM	23.8	26.3	32.5	35.0	23.8	21.3	40.0	27.5	30.0	31.3	33.8	30.0	28.8	23.8	21.3	32.5	27.5	28.8	27.5	27.5
100 mM	6.3	8.8	11.3	11.3	5.0	3.8	15.0	11.3	8.8	11.3	13.8	10.0	11.3	6.3	6.3	15.0	10.0	13.8	8.8	7.5
200 mM	0.0	0.0	1.5	2.0	0.0	0.0	1.8	2.9	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
>200	No growth																			
Control	100% growth																			

S, bacterial strain; C, control; Conc, concentration of metals in millimoles.

e.Lead

Tolerance of bacterial isolates at different concentrations of lead (in %)																				
Conc. (mM)	S1	S2	S3	S4	S5	S6	S7	S8	S8	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
10	93.8	97.5	93.8	93.8	91.3	100.0	96.3	98.8	97.5	91.3	98.8	96.3	95.0	98.8	96.3	100.0	97.5	93.8	98.8	96.3
25	62.5	71.3	46.3	45.0	53.8	40.0	70.0	63.8	47.3	46.3	63.8	49.8	62.5	46.3	41.3	65.0	45.0	48.8	77.5	68.8
50	31.3	33.8	31.3	28.8	36.3	30.0	26.3	32.5	38.8	30.0	32.5	40.0	30.0	31.3	30.0	36.3	30.0	30.0	37.5	32.5
100	20.0	13.8	17.5	15.0	16.3	11.3	12.5	10.0	11.3	8.8	13.8	16.3	7.5	13.8	7.5	15.0	11.3	5.0	15.0	11.3
200	6.3	1.3	0.0	0.0	6.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.8	0.0	0.0	0.0	3.8
>200	No growth																			
C	100% growth																			
Conc. (mM)	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
10	100.0	93.8	97.5	100.0	96.3	100.0	95.0	98.8	93.8	88.8	92.5	95.0	100.0	95.0	95.0	88.8	100.0	91.3	95.0	93.8
25	68.8	49.3	46.3	47.3	61.8	42.5	60.0	58.8	43.8	56.3	58.8	45.0	56.3	65.0	60.0	45.5	52.5	41.3	40.3	57.5
50	30.0	30.0	36.3	36.3	25.0	28.8	41.3	32.5	35.0	36.2	41.3	35.0	33.8	26.3	23.8	35.0	30.0	31.3	30.0	31.3
100	5.0	11.3	15.0	12.5	6.3	7.5	16.3	12.5	10.0	12.5	13.8	8.0	10.0	11.3	8.8	13.8	11.3	16.3	10.0	12.5
200	0.0	0.0	3.8	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	2.5	0.0	1.3	0.0	0.0	0.0	2.5	0.0	0.0
>200	No growth																			
C	100% growth																			

S, bacterial strain; Conc, concentration of metals in millimoles; C, control.

f. Cobalt

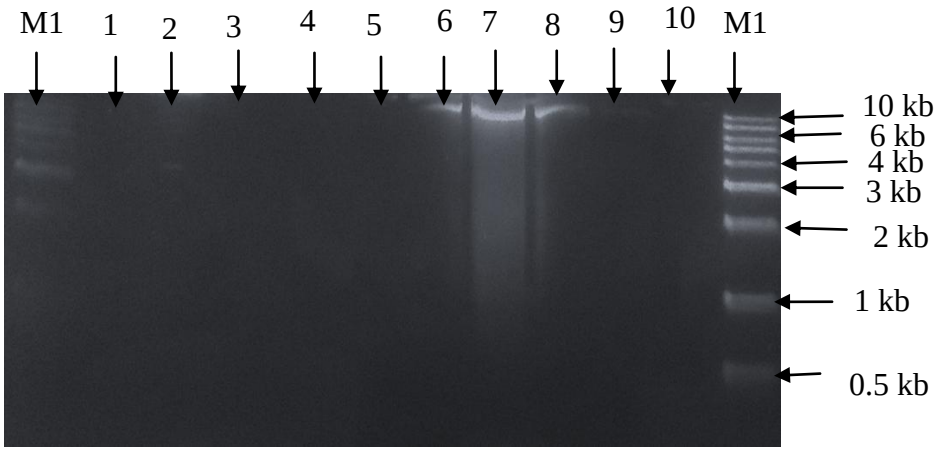
Tolerance of bacterial isolates at different concentrations of cobalt (in %)																				
Conc. (mM)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
10	90.0	97.0	98.8	98.8	97.5	97.5	89.0	97.0	97.5	96.3	98.8	100.0	98.8	96.3	97.5	90.0	97.5	95.0	90.0	98.0
25	42.5	68.8	48.8	40.0	60.0	48.8	47.5	62.5	45.0	47.5	55.0	52.5	48.8	42.5	43.8	45.8	43.8	47.5	52.8	53.8
50	36.5	30.3	37.3	33.3	35.0	38.8	31.3	40.5	25.0	26.2	31.3	40.0	32.8	36.3	23.4	35.7	31.7	35.3	31.9	32.5
100	7.0	14.3	13.0	15.5	7.3	7.5	10.3	11.5	12.0	11.5	14.8	9.0	10.0	11.3	9.8	5.8	10.3	15.3	12.0	13.5
200	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	2.5
>200			No growth																	
C			100% growth																	
Conc. (mM)	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
5	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
10	95.0	93.8	97.5	97.5	96.3	98.8	95.0	98.8	93.8	88.8	92.5	95.0	93.7	94.7	99.0	88.0	98.8	91.3	95.0	93.8
25	48.8	41.3	46.3	41.3	41.3	42.5	60.0	48.8	43.8	46.3	58.8	40.0	56.3	45.0	49.0	45.0	52.5	41.3	41.3	57.5
50	30.0	30.0	36.3	36.3	25.0	28.8	41.3	32.5	35.0	36.2	41.3	35.0	33.8	26.3	23.8	35.0	30.0	31.3	30.0	31.3
100	5.0	11.3	15.0	12.5	6.3	7.5	16.3	12.5	10.0	12.5	13.8	8.0	10.0	11.3	8.8	13.8	11.3	16.3	10.0	12.5
200	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0	0.0	2.5	0.0	2.5
>200			No growth																	
C			100% growth																	

S, bacterial strain; C, control; Conc, concentration of metals in millimoles.

APPENDIX II

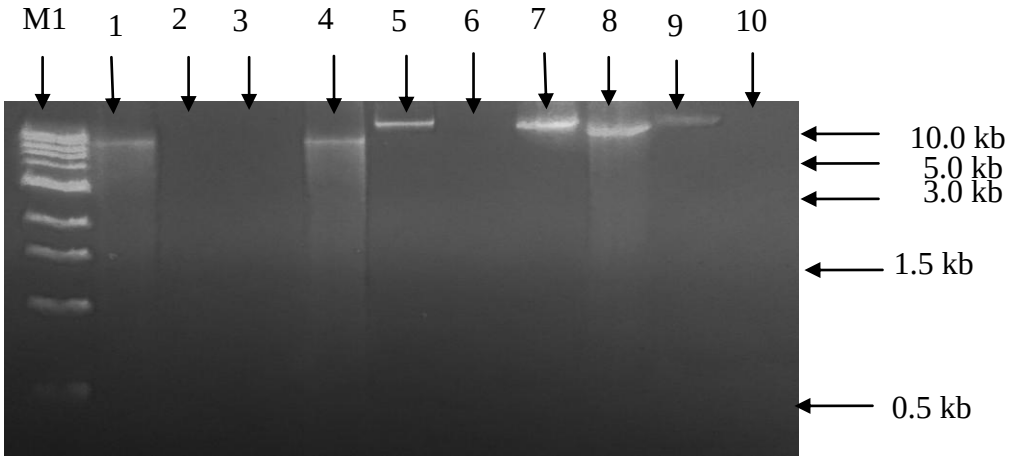
FIGURES REPRESENTING PLASMID PROFILES OF BACTERIAL ISOLATES

Plasmid profile of fecal streptococci (S11-S20)



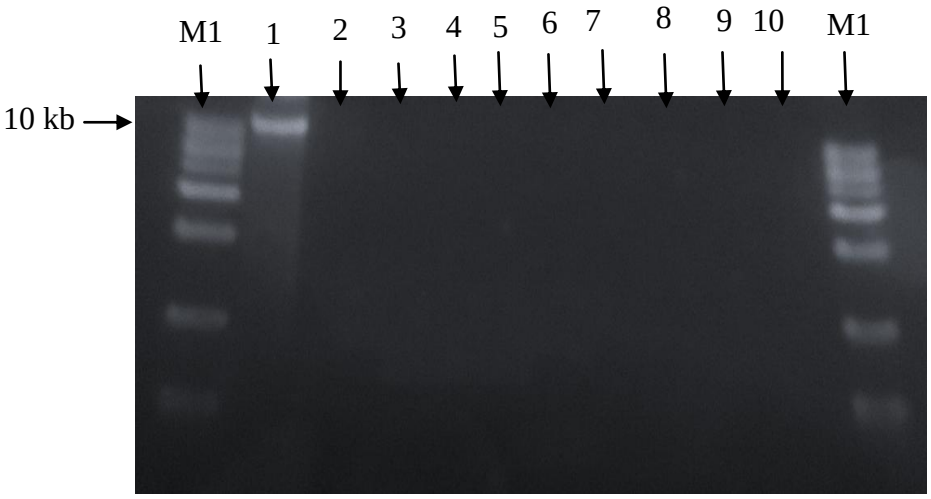
M1, DNA marker (1 kb, GenStat); Lanes 1 - 10, fecal streptococci species (S11 – S20).

a. Plasmid profiles of *Vibrio* species (S21-S30)



M1, DNA marker (1 kb, GenStat), Lanes 1 - 10, *Vibrio* species (S21 – S30).

b. ◄ Plasmid profiles of *salmonella* species (S31 - S40)



M1, DNA marker (1 kb, GenStat); Lane 1 - 10, *Salmonella* species (S31 – S40).